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Characterization of a carbon composite electrode for an electrochemical immunosensor

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Abstract−A bioactive platform with a carbon composite electrode was developed for rapid detection of *Escherichia coli* O157:H7. The porous carbon composite electrode was prepared by a sol-gel method with a mixture of graphite powder and tetraethyl orthosilicate/ethanol. *Escherichia coli* O157:H7 antibodies were physically adsorbed onto the carbon composite electrode. Direct measurements by cyclic voltammetry and electrochemical impedance spectroscopy in the presence of $[Fe(CN)_6]^{3-/4-}$ as a redox probe showed that the immobilization of antibodies onto the carbon composite electrode surface and the binding of *Escherichia coli* O157:H7 cells with antibodies systematically increased the electron-transfer resistance. Those results suggest that a sol-gel derived graphite composite electrode might be utilized as a label-free electrochemical immunosensor for diagnosis, biochemical research, food industry, and so on.

Key words: Immunosensor, Carbon Electrode, Sol-gel, *E. coli* O157:H7, Cyclic Voltammetry, Electrochemical Impedance Spectroscopy

INTRODUCTION

During the past decade, immunosensors with molecular recognition system such as antigen-antibody interaction have been successfully developed and applied to many fields including food, environmental and biomedical processes [1-3]. In the development of immunosensors, the antibody immobilization process for stability and activity of antibody and the signal transfer process for sensing the specific antigen-antibody interaction at the receptor are two major processes. In general, the antibody immobilization on a substrate is achieved by chemisorption or physisorption [4-8]. The signal transfer process that is the most important part in the molecular recognition has been developed into the signal amplification or changing the optical signal at the antigen-antibody interaction to exploit the secondary antibodies [9]. Therefore, level-free immunosensors without secondary antibodies are desirable and have gained considerable interest as bioanalytical devices because they are robust, economical to mass produce, and can achieve excellent detection limits with small analyte volumes [3,10].

Escherichia coli O157:H7 (*E. coli* O157:H7) is one of the human pathogenic species that has emerged as a significant food borne pathogen. *E. coli* O157:H7 infection often causes severe bloody diarrhea and abdominal cramps [11,12]. Currently, several immunosensors for detection of *E. coli* O157:H7 have been reported in the literature. Youngcheng and Yanbin [13] developed a capillary-column bioseparator for separation and detection of *E. coli* O157:H7 by chemically immobilizing antibody against *Escherichia coli* O157:H7 antibodies (anti-*E. coli* O157:H7 antibodies) onto the inner wall of the column. Lee *et al.* [14] fabricated a bioactive platform by immobilizing anti-*E. coli* O157:H7 antibodies by a sol-gel technique. Chuanmin et al. [15] demonstrated a mass-sensitive magnetoelastic immunosensor for detection of *E. coli* O157:H7. However, all those methods are level-immunosensors with fluorescent or enzyme-leveled tracers.

An electrochemical technique is an alternative for developing biosensors for detection of pathogens. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) are techniques working in the presence of any redox probe. These are considered as an effective method for sensing the antigen-antibody interaction on electrode surfaces by probing the features of the interfacial properties such as capacitance and electron-transfer resistance of electrodes [16].

In this study, by using graphite having conductive property in nature, a level-free immunosensor was prepared for detection of *E. coli* O157:H7. A carbon composite electrode was manufactured by a sol-gel method having many advantages, including low-temperature encapsulation of biorecognition elements, tenability of physical characteristics, and mechanical rigidity [17-19]. Anti-*E. coli* O157:H7 antibodies were immobilized onto porous sites of a carbon composite electrode. The immobilization of antibodies onto the carbon composite electrode surface and the binding of *E. coli* O157:H7 cells with antibodies were confirmed by CV and EIS in presence of $[Fe(CN)_6]^{3-/4-}$ as a redox probe. An electrical equivalent circuit was proposed for understanding stepwise changes in impendence components of an immunosensor system.

