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Electrochemical impedance spectroscopic technique with a functionalized microwire sensor for rapid detection of foodborne pathogens

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ABSTRACT

In this study, a label-free biosensor based on electrochemical impedance measurement followed by dielectrophoretic force and antibody–antigen interaction was developed for detection and quantification of foodborne pathogenic bacteria. In our previous work, gold–tungsten wires (25 μm in diameter) were functionalized by coating with polyethyleneimine–streptavidin–anti-*Escherichia coli* antibodies to improve sensing specificity, and fluorescence intensity measurement was employed to quantify bacteria captured by the sensor. The focus of this research is to evaluate the performance of the developed biosensor by monitoring the changes of electron-transfer resistance (ΔR_{et}) of the microwire after the bioaffinity reaction between bacterial cells and antibodies on its surface as an alternative quantification technique to fluorescence microscopy. Electrochemical impedance spectroscopy (EIS) has been used to detect and validate the resistance changes in a conventional three-electrode system in which $[Fe(CN)_6^{3-}]/[Fe(CN)_6^{4-}]$ served as the redox probe. The impedance data demonstrated a linear relationship between the increments of ΔR_{et} and the logarithmic concentrations of *E. coli* suspension in the range of 10^3–10^8 CFU/mL. In addition, there were little changes of ΔR_{et} when the sensor worked with *Salmonella*, which clearly evidenced the sensing specificity to *E. coli*. EIS was proven to be an ideal alternative to fluorescence microscopy for enumeration of captured cells.

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1. Introduction

Rapid sensitive and specific detection of pathogenic microorganisms from food and drinking water are of high interest nowadays, preventing people from risks of being infected by pathogen such as *Escherichia coli* O157:H7 and *Salmonella typhimurium*. According to the literature, traditional methods for pathogen detection are time-consuming, such as the plate counting method and polymerase chain reaction (PCR) including cell enrichment (Invitski et al., 1999; Zourob et al., 2008). It took a few hours or even days for bacteria to be isolated and grown, followed by many biochemical tests for identification. Besides bacteria capture, the quantification and identification step is also very important to a sensing approach, offering the analytical tool to interpret the sensing results. In our previous studies, an antibody-coated microwire sensor coupled with dielectrophoresis (DEP) fluorescence microscopy was used for fluorescence antibody-labeled bacteria quantification, which is rapid but requires a large number of raw measurements to ensure its accuracy.

Electrochemical impedance spectroscopy (EIS) is an effective and reliable method to investigate antibody–antigen interactions on electrode surfaces (Xiao et al., 2007; Vaisocherová et al., 2009; Murat, 2011). The basic approach for EIS is to apply small amplitude sine wave perturbations to an electrochemical system over a wide range of frequencies, and measure the responding signals (e.g. current) as a function of frequencies. The Nyquist plot is one of the most popular formats for interpretation of EIS data. A typical Nyquist plot is generated by comparing an imaginary part of impedance (Z_{im}) versus a real part of impedance (Z_{re}), and consists of a semicircle followed by a straight line. The semicircle part that falls in the high frequency range is dominated by the electron transfer process, while the tail part that falls in the low frequency range is dominated by the diffusion process (Yang and Bashir, 2008). When the imaginary impedance is zero, the intercept of the semicircle with the *x*-axis represents the electrolyte solution resistance (R_s). The diameter of the semicircle is proportional to the electron transfer resistance (R_{et}) of the working electrode. Also, there are certain electron transfer reaction rates that are affected by parameters such as reaction order, temperature, product concentration and potential. The diffusion coefficient of electron transfer reactions can be empirically determined. Several studies have reported that attachment of bacterial cells to the electrode surface could form a blocking layer that inhibits the current flow and thus increases the impedance of the electrode (Chuanmin et al., 2002; Escamilla-Gómez et al., 2009; Huang et al., 2011; Tang et al., 2004; Yang and Bashir, 2008).

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Among various applications, EIS as a tool for characterizing electrodes modified with functional coatings has been well explored by many researchers (Xiao et al., 2007; Vyas and Wang, 2008; Escamilla-Gómez et al., 2009). Films coated on an electrode surface are quite heterogeneous, indicating that the chemistry varies from point to point along the metal surface. Therefore, microelectrodes such as the developed microwire sensor have great advantages over conventional electrodes as a working electrode for electrochemical impedance measurements. A conventional electrode, which has a much larger surface area, takes longer to establish a steady-state current due to the low rate of mass transport. The long time taken for the measurement may introduce problems from drift in the electrode with time. In addition, a working electrode with larger surface area requires a larger area of the counter electrode, which increases the cost of the sensing experiment. Moreover, the small quantity of chemicals reacting at the micro-electrode surface will not change the chemistry significantly within the measurement pool (Bai et al., 2006).

