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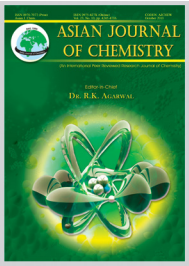

Detection of *Salmonella typhimurium* (LT2) invA Gene Using PNA Probe Biosensor by Electrochemical Impedance

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A peptide nucleic acid sequence with thiol groups at 5-end was covalently linked onto the Au electrode surface *via* S-Au binding for identifying the invasion A gene (invA) of *Salmonella typhimurium* (LT2). The hybridization event was monitored by using electrochemical impedance spectroscopy. The combination of electrochemical impedance spectroscopy technique and peptide nucleic acid probe achieved the ideal sensitivity and selectivity for detection of target DNA and the process of the probe immobilization was simplified. The electrochemical impedance response was linear with the logarithm of invA gene concentrations in the range from 1.0×10^{-13} mol L^{-1} to 1.0×10^{-10} mol L^{-1} with the detection limit of 3.3×10^{-14} mol L^{-1}. Compared with DNA probe, peptide nucleic acid probe provided the sensor with wider linear range and more effective distinguish in mismatch DNAs. The sensor was successfully applied to the detection of invA gene extracted from *S. typhimurium* (LT2) sample.

Key Words: Electrochemical biosensor, Electrochemical impedance spectroscopy, Peptide nucleic acid, *S. typhimurium* (LT2), InvA gene.

INTRODUCTION

Electrochemical biosensor is a promising platform to detect special nucleic acid sequences in humans, viruses and bacteria because of its high sensitivity, selectivity, low cost, small dimensions and fast response. In general, electrochemical biosensors can be divided into two groups, label-free biosensors and labeled biosensors, the latter using organic indicators or metal complexes as redox label to generate electrochemical signals. But there are some challenges for labeled biosensors due to its time-consuming, expensive cost and the redox label would be removed by time extension. Therefore, label-free biosensors attract increasing attentions and show a strong vitality. Electrochemical impedance spectroscopy (EIS) is a convenient and effective electrochemical technique, which has been applied in DNA detection widely. Impedance label-free DNA biosensor was first reported by Lee *et al.*[1]. Wei Zhang and his co-workers used novel nanocomposite membranes modified electrodes to detect DNA hybridization directly by electrochemical impedance spectroscopy[2,3]. Lien *et al.*[4] have fabricated a MWCNTs-polypyrrole sensor to detect CaMV 35S by electrochemical impedance spectroscopy and QCM. Zhang *et al.*[5] have reported gold nanoparticles based sensor by detecting impedance change upon DNA hybridization.

The ability of probe to recognize the specificity target DNA is an essential factor to a sensor. A peptide nucleic acid (PNA) is an analog of DNA with neutral-charged peptide-like backbone consisting of repeated *N*-(2- aminoethyl) glycine units linked by amide bonds and the four natural nucleobases are attached to the central amine of the backbone through methylene carbonyl linkages[6]. Owing to its non-ionic backbone and proper interbase spacing, peptide nucleic acid can form stable duplex with complementary DNA or RNA by high hybridization efficiency and greater specificity. Furthermore, compared to traditional oligonucleotides duplex, peptide nucleic acid/DNA or peptide nucleic acid/RNA possesses remarkable hybridization properties such as low background signal, improved chemical, thermal and enzymatic stability, faster hybridization and tighter binding, especially at low ionic strength[7]. Pournaghi-Azar *et al.*[6] have reported used different indicators to detect DNA by immobilized peptide nucleic acid probe. Luo *et al.*[8] have fabricated a DNA sensor with ferrocene-labeled peptide nucleic acid. Reisberg and co-workers investigated the charge effect on the electrochemical transduction process by peptide nucleic acid and presented a DNA sensor according to the previous result[9].

Salmonella is a universal pathogen responsible for food-borne infections of human gastrointestinal disease, mostly

caused by *Salmonella typhimurium* and *Salmonella enteritdis*[10]. It's very important for food and environment security monitoring, disease diagnosis and pathogen identification to detect *Salmonella* efficiently and rapidly. It has been affirmed that the genetic locus inv allows *Salmonella* to invade the intestinal epithelium which is a vital step in *Salmonella* infections, so the invasion A (invA) gene (the first gene of an operon consisting of at least two additional invasion genes) is considered as a probe in *Salmonella* detection[11]. So far conventional methods for detecting *Salmonella* mainly include DNA hybridization reaction, fluorenscent antibodies, enzyme linked immunosorbent assay (ELISA)[12], polymerase chain reaction (PCR)[13] *etc*. However, the low sensitivity, specificity or the high experiment requirement limits these approaches for routine application. Hence, finding out a new way to detect *Salmonella* is necessary.