EXPERIMENTAL SECTION

1. Materials

Affinity purified mouse anti-*E. coli* O157:H7 antibodies were obtained from Biodesign International USA. A 1 : 1 dilution of antibody was prepared with 50% glycerin solution in water before use. All solutions were prepared in doubly distilled deionized water. Phosphate-buffered saline (PBS; 0.01 M, pH 7.4), hexacyanoferrates (potasium hexacyanoferrate(III) and potasium hexacyanoferrate(II) trihydrate), tetraethyl orthosilicate (TEOS), ethyl alcohol, graphite powers (5,000 mesh (3 μm), 800 mesh (20 μm) and 325 mesh (43

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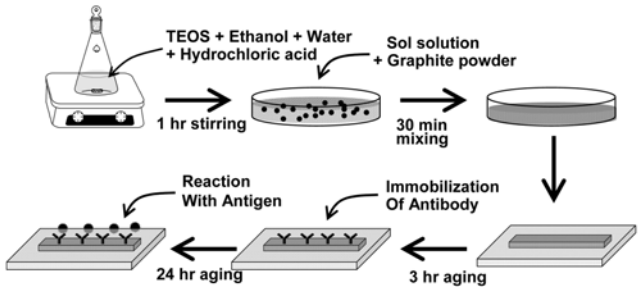

Fig. 1. Typical process for fabrication of a carbon composite electrode and immobilization of anti-*E. coli* O157:H7 antibodies.

μm)) and hydrochloric acid were purchased from Sigma-Aldrich.

E. coli O157:H7 was obtained from American Type Culture Collection (USA) [20]. The pure culture of *E. coli* O157:H7 was prepared in brain heart infusion broth at 37 °C for 20 h. The culture was serially diluted with physiological saline solution. *E. coli* O157:H7 colonies were counted to determine the number of viable cells in cfu/mL (cfu: colony-forming units) by optical density (OD). The culture was then heat-killed in a boiling water bath for 15 min for further use.

2. Fabrication of a Carbon Electrode and Immobilization of Anti-*E. coli* O157:H7 Antibodies

Typical processes for fabrication of a carbon composite electrode and immobilization of anti-*E. coli* O157:H7 antibodies are illustrated in Fig. 1. A sol-gel stock was prepared by mixing 4.5 mL of TEOS, 1.0 mL of ethanol, 2.7 mL of water, and 0.1 mL of 0.05 M HCl. A clear solution was obtained after 1 hr of stirring. One gram of graphite powder was subsequently dispersed into the 1.4 mL sol solution with 30 min mixing. The resulting graphite paste was printed on a slide glass (0.2×3 cm^2) for a working electrode. The printed glasses were cured at 4 °C for 3 hrs. For the immobilization of antibodies, The diluted antibody solution (20 μl) was spread on the surface of the sol-gel derived graphite electrode. The electrode was then kept at 4 °C for 24 hrs. After this process, the electrode was slowly dipped into PBS buffer (pH 7.4) for 30 min, allowing the diffusion of unbound antibodies away from the electrode surface. The electrode was then rinsed extensively with PBS (pH 7.4) containing 1% BSA (bovine serum albumin) and deionized water, and then it was dried with nitrogen.

E. coli cultures with different cell numbers (20 μl) were dropped 3 times onto the surfaces of the sensors, and the sensors were incubated at 37 °C until the solution was evaporated off. To remove nonspecifically bound proteins and cells, the sensors were washed thoroughly with PBS followed by several rinses with deionized water.

3. Cyclic Voltammetry (CV) and Electrochemical Impedance Spectroscopy (EIS) Measurements

All CV and EIS measurements were performed with a 660 series potentiostat from CH Instruments (USA) with a conventional electrochemical setup. The electrode was immersed in 20 mL PBS buffer (pH 7.4) for 30 min. Buffer solution was prepared 65 mL of 0.01 M PBS (pH 7.4) containing 10 mM $[Fe(CN)_6]^{3-/4-}$. Ag/AgCl was used as a reference electrode and a platinum plate as a counter electrode. Potential was scanned from −0.4 to 0.7 V with a scan rate of 100 mV/s [21]. The impedance spectra were recorded in the frequency range from 0.01 to 10,000 Hz at the formal potential of the $[Fe(CN)_6]^{3-/4-}$ redox couple. The amplitude of the alternating voltage was 5 mV. Bode plots, the magnitude of impedance versus log (frequency), were chosen for representation of the data.

