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A sensitive and label-free impedimetric biosensor based on an adjunct probe

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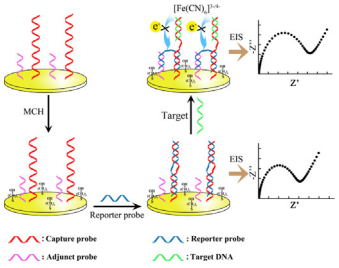
HIGHLIGHTS

- A label-free impedimetric sensor is constructed for DNA sequences detection.
- The key point is adjunct probes immobilized nearby capture probes.
- The biosensor achieves simple and highly selective detection for DNA sequence.
- The sensor is readily extended for other biomolecules, proteins and mRNAs detection.

GRAPHICAL ABSTRACT

Using an adjunct probe for highly sensitive and label-free detection of DNA sequence based on electrochemical impedance spectroscopy was achieved.



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ABSTRACT

A highly sensitive and label-free impedimetric biosensor is achieved based on an adjunct probe attached nearby the capture probe. In this work, the adjunct probe was co-assembled on the surface of gold electrode with the capture probe hybridized with the reporter probe, and then 6-mercapto-1-hexanol was employed to block the nonspecific binding sites. When target DNA was added, the adjunct probe functioned as a fixer to immobilize the element of reporter probe displaced by the target DNA sequences and made the reporter probe approach the electrode surface, leading to effective inhibition of charge transfer. The increase in charge transfer resistance is related to the quantity of the target DNA in a wide range. The linear range for target DNA with specific sequences was from 0.1 nM to 0.5 μM with a good linearity ($R = 0.9988$) and a low detection limit of 6.3 pM. This impedimetric biosensor has the advantages of simplicity, sensitivity, good selectivity, and large dynamic range.

1. Introduction

As the completion of the human genome project, a large number of sequence data have been generated. Human genome analysis is becoming more and more important in the diagnosis of hereditary diseases, detection of infectious agents, and forensic and paternity testing due to the important functions of DNA in gene expression profiling, and the frequent occurrence of some tumors and pathogens diseases. Consequently, the development of low-cost and efficient biosensors capable of highly sensitive detection of human genes has gained much attention worldwide [1–3]. The biosensor technology is mainly used for determining specific DNA sequences for various applications in drug studies, environmental monitoring, forensics, and food safety. And great efforts have been put into developing a variety of such biosensors, including fluorescence [4–6], surface plasmon resonance [7–9], quartz microbalance [10–13], atomic force microscopy [14–16], and electrochemical techniques [17–19]. Among them, electrochemical biosensors have well development potentials owing to their attractive features such as low cost, simplicity, fast response,

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high sensitivity, and ease of miniaturization. Yang and Zhang [20] reported a sensitive electrochemical biosensor with an adjunct probe. They used a redox-labeled (methyl blue, MB) probe and square wave voltammetry (SWV) to detect MB signal, and the detected limit for target DNA can be as low as 2.0 pM. In addition, electrochemical impedance spectroscopy (EIS) attracts more and more attention because of its high sensitivity. It is a technique that can transduce changes in interfacial properties between the electrode and the electrolyte induced by DNA hybridization, conformational changes, or DNA damages to an electrical signal. It is very effective for the characterization of bio-functionalized electrodes and the detection of DNA hybridization. EIS has been the most frequently used methodology in the design of electrochemical sensors [21,22]. Compared with other redox-labelled electrochemical methods such as cyclic voltammetry (CV), differential pulse voltammetry (DPV), and SWV, EIS provides unique advantages, such as high sensitivity, signal quantification ease, less destructive effect on the measured biological interactions and ability to separate the surface binding events from the solution impedance. Impedance data are recorded in the range of frequencies, using alternating current of small amplitude. In addition, EIS is also used to characterize molecular interactions on the electrode surface due to less destruction to the measured biological interactions. Electrochemically inert species can be conveniently measured by EIS when the measurement is performed in the presence of a redox probe, such as ferricyanide, which undergoes oxidation and reduction at the surface of the electrode at a certain potential applied during the measurement. The surface availability for redox reaction decreases with the increased binding of the analyte [23]. Therefore, the EIS technique is a powerfully label-free tool for probing molecular binding events such as DNA hybridizations [24–27].

