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An aptamer-based electrochemical biosensor for the detection of *Salmonella*

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ABSTRACT

Salmonella is one of the most common causes of food-associated disease. An electrochemical biosensor was developed for *Salmonella* detection using a *Salmonella*-specific recognition aptamer. The biosensor was based on a glassy carbon electrode modified with graphene oxide and gold nanoparticles. Then, the aptamer ssDNA sequence could be linked to the electrode. Each assembly step was accompanied by changes to the electrochemical parameters. After incubation of the modified electrode with *Salmonella*, the electrochemical properties between the electrode and the electrolyte changed accordingly. The electrochemical impedance spectrum was measured to quantify the *Salmonella*. The results revealed that, when more *Salmonella* were added to the reaction system, the current between the electrode and electrolyte decreased; in other words, the impendence gradually increased. A detection limit as low as 3 cfu/mL was obtained. This novel method is specific and fast, and it has the potential for real sample detection.

1. Introduction

Salmonella is one of the most frequently occurring pathogens in food that affects people's health (Newell et al., 2010; Scallan et al., 2011; Wong et al., 2009). It is widely distributed in nature. Meat, eggs, milk and other animal products contain a rich variety of nutrients that are very suitable for the growth and spread of *Salmonella* (Guard-Petter, 2001). The conventional methods for *Salmonella* detection are usually classical culture methods that include the sequential steps of pre-enrichment, selective enrichment and selective differential plating. These methods are time-consuming, labor-intensive and impractical for real-time applications. The more recently developed PCR and ELISA methods have their own drawbacks that limit their further application (Gehring et al., 1996; Jaradat et al., 2004; Miller et al., 2011; Proux et al., 2000). The development of new techniques with faster response time, better sensitivity and selectivity and no need for pre-enrichment remains a challenge of research interest.

Nucleic acid aptamers are single-stranded DNA or RNA molecules that form sequence-defined unique structural forms with binding affinities for specific targets. Several characteristics of aptamers make them attractive for pre-analytical sample processing and biodiagnostic assay development, including their small size, ease of synthesis and labeling, lack of immunogenicity, low cost of production and target binding affinity and specificity (Bruno and Kiel, 1999; Hamaguchi et al., 2001; Joshi et al., 2009; Tombelli et al., 2005a; Wilson et al., 2001). The target molecules of an aptamer can be drugs, proteins, organic dyes, antibiotics, and even virus particles and complete cells. Biosensor technologies have been used as potential alternatives to circumvent the bottlenecks of traditional methods because of their rapid response time; furthermore, they are sensitive, robust, portable and easy to use. Biosensors that use an aptamer as a specific recognition part known as an aptasensor have become an area of research interest. Currently, a number of aptasensors have been developed to detect and quantify proteins. They provide a quick, cost-effective and sensitive platform for screening methods with minimal sample preparation (Bai et al., 2012; Chang et al., 2012; Lee et al., 2008; Tombelli et al., 2005b).

Electrochemical detection methods possess several advantages, such as easy operation, low cost, high sensitivity, simple instrument and suitability for portable devices. An ideal biosensor should have a high signal/noise (S/N) ratio, a low detection limit and a broad linear range of the analyte concentration (Ronkainen et al., 2010). Nanomaterials have received special attention in the development of novel biosensing systems (Huang et al., 2013; Jain, 2003; Kikkeri et al., 2013; Singh et al., 2010; Wang and Qu, 2013). Extensive studies have shown gold nanoparticles (GNPs) to be promising candidates for modifying electrochemical biosensors (Ding et al., 2013; Wang et al., 2013). These modified electrodes can then host the bio-recognition layer. GNPs introduce many advantages to these sensors, such as their ability to provide a robust and efficient loading platform for further improvement in electron transfer between the active site and the electrode (Hanefeld et al., 2009).