4. Optical Density Measurements for Determination of *E. coli* O157:H7 Concentration

Optical density (OD) was measured by using UV to confirm the *E. coli* O157:H7 concentrations. When the absorbance of the cell is 0.4, the concentration of the *E. coli* O157:H7 is 6×10^8 cfu/mL. Using this data as the criterion, the concentrations of the cells could be confirmed. Concentrations of *E. coli* O157:H7 cells prepared were from 1.68×10^7 to 1.68×10^9 cfu/mL.

RESULTS AND DISCUSSION

1. Cyclic Voltammetry (CV) Measurements

Assay time, sensitivity, selectivity and reproducibility are the major concerns for the rapid detection of *E. coli* O157:H7 in food, medical, or other samples. The transduction mechanism for detection of *E. coli* O157:H7 is shown in Fig. 2. Hexacyanoferrates are often used as a redox probe for the characterization of a sol-gel derived carbon paste electrode [22,23]. In this study, the surface immobilization reaction and the antigen-antibody reaction by cyclic voltammetry were monitored with $[Fe(CN)_6]^{3-/4-}$ as a redox probe. The changes in peak current and the separation of peak potentials in the voltammogram with electrode surface were related to the electron-transfer resistance.

1-1. Effect of Particle Size of Carbon on CV

Carbon composite electrodes with three types of graphite were fabricated to determine the effect of particle size of carbon on CV. Fig. 3 shows the CV obtained from three electrodes with carbon particle sizes of 3, 20 and 43 μm, respectively. Fig. 3a shows that the electrode with 3 μm sized carbon powder is a reversible system since the potential difference is less than 0.6 V. However, the potential difference measured in the electrode with 20 μm sized carbon powder is about 0.6 V (Fig. 3b), and that with 43 μm sized car-

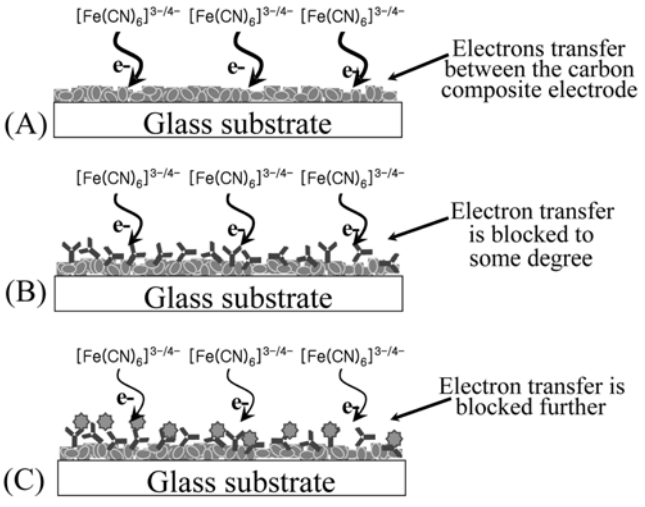

Fig. 2. Schematic of an electrode system: (A) Bare electrode, (B) with antibody immobilization and (C) with cell binding, (Oval: *E. coli* O157:H7 cell, Y: anti-*E. coli* antibody).