However, one of the challenges in EIS-based detections is to amplify the signal output. Common methods for amplified EIS detection of a trace quantity of targets require modifications of either the target or the probe molecules with nanomaterial labels [28–30], which sacrifice the inherent advantages of this technique such as simplicity and label-free capability. Very recently, target recycling based on the DNA enzyme has become an interesting alternative for sensitive detection of DNA and is used to amplify EIS signal [31–33]. Despite the great progress made by these target recycling DNA sensors, some limits (e.g. recognition units and specific ions) still exist. For example, the restriction endonucleases require target sequences to contain the recognition units, while exonuclease III requires 3′-hydroxyl termini of double-stranded DNA. Furthermore, enzymes can be attached to catalyze the formation of an insoluble product, which precipitates on the sensing surface, further increasing charge transfer resistance (R_{ct}) [34]. Besides, "sandwich-type" structural design is used to amplify signal [35–39]. However, these additional amplification steps increase the complexity, cost of the detection system, and frequent interference from the detection environment.

In this work, we proposed a label-free impedimetric biosensor for detection of DNA sequence and used an adjunct probe to amplify EIS signal. An adjunct probe, thiol-modified DNA sequence with 14 bases, functioned as a fixer to immobilize the dissociative element of reporter probe to form loop structure. The adjunct probe made the reporter probe collide with the electrode surface, which effectively blocked the charge transfer and amplified EIS signal. The increment of R_{ct} in the presence of the adjunct probe was more than ten times that of R_{ct} without adjunct probe. Based on these results, a simple, low-cost, and label-free biosensor for detection of DNA sequence was constructed. Our method achieved detection for the target without using any redox-label, and the detected concentration of target can be as low as 6.3 pM by the EIS technique and the adjunct probe. This biosensor has remarkable advantages of simple fabrication process, sensitivity, large dynamic range, label-free detection, and less interference from the detection environment.

2. Experimental

2.1. Chemicals and reagents

6-Mercapto-1-hexanol (MCH) and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used as received without further purification. The buffer solutions were as follows: phosphate buffer solution (PBS) (pH 7.4, 0.1 M) prepared with 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4; the DNA immobilization buffer containing 20 mM Tris–HCl, 0.5 M NaCl, and 10 mM $MgCl_2$ (pH 7.4, 25 °C); the hybridization buffer containing 20 mM Tris–HCl, 0.5 M NaCl, and 10 mM $MgCl_2$ (pH 7.4, 25 °C) and 20 mM Tris–HCl, 1.0 M NaCl, 10 mM $MgCl_2$ (pH 7.4, 25 °C). Ferricyanide solution ($[Fe(CN)_6]^{3-/4-}$, 5.0 mM, 1:1) was obtained by dissolving potassium ferricyanide and potassium ferrocyanide with PBS (0.1 M, pH 7.4) and used as the supporting electrolyte medium. All the reagents mentioned above are of analytical reagent grade and were used without further purification. All solutions were prepared using ultrapure water (specific resistance of 18.2 MΩ cm). All the oligonucleotides probes and target DNA (Table 1) used in the experiments were obtained from Shanghai Biochemical Co. (China).

2.2. Apparatus

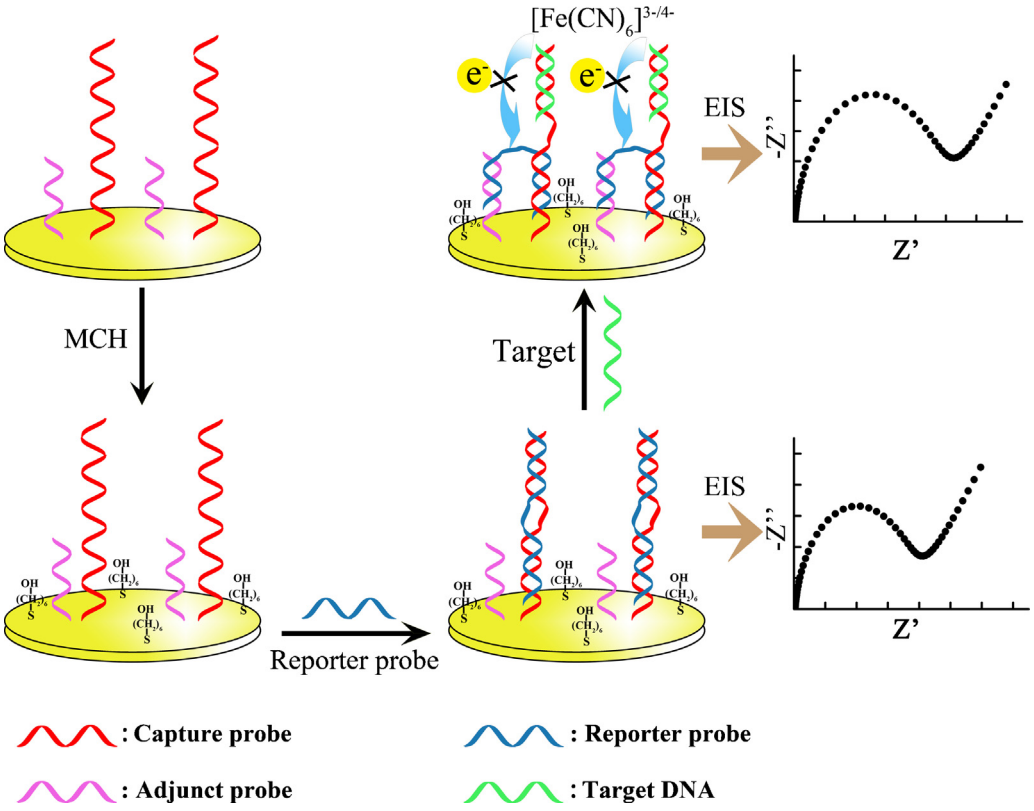
The EIS measurement was monitored on the electrochemical workstation (CHI 660D, CH Instruments, Chenhua Corp, Shanghai, China). The experiment was performed with a typical three-electrode system, in which the functionalized gold electrode (2.0 mm diameter, CHI Co. Ltd., Shanghai, China) was the working electrode, a platinum electrode was the counter electrode, and an Ag/AgCl electrode with saturated KCl was the reference electrode. The measured EIS spectra were analyzed with the help of equivalent circuit using ZSimpWin 3.10 (Princeton Applied Research), and the data were presented in Nyquist plots.