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In this study, an aptamer-based electrochemical biosensor was constructed for the detection of *Salmonella enterica* serovar Typhimurium. First, glassy carbon electrode (GCE) was modified with graphene oxide (GO) and GNPs for their biocompatibility and high electron transfer properties. Then, thiolated aptamer ssDNA sequence was linked to the surface of the GCE. Here, the aptamer sequence was reported by Joshi R. et al. (Joshi et al., 2009). In the published paper, the DNA aptamers of *S. enterica* serovar Typhimurium were selected and evaluated. Due to the high specific recognition effect of the aptamer, *Salmonella* could be captured. During the entire process, the electrochemical parameters were also changed accordingly. Cyclic voltammetry (CV) was used to characterize each electrode modification step, and the quantification of *Salmonella* linked to the modified electrode was measured by electrochemical impedance spectroscopy (EIS). In the range of 2.4–2.4 $\times 10^3$ cfu/mL, *Salmonella* concentration exhibited a good linear relationship with impedance, and the detection limit was 3 cfu/mL. This method showed high sensitivity, good selectivity and short detection time. It has the potential to be improved for daily detection work.

2. Materials and methods

2.1. Chemicals

Potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium chloride (KCl), solid agar, sodium chloride (NaCl), alumina powder (Al_2O_3) (particle size of 1.0 μm, 0.3 μm, 0.05 μm), sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4) and chloroauric acid ($HAuCl_4$) were purchased from Sinopharm Chemical Reagent Co., Ltd. Tryptone and yeast extract were purchased from OXOID Co., England. The thiolated aptamer nucleotide sequence of *Salmonella* was synthesized by Shanghai Sangon Biological Science & Technology Company (Shanghai, China). The ssDNA sequence was as follows: 5′-HS-TAT GGC GGC GTC ACC CGA CGG GGA CTT GAC ATT ATG ACA-G-3′.

The LB media were prepared as follows. Solid LB medium (1 L): 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g solid agar. Liquid LB medium (1 L): 10 g tryptone, 5 g yeast extract, 10 g NaCl.

2.2. Surface modification and characterization of GCE

The bare GCE electrode was first polished sequentially with Al_2O_3 powders containing different particle sizes (1.0 μm, 0.3 μm, and 0.05 μm). Then, it was washed sequentially with ultrapure water, ethanol and ultrapure water for ultrasonic cleaning to remove the adsorbates. Next, the GCE was dried with nitrogen for further use.

Five microliters of the GO suspension was spread on the GCE surface and air-dried to prepare the modified electrode GO/GCE.

The GO/GCE electrode was immersed in 1% chloroauric acid solution, and the constant potential method (Chen et al., 2013) was used for the electrochemical deposition of GNPs on the surface of GO/GCE.

Five microliters of the 5 μM ssDNA sequences was incubated with the modified electrode at 36 °C for 2 h and then washed with ultrapure water and PBS.

Each modification step was characterized by CV technology using a CHI 660D Electrochemical Workstation (Chen Hua Instrument Co., Ltd, Shanghai, China).

2.3. Activation, cultivation and plate counting of Salmonella

The *Salmonella* were inoculated into the LB liquid medium and cultivated under shaking at 37 °C for 12 h enrichment. The enriched bacterial was centrifuged at 5800 r/min for 10 min (25 °C), and the supernatant was discarded. The precipitate was washed with PBS (0.1 M, pH 7.4) three times and resuspended in PBS. The absorbance was measured at 600 nm. The collection was centrifuged and diluted repeatedly to optical density value 0.12. This preparation was used as the original *Salmonella* sample. Then, the original bacterial was diluted to eight concentrations along a gradient from 10^{-1} to 10^{-8} using physiological saline. One hundred microliters of 10^{-5}, 10^{-6}, and 10^{-7} *Salmonella* was coated on the solid LB agar plates. Each plate was coated with three parallel boards. After cultivation at 37 °C for 12 h, the colonies were counted for the calculation of the *Salmonella* sample (cfu/mL).