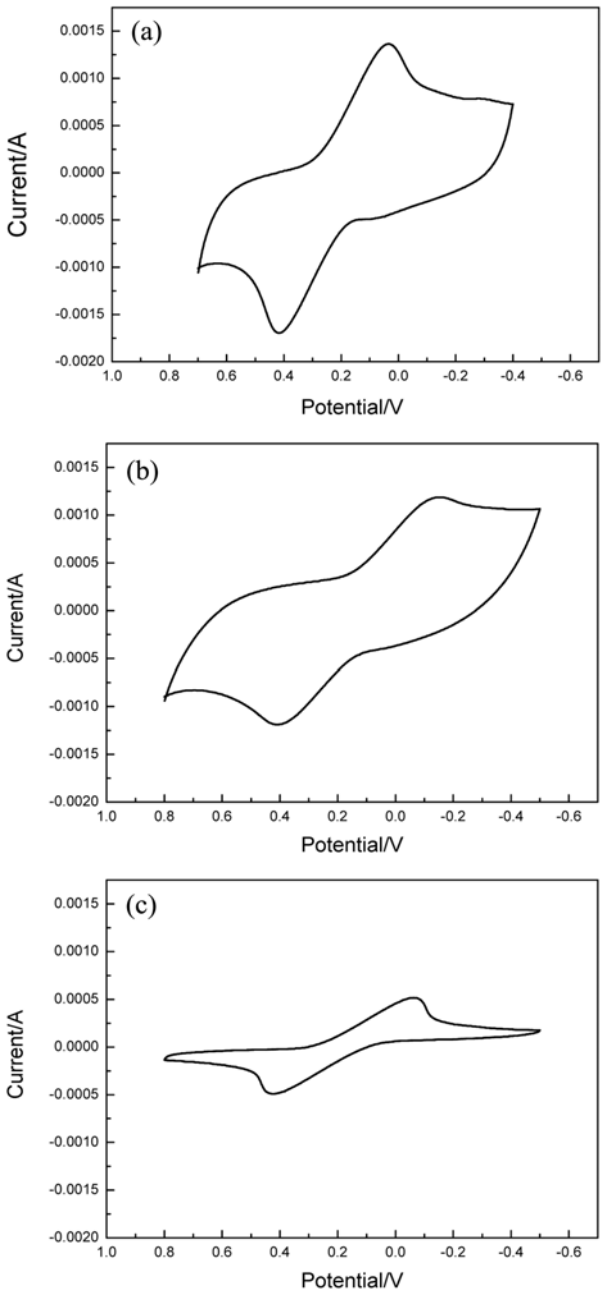

Fig. 3. Effect of particle size of carbon on cyclic voltammetry: (a) 3, (b) 20 and (c) 43 μm. In the presence of 10 mM of [Fe(CN)$_6$]$^{3-/4-}$ in 0.01 M PBS, pH 7.4, Scan rate=100 mV/s; Ag/AgCl as a reference electrode; platinum as a counter electrode.

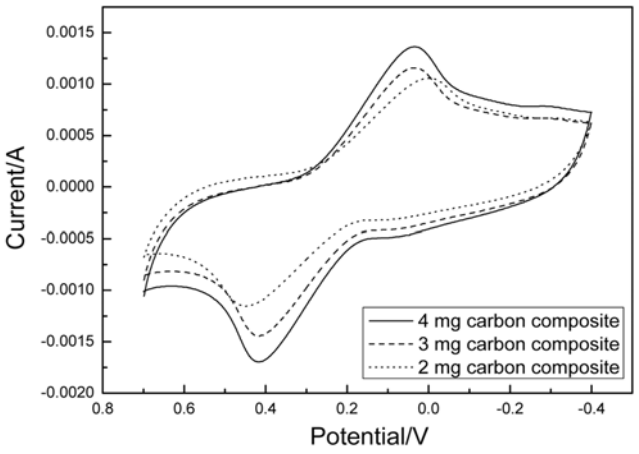

Fig. 4. Influence of amount of carbon on cyclic voltammetry. In the presence of 10 mM of [Fe(CN)$_6$]$^{3-/4-}$ in 0.01 M PBS, pH 7.4, Scan rate=100 mV/s; Ag/AgCl as a reference electrode; platinum as a counter electrode.