In this work, we fabricated a peptide nucleic acid probe biosensor based on electrochemical impedance spectroscopy technique to detect invA sequence of *Salmonella typhimurium* (LT2) directly. The process of probe immobilization was comparatively simple and the hybridization reaction of the peptide nucleic acid probe with complementary DNA was high sensitive and selective. The difference of electron transfer resistance (ΔR_{et}) between peptide nucleic acid/Au electrode and hybrid DNA/peptide nucleic acid/Au electrode was found to be linear with the logarithm of the complementary oligonucleotides concentrations in the range of 1.0×10^{-10} mol L^{-1} - 1.0×10^{-13} mol L^{-1}, with a detection limit of 3.3×10^{-14} mol L^{-1}. We also compared peptide nucleic acid probe and ssDNA probe with their hybridization properties and found that peptide nucleic acid was able to discriminate between complementary DNA and mismatch DNAs remarkably. The biosensor was applied to the determination of invA sequence in *Salmonella typhimurium* samples and the results were satisfactory.

EXPERIMENTAL

Electrochemical impedance spectroscopy and cyclic voltammetry measurements were performed by a CHI660C electrochemical analyzer (Shanghai Chenhua, China). A three-electrode system contained the gold electrode as working electrode, a Pt counter electrode and a Ag/AgCl reference electrode. All the potentials given in this paper were referred to Ag/AgCl.

Salmonella Typhimurium (LT2) was provided from Genomics research center of Harbin Medical University, China. The restriction enzyme Hpall was purchased from Fermentas (USA) and the TIANamp bacteria DNA kit was obtained from Tiangen Biotech Co., (Beijing, China). All the chemicals were analytical grade. All aqueous solutions were prepared using Milli-Q water.

The specific sequence of peptide nucleic acid probe (5' SH-(CH_6)-GATGAGTATTGATGCCGA 3') was synthesized by PANAGENE (Korea). ssDNA probe (5' SH-(CH_6)-GATGAGTATTGATGCCGA 3'), complementary target DNA (5' TCGGCATCAATACTCATC 3'), single-base-mismatch DNA (5' TCGGCAACAATACTCATC 3') and three-base-mismatch DNA (5' TCGGCTAAAATACTCATC 3') were all purchased from Shanghai Sangon Biotech Co., (Shanghai, China). All the oligonucleotides were stored in 4 ºC with TE.

The Au electrode was purified with piranha solution [a mixture solution with 3:7 (v/v) of 30 % hydrogen peroxide and concentrated sulfuric acid] for 10 min, followed by rising with doubly distilled water. Then the surface was polished till mirror-like with 1 μm α-Al_2O_3. After that, the electrode was ultrasonically washed with ethanol and doubly distilled water for 3 min, respectively. Then a 10 μL peptide nucleic acid or ssDNA (1 μmol L^{-1}) was dropped onto the clean bare Au electrode surface and stored in 4 ºC for 12 h. After washing thoroughly with TE, the electrode was immersed into 2 × SSC buffer at 60 ºC for 45 min with hybridization shaking and washed successively with 2 × SSC-0.1 % SDS and 0.2 × SSC-0.1 % SDS. Then, it was immersed in the boiling water for 5 min and transferred into ice-water for 180 s at once for reuse. After 5 times repeatedly reusements, the measurement signals were 94 % of original signals. In this work, all the electrochemical impedance spectroscopy measurements were recorded in 5 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) solution containing 0.01 mol L^{-1} KCl. The AC voltage amplitude was 5 mV and the voltage frequencies ranged from 1 to 104 Hz. The applied potential was 110 mV *vs*. Ag/AgCl. The difference of electron transfer resistance was calculated as $\Delta R_{et} = R_{et\ hybrid\ electrode} - R_{et\ immobilized\ electrode}$.