2.4. Electrochemical detection of Salmonella

Three-electrode system was used for detection including the working electrode (GCE electrode), reference electrode and platinum electrode. The modified GCE electrode was first incubated with different dilutions of *Salmonella* at 37 °C for 1.5 h until the bacteria were fully reacted with the electrode's corresponding thiolated aptamer. Then, the electrode was rinsed with PBS and ultrapure water to remove the unbound *Salmonella*. Finally, the electrode was immersed in the $[Fe(CN)_6]^{3-/4-}$ electrolyte (10^{-3} M) for EIS detection using an Electrochemical Workstation (CHI 660D, Chen Hua Instrument Co., Ltd, Shanghai, China).

Specificity experiments were conducted using *Listeria monocytogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Enterobacter sakazakii* as control bacteria. Between different bacteria, the GCE electrode was polished, washed and dried (experimental procedure was described in detail in 2.2) for each bacteria detection. Their activation and cultivation processes were performed the same as those for *Salmonella*. Then, a 10^{-6} dilution of each bacteria was used for the electrochemical detection in place of *Salmonella*, as described above.

2.5. Recovery experiments for pork sample

In this experiment, the commercial pork was used as realistic samples for recovery experiments in the detection of Salmonella. Pork was first sterilized in boiling water for 5 s. And then the deep muscle was clipped by sterile scissors (25 g). After grinding, 225 mL sterile water was added to make 10^{-1} dilution. 100 μL *Salmonella* (10^2 cfu/mL, 10^3 cfu/mL and 10^4 cfu/mL respectively) was added to 900 μL pork sample. Then, the electrochemical detection was conducted to calculate the detectable amount of *Salmonella*.

3. Results and discussion

The electrochemical detection process of *Salmonella* is shown in Fig. 1. The GCE electrode was first modified with GO and GNPs to improve the electron transfer effect. Then, it was incubated with the *Salmonella*-specific recognition aptamer to construct the aptamer-based electrochemical biosensor. Finally, this composite structure was immersed in different dilutions of *Salmonella*. With more *Salmonella* in the detection system, the electron transfer between the electrode and electrolyte was more inhibited and resulted in a smaller current and a higher resistance. These values could be quantified by EIS measurement.

3.1. Salmonella plate counting

In our study, three dilutions (10^{-5}, 10^{-6}, and 10^{-7}) of *Salmonella* were selected for the plate counting experiment. The numbers of colonies for two consecutive dilutions (10^{-5} and 10^{-6} dilutions) was in the appropriate range (30–300). The colonies were 235, 225, 223 for 10^{-5} dilution and 46, 33, 38 for 10^{-6} dilutions. Thus, the *Salmonella* was calculated using the following formula (Chinese national standard GB4789.2-94):

$$N = \Sigma C / (n_1 + 0.1 n_2) d$$

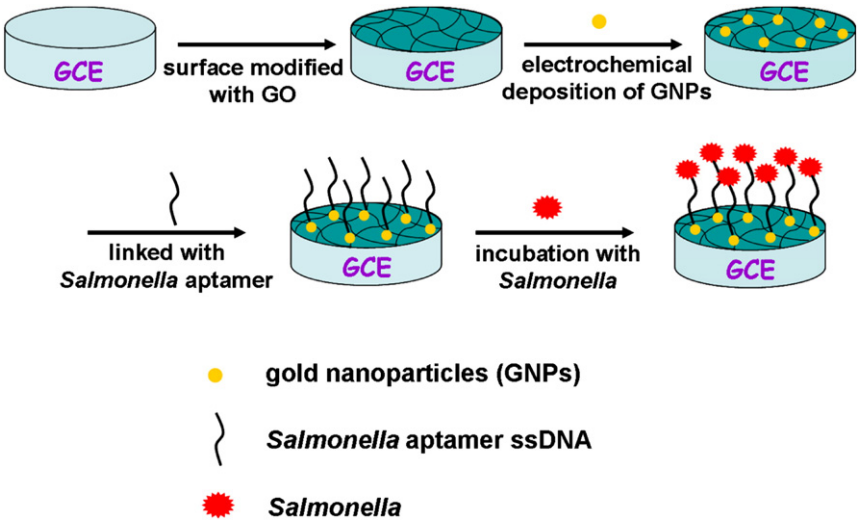

Fig. 1. Schematic illustration for the aptamer-based electrochemical detection of *Salmonella*.