In our previous study, we developed a functionalized microwire sensor in which gold–tungsten wires (25 μm in diameter) were coated with polyethyleneimine–streptavidin–anti-*E. coli* antibodies to improve the sensing specificity, and fluorescence intensity measurement was employed to quantify bacteria captured by the sensor (Lu and Jun, 2012). In comparison to other pathogenic detection studies using the EIS technique (Geng et al., 2008; Mantzila et al., 2008; Guo et al., 2012), this study explored integration of EIS into the microwire sensing platform for further enhanced sensing accuracy and rapidity. The objectives were to establish an electrochemical system for impedance measurement in which the microwire served as the working electrode, and to evaluate the performance of the microwire sensor by the electrochemical impedance method as an alternative to fluorescence microscopy.

2. Material and methods

2.1. Materials

7% gold–tungsten plate wire 25 μm in diameter was supplied by ESPI Metals (Ashland, OR). Stock cultures of *E. coli* K12 and *S. typhimurium* were obtained from the Food Microbiology Laboratory (University of Hawaii, Honolulu, HI). Polyethyleneimine (PEI), streptavidin and biotinylated mouse monoclonal anti-β-galactosidase antibodies were purchased from Sigma (Product# S4762, # S4762 and # 408700, Saint Louis, MO). Electrolyte solution used for EIS and CV measurements was prepared by dissolving 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ in 0.1 M KCl solution (product # 244023, # P3289, and # P9541, Sigma-Aldrich Co., Saint Louis, MO).

2.2. Instrumentation

An automated *xyz* stage controlled by the COSMOS program (Franklin Mechanical & Control Inc., Gilroy, CA; Velmex, Inc., Bloomfield, NY) was applied for manipulating the motion of the wire during functionalization and bacteria deposition on wire surface. Electrochemical tests were performed using a μ-Autolab type III potentiostatic frequency response analyzer (FRA) equipped with NOVA software version 1.6 (Metrohm Autolab USA Inc., Riverview, FL) (Fig. 1). The conventional three-electrode configuration was utilized for constructing the electrochemical cell. The working electrode (WE) was the microwire modified with various coatings and a platinum wire (product # CHI 115, CH Instruments, Inc., Austin, TX) with a diameter of 0.5 mm served as the counter electrode (CE). The Ag/AgCl (3 M KCl) reference electrode (RE) was purchased from VWR (catalog # A 57194, Brisbane, CA).

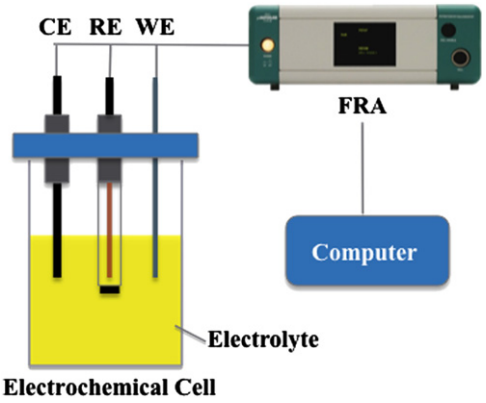

Fig. 1. Electrochemical cell and potentiostat for EIS measurements. CE: counter electrode; RE: reference electrode; WE: working electrode; FRA: frequency response analyzer.

2.3. Bacterial cultivation

All bacterial strains were transferred into tryptic soy broth (TSB; BD diagnostic systems, Franklin Lakes, NJ) twice and incubated at 37 °C for 24 h prior to use. The initial concentrations of the *E. coli* K12 and *S. typhimurium* stock cultures were obtained using serial dilutions and plate counting methods.

2.4. Preparation of functionalized microwires

Microwires were sanitized in an ultrasonic washer, rinsed with 95% alcohol, dried in air and mounted on the automated *xyz* stage. A polydimethylsiloxane (PDMS) coated plate was used as a substrate for maintaining the shape of reagent droplets throughout the immobilization process. The sanitary wire tip was immersed into 5 μL PEI for 5 min, and then dried in air for 2 min. Thereafter, the wire was soaked in 5 μL streptavidin for 1 min and then pulled out. Biotinylated anti-β-galactosidase antibodies were applied afterward in the same manner for 5 min to allow antibodies to bind to streptavidin. The biotinylated antibodies were linked to the streptavidin and ready to cross-link with bacterial cells.