The enriched *S. typhimurium* (LT2) was extracted with TIANamp bacteria DNA kit and the extracted genomic DNA was digested by restriction enzyme Hpall. After digestion, the mixture was denatured in boiling water for 10 min and chilled in ice-water immediately to obtain single-stranded DNA. The single-stranded DNA was diluted 10 times in order to hybridize with peptide nucleic acid.

RESULTS AND DISCUSSION

Fig. 1 shows the Nyquist diagrams of 5 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ at (a) bare Au electrode, (b) peptide nucleic acid/Au electrode and (c) hybrid DNA/peptide nucleic acid/Au electrode, respectively. Compared to bare Au electrode (curve a), the electron transfer resistance (R_{et}) of curve b was much larger. The change of the R_{et} indicated that probe peptide nucleic acid was immobilized onto the Au electrode surface *via* S-Au connection and the peptide nucleic acid layer prevented the $[Fe(CN)_6]^{3-/4-}$ anions from reaching the electrode surface. After peptide nucleic acid and complementary DNA hybridization (curve c), the negative charged phosphate backbone of DNA repelled the $[Fe(CN)_6]^{3-/4-}$ anions, resulting in the R_{et} increased greatly to 1.223×10^3 Ω in contrast to curve b. Obviously, the characterization of electrochemical impedance spectroscopy confirmed that the peptide nucleic acid/Au electrode could successfully recognize target DNA.

Hybridization temperature and hybridization time were optimized by impedance measurement. Fig. 2 shows the peptide nucleic acid/Au electrode hybridized with 1.0×10^{-10} mol L^{-1} target DNA at 40, 50, 60 and 70 ºC for 45 min, respectively. It could be found that the difference of electron transfer resistance (ΔR_{et}) increase with hybridization temperature increased in the range from 40 to 60 ºC. When hybridization temperature was higher than 60 ºC, the ΔR_{et} decreased with the further increase of hybridization temperature. The reason might be that the repulsive interaction increased between the

negatively charged DNA backbone and $[Fe(CN)_6]^{3-/4-}$ anions when DNA/peptide nucleic acid duplex was formed on the electrode. Therefore, 60 ºC was chosen as optimum hybridization temperature.

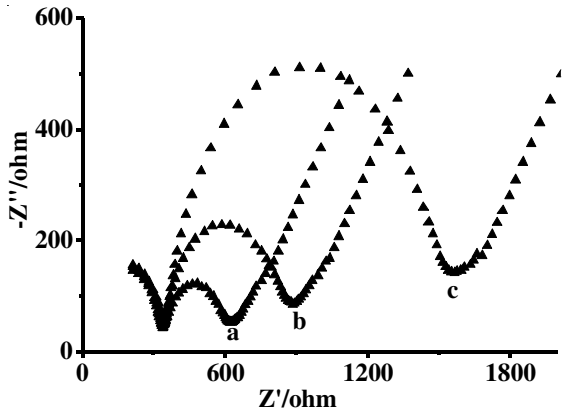

Fig. 1. Nyquist diagrams recorded at (a) bare Au electrode, (b) probe peptide nucleic acid/Au electrode, (c) hybrid DNA/peptide nucleic acid/Au electrode. DNA concentration: 1.0×10^{-10} mol L^{-1}

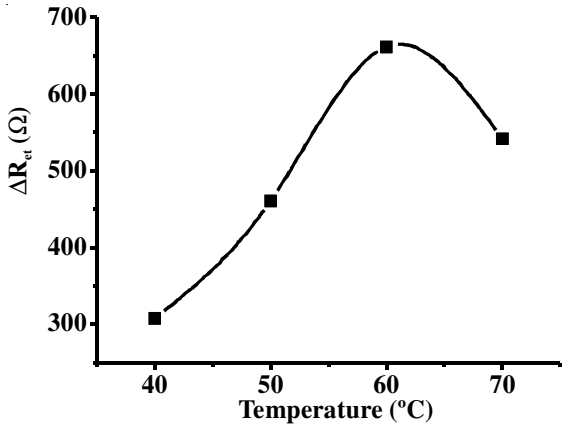

Fig. 2. Effect of the hybridization temperature on measurement signals

Hybridization time is another factor affecting the measurement signal. The ΔR_{et} increased with the hybridization time increased up to 45 min (Fig. 3), however, at 60 min ΔR_{et} decreased little compared to 45 min. This indicated that the hybridization reaction on the electrode surface was completed. Considering saving experiment time, 45 min was more suitable for hybridization reaction.