ΣC — number of total colonies within the appropriate dilutions
n_1 — number of plates for the low dilution
n_2 — number of plates for the high dilution
d — low dilution

According to the above results, the concentration of the original *Salmonella* was calculated as 2.4×10^8 cfu/mL.

3.2. Surface modification and characterization of GCE

In a typical electrochemical experiment, the electrochemical parameters such as current, electric potential and resistance are usually measured to characterize different reaction steps that occur on the working electrode.

Fig. 2 depicts the CV curves measured to characterize the GCE surface modification steps. Impurities and other contaminations could be adsorbed easily on the surface of the working electrode to form a layer of oxide film. The existence of oxygen-containing groups would directly influence the efficacy of the electrode, influencing reproducibility, stability, sensitivity and selectivity. Thus, the GCE was first polished with Al_2O_3 powder to remove contaminants. The redox peak is depicted in curve a. After modification with GO, the CV curve redox peak exhibited a notable decrease (curve b) because GO occupied the active sites of the GCE, which affected the diffusion of $[Fe(CN)_6]^{3-/4-}$ to the electrode surface, resulting in the increased resistance. Therefore, the redox current decreased visibly. Then, the GCE electrode was immersed in the chloroauric acid solution, and GNPs were electrodeposited on the surface of the GO-modified GCE electrode using an electrochemical workstation. GNPs have large surface areas, and their surface free energies are high. Thus, they can cause a signal amplification effect. Moreover, because of their good biocompatibility, they can be used in the detection of biological molecules. The CV properties of the electrode after modification with GNPs were characterized in curve c. We can see that the redox peak increased significantly. Subsequently, when the aptamer was fixed on the electrode, a sharp decline in peak current occurred in curve d. This phenomenon could be ascribed to the electrostatic repulsion between the negatively charged aptamer sequence and $[Fe(CN)_6]^{3-/4-}$. The results obtained from the changes to the CV curves in Fig. 2 indicated that the GO, GNPs and *Salmonella* aptamer had been successfully attached to the GCE electrode.

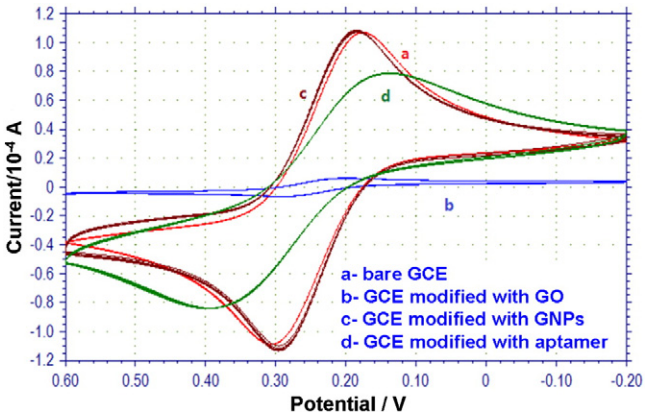

Fig. 2. Cyclic voltammetry curves for different modification steps of GCE.

3.3. Optimization of incubation time for Salmonella detection

We investigated the optimal incubation time for the electrochemical detection of *Salmonella*. The modified GCE electrode was incubated with a 10^{-6} dilution (2.4×10^2 cfu/mL) of *Salmonella*. EIS measurement was conducted at different incubation times. The resistance could be obtained from the EIS diagram through the electrochemical workstation's software. Fig. 3 showed the calibration curve between the resistance and incubation time. It could be observed that, when the time increased from 0 min to 35 min, the resistance also increased as a result of the strengthened interaction between the aptamer and *Salmonella*. After