2.5. Detection of E. coli cells

5 μL of each *E. coli* K12 cell suspension with different cell concentrations were deposited on the surface of functionalized microwires using the DEP and antibody–antigen interaction-governed sensing technique that was developed in our previous study (Lu and Jun, 2012). Electrochemical impedance measurements were carried out within a frequency range of 10^{-1}–10^5 Hz. The applied DC potential was 200 mV and the amplitude of the applied sinusoidal perturbation was 10 mV. A 10 min interval was allowed between each of the two measurements to ensure the steady-state of the electrochemical reactions.

2.6. Data analysis

Experimental data were displayed by Nyquist plots. The Nyquist plots were then fitted by the built-in analytical tool "Electrical Circle Fit" in the NOVA software, and both solution resistance (R_s) and electron transfer resistance (R_{et}) were collected from the fitted data. The changes of electron transfer resistance (ΔR_{et}) for the redox reaction happening at the electrode-film

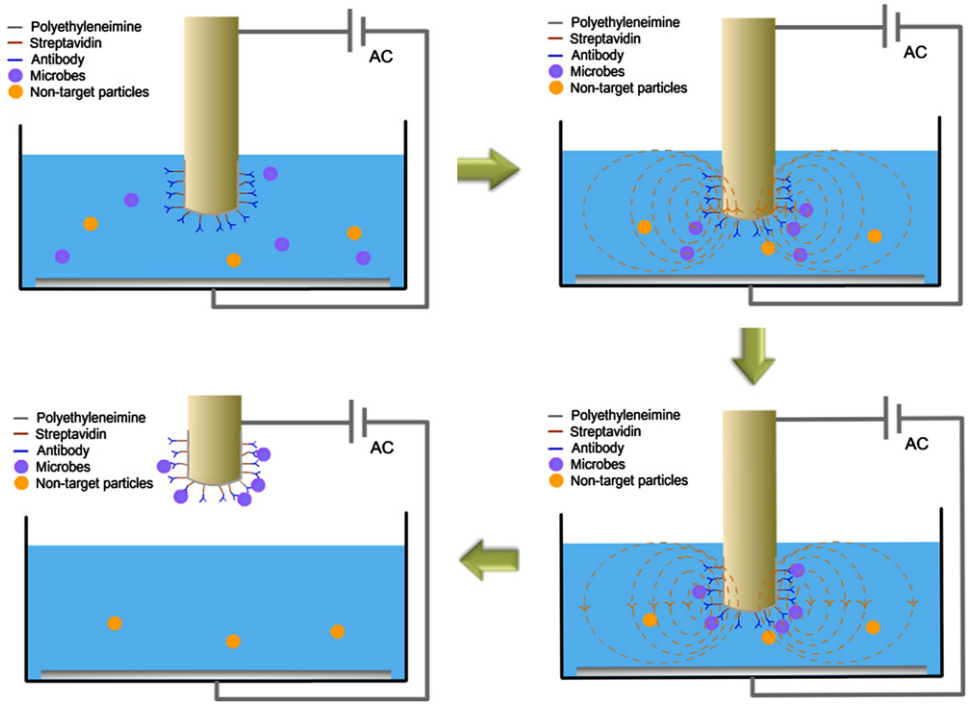

Fig. 2. Schematic strategy of target microbes sensing based on dielectrophoresis and antibody–antigen interactions.

interface before and after bacteria binding with the antibodies were calculated as follows:

$$\Delta R_{et} = R_{et}(\text{antibody}-\text{bacteria}) - R_{et}(\text{antibody}) \quad (1)$$

3. Results and discussion

Fig. 2 describes the stepwise process of selective bacteria detection. The first functional layer was PEI, which was capable of directly binding to streptavidin via electrostatic interaction. One mole of streptavidin is able to absorb 4 mol of biotin through the well-known streptavidin–biotin interaction, making streptavidin a powerful tool to increase the biotinylated antibody loading during immobilization (Xiao et al., 2007).