Fig. 4(A) presents the Nyquist diagrams of the probe peptide nucleic acid/Au electrode before and after hybridization reaction with different concentrations of target DNA. It could be easily observed that the the semicircle part of the impedance spectra increased with the concentration of target DNA increasing. As Fig. 4(B) shown, the calibration curve illuminated that the ΔR_{et} was linear with the logarithm of the complementary DNA concentrations in the range from 1.0×10^{-13} mol L^{-1} to 1.0×10^{-10} mol L^{-1}. The regression equation was $\Delta R_{et} = 95.26 + 141.51 \log C_{DNA}$ (unit of C was mol L^{-1}) with the regression coefficient of 0.9923. The detection limit was 3.3×10^{-14} mol L^{-1}. These results demonstrated that the sensitivity of the present peptide nucleic acid biosensor was good enough to recognize the complementary invA gene.

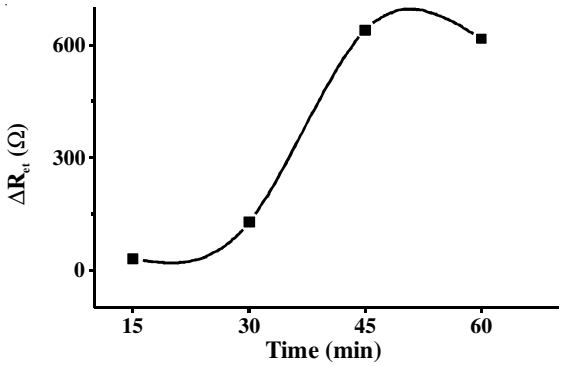

Fig. 3. Effect of the hybridization time on measurement signals

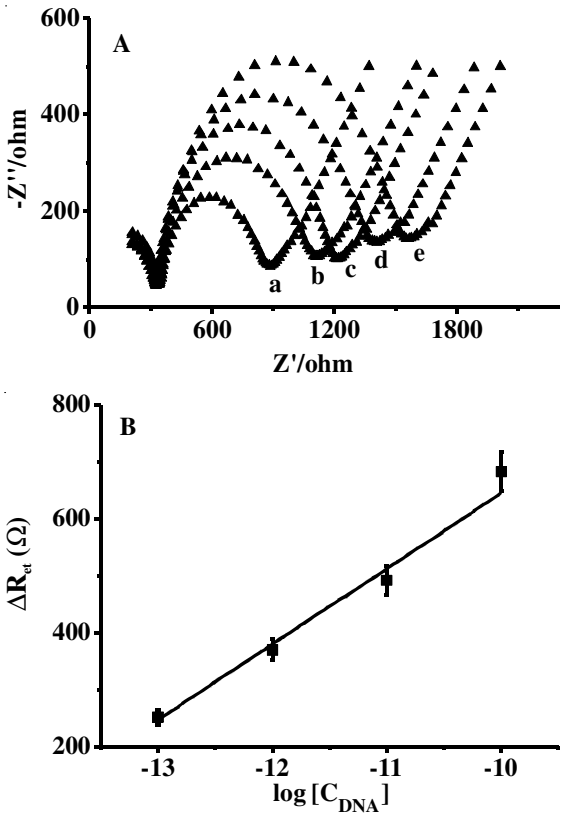

Fig. 4. (A) Nyquist diagrams recorded (a) at the probe peptide nucleic acid/Au electrode andafter hybridization reaction with different concentrations of complementary invA gene: (b) 1×10^{-13} mol L^{-1} (c) 1×10^{-12} mol L^{-1} (d) 1×10^{-11} mol L^{-1} (e) 1×10^{-10} mol L^{-1}. (B) Calibration graph for the logarithm of invA gene concentrations, ranging from 1×10^{-13} mol L^{-1} to 1×10^{-10} mol L^{-1}

The selectivity of the peptide nucleic acid biosensor was investigated with single-base-mismatch, three-base-mismatch and complementary DNA, respectively.

Fig. 5 showed, a pronounced increase in electron transfer resistance (R_{et}) after the peptide nucleic acid probe. It was hybridized with its complementary DNA (curve d) and a slight increase was also observed after the peptide nucleic acid probe when hybridized with single-base-mismatch DNA (curve c). However, the increase of R_{et} was negligible after the probe was hybridized with three-base-mismatch DNA (curve b). It suggested that the hybridization reaction was not achieved. Thus, the sensor could discriminate between complementary DNA and mismatch DNAs *via* comparing the increase of the R_{et} with high selectivity.