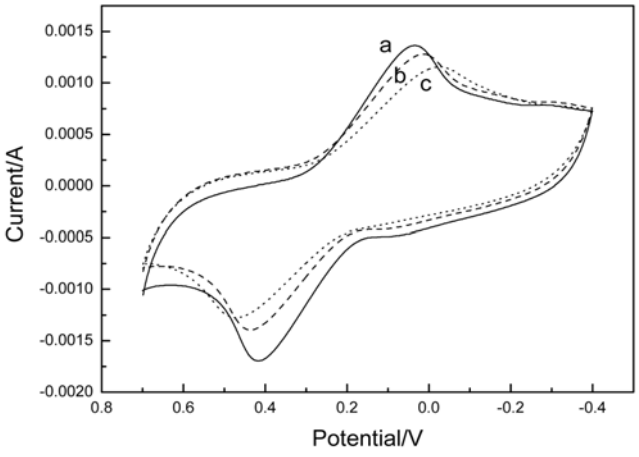

Fig. 5. Cyclic voltammetry of (a) a bare sol-gel derived carbon composite electrode, (b) after antibody immobilization, and (c) after *E. coli* cells binding in the presence of 10 mM [Fe(CN)$_6$]$^{3-/4-}$; *E. coli* O157:H7 1.68×10^8 cfu/mL. In the presence of 10 mM of [Fe(CN)$_6$]$^{3-/4-}$ in 0.01 M PBS, pH 7.4, scan rate=100 mV/s; Ag/AgCl as a reference electrode; platinum as a counter electrode.

bon powder is much larger than 0.6 V, as can be seen from Fig. 3c. Therefore, the carbon composite electrode with 3 μm sized powder was adopted in the present experiments.

1-2. Effect of Amount of Carbon on CV

Before the antibodies were immobilized on the surface of an electrode, CV was measured as a function of the amount of carbon applied onto an electrode (0.2×3 cm^2). As can be seen from Fig. 4, the voltammogram shows that the resulting current was also increased from 1.06 to 1.36 mA, when the weight of carbon paste was increased from 2 to 4 mg. From those results, the electrode fabricated with carbon paste of 4 mg was selected in the present experiments.

1-3. Effect of Antibody and Antigen on CV

Fig. 5 shows that the voltammetric behavior of a redox probe is clearly influenced by surface modification of the electrode. The permeability of ions through a sol-gel derived carbon composite electrode is so high that a redox couple can penetrate it (Fig. 5(a)). As expected, immobilization of anti-*E. coli* O157:H7 was observed to reduce the penetration of the redox couple (Fig. 5(b)). About 6% decrease in the separation of peak currents from 1.36 to 1.28 mA was clearly shown upon the binding of antibody immobilization to the electrode surface. After the treatment of the antigen-antibody interaction was made, the electron transfer of the electrode was shown to be decreased further from 1.28 to 1.16 mA (9%) as can be seen from Fig. 5c. From those results, it can be summarized that the carbon paste electrode derived by a sol-gel method may be applied as