Fig. 3a is the impedance spectra of the sensing wires that concentrate and capture *E. coli* cells at different initial concentrations. According to Vyas and Wang (2008), layer-by layer buildup of thin films on a substrate is not always homogeneous and sometimes fails to fully cover the substrate surface. However, the EIS system is able to catch and monitor subtle but critical electrochemical changes obtained from the working electrode, irrespective of whether it is homogeneous or heterogeneous by nature. The Nyquist plot can be interpreted by measuring the magnitude of each semicircle, which is proportional to the impedance of the sensing electrode under investigation in the entire electrochemical cell (Fig. 1). The impedance data were fitted to a Randles equivalent circuit to interpret electrochemical properties of the microwire biosensor (Fig. 3b; Mantzila et al., 2008). The Randles equivalent circuit consists of R_S in series with the parallel combination of the double-layer capacitance (C_{dl}) and an impedance of a faradaic reaction, which is composed of the electron transfer resistance (R_{et}) and the Warburg element of diffusion (Z_w). It is noted that the R_{et} includes the resistance of the non-functionalized area (R_{nf}) and the resistance of the functionalized area (R_f) of the single microwire in a series combination. Since the functionalized area of the wire acts as an insulating layer to resist the current flow, the value of R_f is much larger than R_{nf}, thus mainly representing the resulting impedance of the electrode being modified (Xiao et al., 2007).

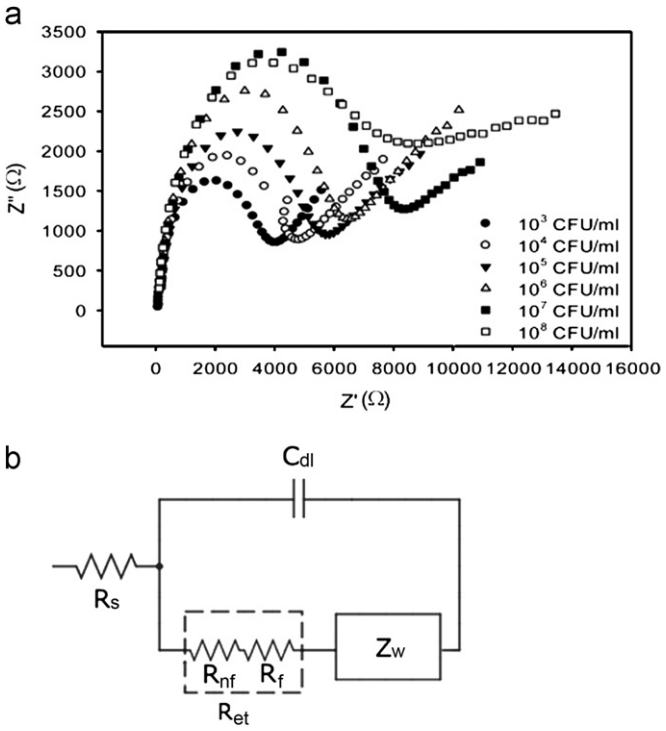

Fig. 3. (a) Impedance spectra of functionalized microwires with *E. coli* K12 cells binding to surfaces. Operating parameters: EIS, 5 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) in 0.1 M KCl, 0.1–10,000 Hz frequency range with 0.01 V amplitude sinusoidal perturbation; *E. coli* concentrations: 10^3–10^8 CFU/mL. (b) Randles equivalent circuit of faradaic EIS measurement: R_s: electrolyte solution resistance; C_{dl}: double-layer capacitance; R_{et}: electron transfer resistance; R_{nf}: resistance of non-functionalized area; R_f: resistance of functionalized area; Z_w: Warburg element of diffusion.

Fig. 4 shows that the electron transfer resistance increased as the logarithmic value of *E. coli* concentrations increased from 10^3 to 10^8 CFU/ml. An increase in *E. coli* cells attached to the microwire created a more effective barrier to electron transfer, thus leading to a larger diameter of the corresponding semicircle in the Nyquist plot. Regarding the slower increasing trend from 10^7 to 10^8 CFU/ml, one possibility could be that the active sites of immobilized antibodies for binding bacteria were close to saturation, resulting only in a small impedance change. Since 5 μL bacterial suspension was used for each detection, the detection limit could be estimated as $5\ \mu L \times (n \times 10^3\ \text{CFU/ml}) = 5 \times n\text{CFU}$, where the index, n could be 1–10, depending on the initial number of bacterial stock culture.