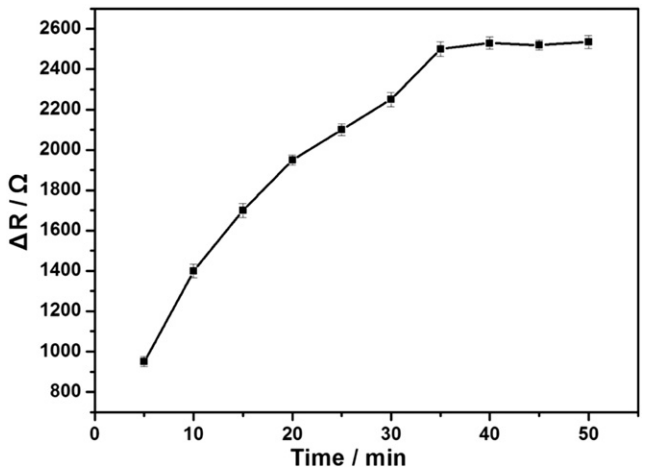

Fig. 3. Calibration curve between the resistance (obtained from EIS measurement) and incubation time.

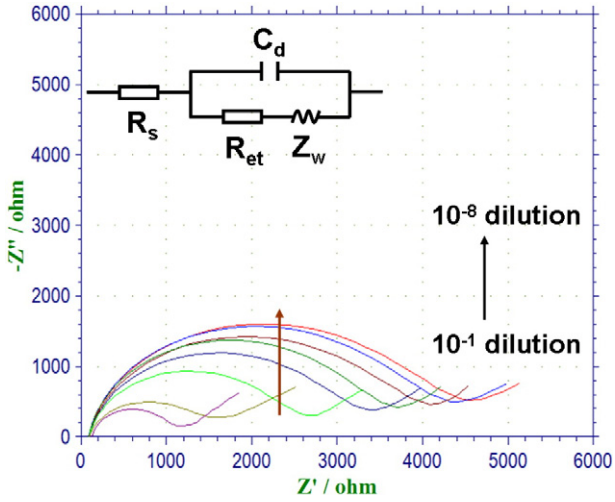

Fig. 4. EIS characterization for different dilutions of *Salmonella* (inset: the equivalent circuit).

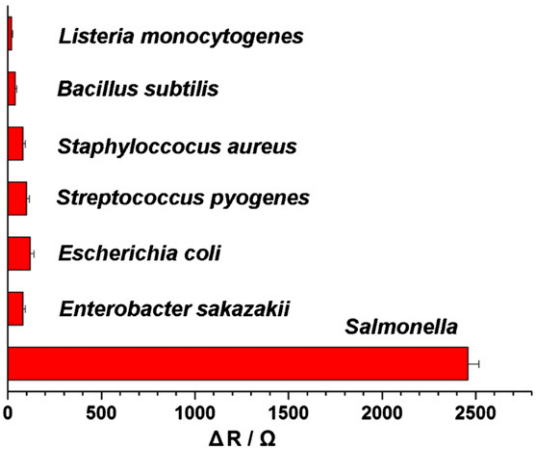

Fig. 6. The electrochemical results of EIS measurement for six different strains of bacteria and *Salmonella*.

35 min, the resistance remained almost the same. Therefore, the optimal incubation time is 35 min. If the time is too short, the *Salmonella* will not be fully combined with the aptamer, affecting the results.

3.4. Electrochemical detection of Salmonella

Different dilutions of *Salmonella* (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} with corresponding concentrations of 2.4×10^7 cfu/mL, 2.4×10^6 cfu/mL, 2.4×10^5 cfu/mL, 2.4×10^4 cfu/mL, 2.4×10^3 cfu/mL, 2.4×10^2 cfu/mL, 24 cfu/mL, and 2.4 cfu/mL, respectively) were incubated with the surface-modified GCE electrode for 35 min. The EIS characterization is shown in Fig. 4, and the inset shows the equivalent circuit. When the amount of *Salmonella* added to the detection system increased, the radius of the semicircle also increased. During the reaction process, *Salmonella* were attached to the surface of the GCE electrode via the specific recognition of the aptamer. With more *Salmonella* in the reaction system, more became attached to the electrode, and the electron transfer between the $[Fe(CN)_6]^{3-/4-}$ electrolyte solution and the electrode was greatly inhibited. Therefore, the resistance increased accordingly.