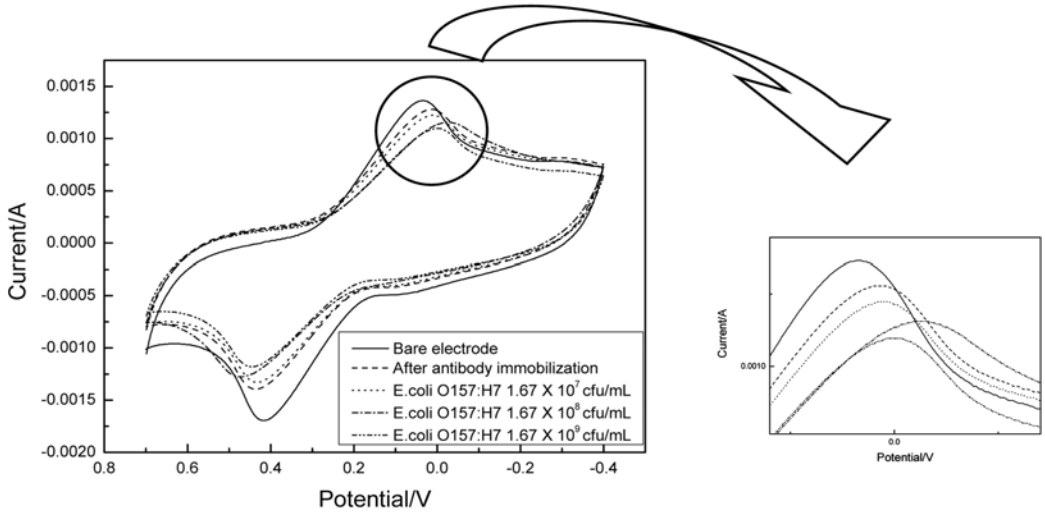

Fig. 6. Effect of antigen concentration on cyclic voltammetry. (a) 1.68×10^7, (b) 1.68×10^8, (c) 1.68×10^9 cfu/mL *E. coli* O157:H7 cells. In the presence of 10 mM of $[Fe(CN)_6]^{3-/4-}$ in 0.01 M PBS, pH 7.4, Scan rate=100 mV/s; Ag/AgCl as a reference electrode; platinum as a counter electrode.

an electrochemical immunosensor for detection of pathogens such as *E. coli* O157:H7.

1-4. Effect of Antigen Concentration on CV

Fig. 6 is a cyclic voltammogram measured with respect to the concentration of *E. coli* O157:H7. In this figure, peak currents of the electrodes are shown to decrease as concentrations of *E. coli* O157:H7 increase. The peak currents are 1.22, 1.16, and 1.1 mA, when the concentrations of the *E. coli* O157:H7 are 1.68×10^7, 1.68×10^8, and 1.68×10^9 cfu/mL, respectively. Approximately 6% decreases were observed by an about ten times increase in the *E. coli* O157:H7 concentration. The decrease of the current by increasing the concentration of antigen is the same effect with the increase of the electron transfer resistance by the antibodies adsorbed on the carbon paste electrode surface.

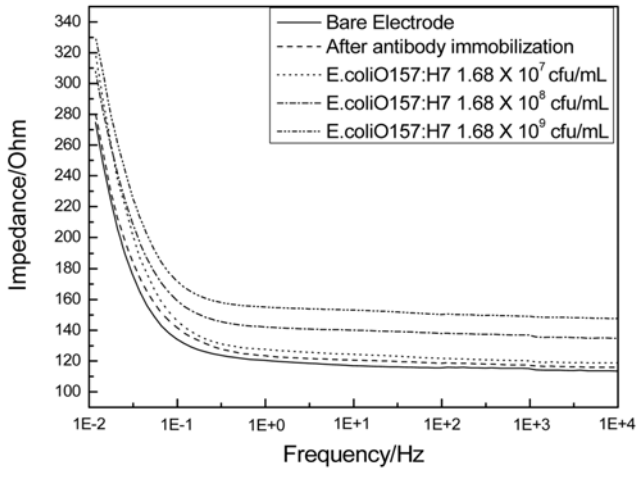

Fig. 7. The bode amplitude plots of impedance spectra for (a) a bare carbon composite electrode, (b) an antibody immobilized carbon electrode, after *E. coli* O157:H7 binding: (c) 1.68×10^7, (d) 1.68×10^8, (e) 1.68×10^9 cfu/mL in the presence of $[Fe(CN)_6]^{3-/4-}$ as a redox probe in PBS. Frequency range: 0.01 to 10,000 Hz. Amplitude: 5 mV.

2. Electrochemical Impedance Spectroscopy (EIS) Measurements

EIS is another effective method to monitor the feature of a surface-modified electrode and theoretical analysis of impedance property of an electrode, allowing an understanding of chemical transformation and processes associated with the conductive electrode surface [24]. Fig. 7 is the impedance responses of the $[Fe(CN)_6]^{3-/4-}$ as a redox probe in PBS on a bare carbon composite electrode, an antibody immobilized carbon electrode and three cell bound electrodes with the cell concentrations of 1.68×10^7, 1.68×10^8 and 1.68×10^9 cfu/mL, in the frequency range from 0.01 to 10,000 Hz. This figure also clearly shows that the electron-transfer resistance is systematically increased with the immobilization of antibodies onto the surface of a carbon composite electrode and increasing concentration of *E. coli* O157:H7 cells bound with antibodies.