Fig. 5 shows the specificity of the functionalized microwire sensor towards *E. coli* when *Salmonella* was used as a negative control. Both *E. coli* and *Salmonella* suspensions at 10^4 CFU/ml were employed for the test. After binding with *Salmonella,* the electron transfer resistance of the wire increased by 126.5 Ω compared to that in the absence of any bacteria. This may be due to likely non-specific adsorption of *Salmonella* cells onto the electrode surface. However, a much greater R_{et} increment of 2066.5 Ω was observed with *E. coli* cells captured onto the wire surface, indicating high selectivity of the modified microwire sensor immobilized with antibodies specific for *E. coli* K12.

In comparison with the bacterial quantification method used in our previous research, EIS demonstrates several advantages over fluorescence microscopy. According to FESEM images, bacterial cells captured on a wire were not uniformly distributed on the surface. Fluorescence intensity measurement highly depends on the area selected for measurement and thus requires a large amount of measurements and finally an average must be made to improve the accuracy. Also, the fluorescence intensity is quantified based on two-dimensional images taken by the microscope, which is unable to represent all characteristics of the cylindrical sensing wire; however, EIS allows for integrated and dynamic analysis of the microwire without any blind spots since it could sense even minute changes in the impedance value of the entire wire surface. In addition, the mechanism of bioaffinity reactions between bacterial cells and antibodies that occurred at the interface could be better understood by further interpreting electrochemical diffusion processes.

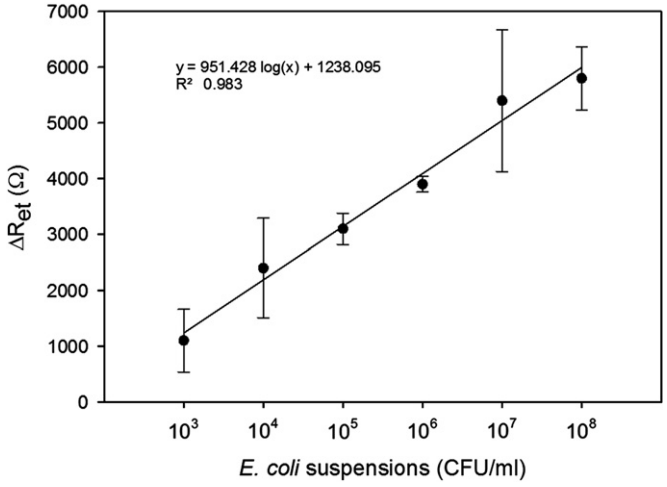

Fig. 4. Changes of electron transfer resistance of *E. coli* K12 cells captured on the electrode surface. Cell concentrations vary from 10^3 to 10^8 CFU/mL. The error bars represent one standard deviation where the number of replications is 3.

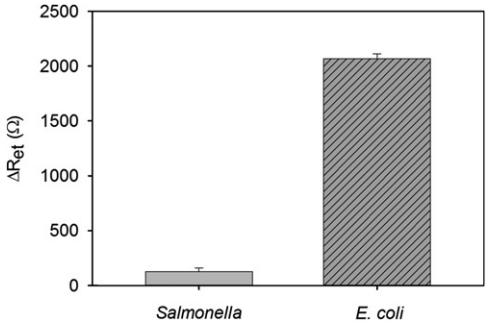

Fig. 5. Sensing specificity of the functionalized microwire sensor validated for *E. coli* K12. *E. coli* K12 and *Salmonella* concentrations are 10^4 CFU/mL.

4. Conclusions

Label-free, rapid and specific detection of *E. coli* K12 by a microwire sensor functionalized with a three-layer (PEI–streptavidin–antibodies) was demonstrated in this study. Sensitivity and specificity of the developed sensor were evaluated by monitoring the changes in electrochemical impedance of the wire after bacterial cells were deposited on its surface. A linear trend of increasing impedance was obtained when the *E. coli* concentration increased logarithmically from 10^3 to 10^8 CFU/ml. EIS analysis has proved that the sensor was able to detect about five CFUs of *E. coli* cells per microwire from 1000 CFU/ml microbial suspension. In addition, little change of ΔR_{et} was observed when the sensor worked with *Salmonella*, which clearly evidenced the sensing specificity towards *E. coli* K12.

Future studies could include the improvement of current detection limit and sampling volume. For example, a "pen-sized" portable biosensor equipped with multiplexed microwires can be developed for effective cell concentration via dielectrophoretic force and a larger sample volume to be examined.

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