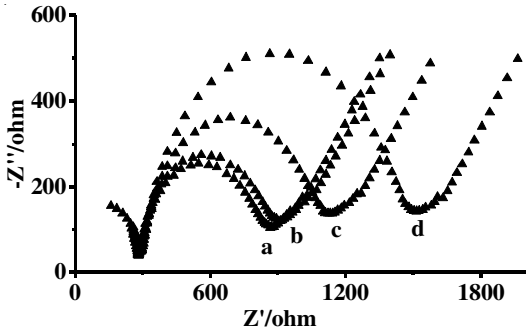

Fig. 5. Nyquist diagrams recorded at (a) peptide nucleic acid/Au electrode, the electrode hybridized with (b) three-base-mismatch DNA, (c) single-base-mismatch DNA and (d) complementry DNA, respectively. All DNAs concentration: 1×10^{-10} mol L^{-1}

We also compared hybridization properties between probe peptide nucleic acid and probe ssDNA. Probe ssDNA hybridized with target DNA at 50 ºC for 0.5 h in this part. Table-1 showed the contrast to ssDNA, probe peptide nucleic acid provided the sensor with wider linear range and more remarkable change of ΔR_{et} for mismatch DNAs. These results suggested the outstanding hybridization properties of probe peptide nucleic acid such as higher affinity and specificity offered the sensor with better performance.

TABLE-1
HYBRIDIZATION PROPERTIES BETWEEN PROBE PNA AND PROBE ssDNA

Probe	Linear range (mol L^{-1})	ΔR_{et} decrease (%) single-base-mismatch DNA	ΔR_{et} decrease (%) three-base-mismatch DNA
PNA	1.0×10^{-13} – 1.0×10^{-10}	72.98*	81.63*
ssDNA	1.0×10^{-12} – 1.0×10^{-10}	43.47*	52.81*

*Correspond to the ΔR_{et} value of complementry target DNA

Fig. 6 shows the impedance response of peptide nucleic acid/Au electrode hybridized with different percentage of invA gene DNA extracted from *S. typhimurium* (LT2) and digested by restriction enzyme Hpall. The R_{et} increased gradually with the amount of invA gene in the hybridization solution. It indicated that the biosensor could recognize target DNA extracted from real samples effectively.

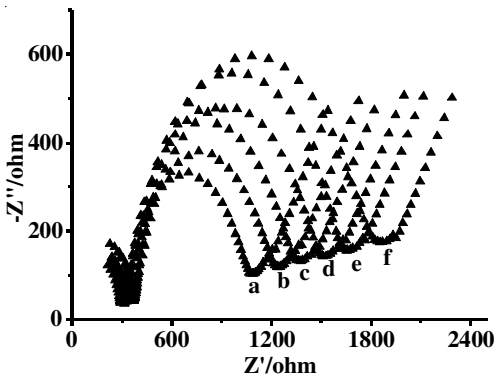

Fig. 6. Nyquist diagrams recorded at (a) peptide nucleic acid/Au electrode and after hybridization reaction with different percentage of invA gene : (b) 20 %, (c) 40 %, (d) 60 %, (e) 80 %, (f) 100 %

Reusability of the peptide nucleic acid/Au electrode was performed by immersed the electrode in the boiling water for 5 min followed by immersion in an ice-water bath for 180 s to make the peptide nucleic acid/DNA duplex denature. And the denatured biosensor could be used for 5 times repeatedly without the losen of ΔR_{et} value compared with the original response. In a typical mesaurement, the ΔR_{et} was 6.45×10^2 Ω in the first measurement, while, the response of the sensor after using 5 times was 6.03×10^2 Ω.

Conclusion

In this study, we immobilized peptide nucleic acid on the Au electrode to detect invA gene of *S. Typhimurium* (LT2) using electrochemical impedance spectroscopy technique directly. The experimental results demonstrated that the peptide nucleic acid biosensor could recognize invA gene with higher sensitivity, lower detection limit, wider linear range and could discriminate between complementary DNA and mismatch DNAs with higher selectivity contrast to ssDNA probe. In a word, the biosensor provided a simple, label-free method to detect *S. Typhimurium* (LT2) and it would be applied foreground for real sample detection.

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