2.3. Fabrication of the biosensor

The process for the fabrication of the biosensor is shown in Scheme 1. According to the reported protocol [40], the biosensor was fabricated by the modification of polycrystalline gold electrode with the DNA probes. Briefly, the gold electrode was first polished carefully on microcloth with alumina suspensions (0.3 and 0.05 μm in diameter) in sequence, and then sonicated sequentially in ultrapure water, ethanol, and ultrapure water for 3 min to remove residual alumina powder. The above electrode was further electrochemically cleaned by a redox cycling in 0.5 M H_2SO_4 with the potential between −0.2 and +1.5 V (vs. Ag/AgCl) at 0.1 V s^{-1} until a representative cyclic voltammogram of a clean gold electrode was obtained, and finally rinsed with ultrapure water. After drying with nitrogen, the electrode was incubated in 30 μL of buffer (20 mM Tris–HCl, 0.5 M NaCl, 10 mM $MgCl_2$, pH 7.4) containing 0.5 μM capture probes and 0.08 μM adjunct probes at room temperature for 16 h. Subsequently, the self-assembled electrode was immersed in 30 μL of 2 mM MCH for 1 h. Next, 30 μL of 0.5 μM reporter probes was added and incubated with the electrode in the buffer (20 mM Tris–HCl, 0.5 M NaCl, 10 mM $MgCl_2$, pH 7.4) at 25 °C for 4 h. Then, the capture/adjunct/reporter probe-modified electrode was treated with various concentrations of the target DNA (30 μL in volume) in the buffer (20 mM Tris–HCl, 1 M NaCl, 10 mM $MgCl_2$, pH 7.4) at 25 °C for 4 h. After each step, the modified electrode was thoroughly cleaned with ultrapure water. In order to make the system to reach stable state and obtain an accurate value

Table 1
Synthesized oligonucleotides probes and target DNA used in the experiments.

Name	Sequence
Capture probe	5′-SH-$(CH_2)_6$-TGACAGGATCCAAAAGGACCCCCCTACGCCACCAGCTCCAA-3′
Reporter probe	5′-TGGAGCTGGTGGCCCCCTTTCCTTTTGGATCCTGTCA-3′
Adjunct probe	5′-CCAGCTCCATAAAA-$(CH_2)_3$-SH-3′
Target DNA	5′-TTGGAGCTGGTGGCGTA-3′
Mismatched DNA	5′-GTGGAGCTTGTGGCGTC-3′
Non-complement DNA	5′-GGCGTTTTATTCTTGTT-3′



Scheme 1. A schematic illustration of using an adjunct probe for sensitive and label-free electrochemical impedance spectroscopy detection of DNA sequence.

of R_{ct}, the modified electrode was left in $[Fe(CN)_6]^{3-/4-}$ for 6 min before each EIS measurement.

3. Results and discussion

3.1. Design strategy of adjunct probe-aided electrochemical impedance biosensor

As shown in Scheme 1, the thiol-terminated capture probe was immobilized on the surface of gold electrode via gold–sulfur bond. The biosensor was demonstrated by attaching a label-free reporter probe onto the capture probe. In the absence of the target DNA, the double-strand DNA (dsDNA) consisting of the capture probe and the reporter probe blocked the $[Fe(CN)_6]^{3-/4-}$ from the electrode surface, generating a relatively low electrochemical signal. With the addition of a 17-base complementary target DNA, the hybridization of target DNA with the capture probe displaced the 14-base element from the reporter probe, producing a relatively dissociative single-strand DNA (ssDNA). The flexible ssDNA possibly approaches the electrode surface, generating an increased EIS signal. Actually, the dissociative ssDNA difficultly bends to the electrode surface. This fact is basically attributed to two reasons. First, the dissociated reporter probe might part from the electrode surface with a relatively long distance, leading to incomplete contact between the reporter probe and the electrode. Second, the steric hindrance of capture probes prevents reporter probes from approaching the electrode. These two factors limit the enhancement of EIS signal.