From the EIS test, the resistance value ΔR (the resistance difference between the working electrode and the bare electrode, the working electrode is the modified GCE cultivated with *Salmonella*, and the bare electrode is only the modified GCE without *Salmonella*) could be calculated using the electrochemical workstation. The calibration curve between the value of ΔR and the concentration of *Salmonella* is shown in Fig. 5. From the data, we can see that when the concentration changed from 2.4 cfu/mL to 2.4×10^3 cfu/mL, a good linear relationship could be obtained. The equation of this relationship is $y = 1041 + 706x$, $R^2 = 0.9975$. The detection limit is 3 cfu/mL.

To investigate the specificity of the *Salmonella* aptamer sensor, six different strains of bacteria (*L. monocytogenes*, *B. subtilis*, *S. aureus*, *S. pyogenes*, *E. coli*, *E. sakazakii*) were selected for the comparison experiment. A 10^{-6} dilution of each bacterium was incubated with the surface-modified GCE electrode for 35 min. Then, EIS detection was conducted. After calculating the resistance value, the corresponding ΔR was determined, as shown in Fig. 6. It can be seen clearly that the ΔR values of the six strains of bacteria are apparently much lower than the value obtained for the *Salmonella*. These comparative experiments showed that the experimental design of the *Salmonella* aptamer sensor had good specificity and selectivity toward *Salmonella* detection.

The accuracy of *Salmonella* detection in agricultural commodities was also evaluated by determining the recovery of *Salmonella* by a standard addition method, into which a known quantity of *Salmonella* was added. As shown in Table 1, the recoveries were between 97.3% and 105%, indicating good accuracy of the proposed aptamer-based electrochemical bioassay for *Salmonella* detection.

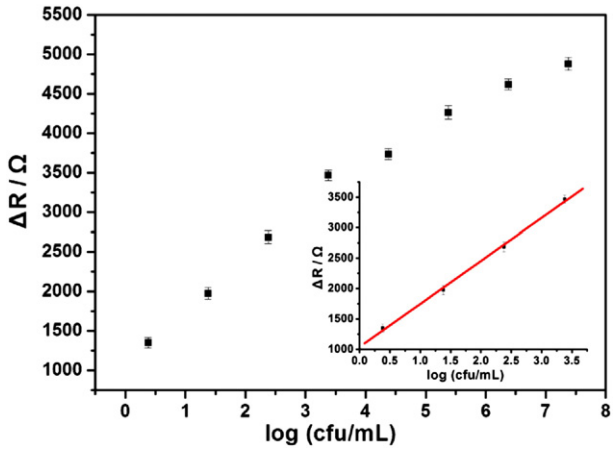

Fig. 5. Calibration curve between the resistance and the concentration of *Salmonella*.

4. Conclusion

In this study, a GCE electrode was modified with GO and electrodeposited with GNPs. The thiolated *Salmonella* aptamer ssDNA sequence could be linked to the electrode via the effect between thiol groups and GNPs. An aptamer-based electrochemical biosensor was successfully fabricated for the detection of *Salmonella*. Measurement was conducted in a three-electrode system. When more *Salmonella* were incubated in the detection system, the current generated between the electrode and the electrolyte decreased and resulted in higher resistance. Due to the high specificity and selectivity of the aptamer, this constructed sensor could achieve a good linear relationship between 2.4 cfu/mL

Table 1
Recovery results for the added *Salmonella* from pork samples obtained by the developed method.

Sample	Added concentration (cfu/mL)	Detected concentration (cfu/mL)	Recovery ratio (%)
No. 1	10	10.5	105
No. 2	100	97.3	97.3
No. 3	1000	986.5	98.7

and 2.4×10^3 cfu/mL ($y = 1041 + 706x$, $R^2 = 0.9975$), and a high efficiency with a detection limit of 3 cfu/mL was obtained.

Acknowledgments

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