Generally, an electrochemical cell can be considered simply impedance to a small sinusoidal excitation; hence, it is interesting to represent the cell performance by an equivalent circuit of resistors and capacitors that pass current with the same amplitude as the real cell does under a given excitation [25]. A frequently used circuit,

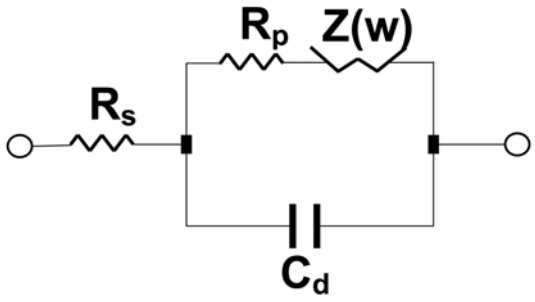

Fig. 8. Equivalent circuit on an electrochemical impedance spectroscopy measurement for a carbon composite electrode. C_d: Double layer capacitance; R_p: the electron transfer resistance; Z_w: the warburg impedance; R_s: the resistance of the electrolyte solution.

Table 1. Simulated values of electrical elements in the equivalent circuit by fitting the experimental data for a bare carbon composite electrode, the antibody immobilized electrode, and after cell binding to the equivalent circuit

	R_s (Ω)	C_d (μF)	R_p (Ω)	Z_w (Ω)
Bare electrode	110.5	9.64	164.1	6.40
Antibody immobilization	116.3	9.49	164.3	10.58
Percentage change	5.25%	−1.56%	0.12%	65.3%
E. coli binding (1.68×10^7 cfu/mL)	121.9	9.45	169.2	12.41
Percentage change	4.82%	−0.42%	2.98%	17.3%
E. coli binding (1.68×10^8 cfu/mL)	131.2	8.70	176.7	15.86
Percentage change	7.63%	−7.94%	4.43%	27.8%
E. coli binding (1.68×10^9 cfu/mL)	148.4	8.49	181.7	26.57
Percentage change	13.1%	−2.41%	2.83%	67.5%

called the Randles equivalent circuit, is shown in Fig. 8. The impedance spectra of the electrodes that were used in the present experiments could be interpreted by this equivalent circuit model. The circuit includes ohmic resistance of the electrolyte solution, R_s, Warburg impedance, Z_w, which is resulted from the diffusion of ions to the electrode interface from the bulk of the electrolyte, double layer capacitance, C_d, and electron transfer resistance, R_p [25]. Ideally, Z_w and R_s indicate the properties of the electrolyte solution and diffusion of the redox probe; therefore, they are not affected by modifications occurring on the electrode surface. On the other hand, R_p are related to the dielectric and insulating features at the electrode/electrolyte interface, and hence they are affected by modification of the electrode surface.

Simulated results with the experimental data to an equivalent circuit are summarized in Table 1. For the bare carbon composite electrode, the values for R_s, C_d, R_p and Z_w are 110.5 Ω, 9.64 μF, 164.1 Ω and 6.40 Ω, respectively. After antibody immobilized on the electrode, increases of 5.25 in R_s, 0.12 in R_p, and 65.3 in Z_w were observed, respectively. This is consistent with the results in the CV that the antibody immobilized on the bare carbon electrode surface led to a large increase in charge-transfer resistance. Similarly, with the occurrence of antigen-antibody interaction onto the electrode surface, an increase of the electron transfer resistance was observed as well. This was great change among all these electrical elements, suggesting that antibody and antigen layer created additional barriers and further prevented the access of the redox probe to the electrode surface with an increase in R_p.

CONCLUSIONS

A bioactive platform with a sol-gel derived carbon composite electrode was fabricated and antibodies were immobilized onto the porous surface of a carbon composite electrode via physical adsorption. The detecting biomaterial, *E. coli* O157:H7, was immobilized on the carbon composite electrode by the antigen-antibody recognition. The electrode was investigated by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) at the presence of hexacyanoferrates as a redox probe. Results of CV and EIS clearly demonstrate that the electron-transfer resistance is systematically increased with the immobilization of antibodies and antigens onto surface of a carbon composite electrode and also increasing concentration of antigens bound with antibodies. From those results, it is concluded that the system proposed in the present study can be used as a label-free immunosensor which is a fast, reliable, and convenient technique for detection of pathogenic biomaterials.

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