To improve the sensitivity of this biosensor, we adopted the co-assembly of capture probe and adjunct probe on the gold electrode, followed by surface blocking with MCH and incubation of the reporter probe, which was used to form a dsDNA. Then the target DNA was added and hybridized with the top of capture probe, instead of 5′ end of reporter probe. Meanwhile, the displaced element of reporter probe bended to hybridize with the adjunct probe. The adjunct probe made the reporter probe collide with the electrode surface, and functioned as a fixer to immobilize the dissociative element of reporter probe, which effectively blocked the charge transfer. As EIS allows a label-free measurement of the binding event, the resulting electrode is subjected to the measurement by EIS technique. Consequently, a substantial increase in R_{ct} is monitored by EIS.

3.2. Effect of adjunct probe concentration

According to the reported protocol [41], middle concentration (~0.5 μM) of probe self-assembled on electrode could improve efficiency of hybridization. Thus, the concentration of capture probe

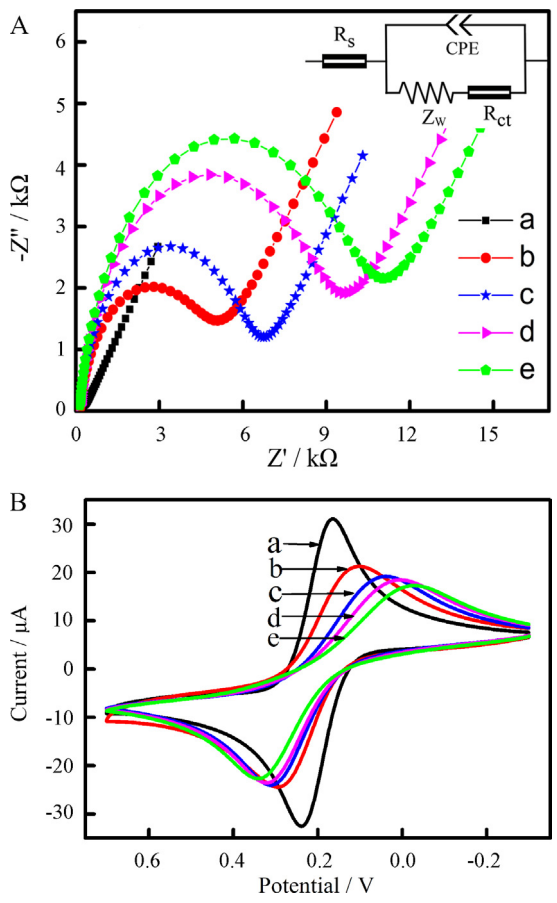


Fig. 1. (A) Nyquist diagrams (Inset: equivalent circuit used to fit the EIS data) and (B) cyclic voltammograms of the sensor at different stages: (a) bare gold electrode, (b) capture and adjunct probes self-assembled gold electrode, (c) MCH modified capture/adjunct/gold electrode, (d) reporter probe functioned capture/adjunct/MCH/gold electrode, and (e) target DNA hybridized capture/adjunct/MCH/reporter/gold electrode. EIS measurements were implemented with the frequency range from 10 kHz to 0.1 Hz and an alternate voltage of 5 mV. The cyclic voltammograms were recorded at a scan rate of 50 mV s^{-1}. All measurements were carried out in 0.1 M PBS (pH 7.4) containing 5.0 mM (1:1) $[Fe(CN)_6]^{3-/4-}$.

was fixed at 0.5 μM. In order to avoid excessive assembly concentration, we changed the concentration of adjunct probe from 0.02 to 0.1 μM, and ensured that the assembly concentration was less than 0.6 μM and the concentration of capture probe was higher than that of adjunct probe. As can be seen from Fig. S1 (see Supplementary Material), the decrease of electrochemical signal with the adjunct probe concentration beyond 0.08 μM might result from the following two factors: first, the high-concentration adjunct probes might weaken the density of the capture probes on the electrode surface due to the existence of competitive reactions between them with the electrode surface. Second, the high-concentration adjunct probes might increase the steric hindrance of the microenvironment, adversely preventing the dissociative reporter probes from colliding with the electrode surface. In this experiment, the optimum concentration of adjunct probe was 0.08 μM.

3.3. Characterization of the electrochemical impedance biosensor

The stepwise sensor fabrication process was characterized by EIS, and the resulting Nyquist plots are displayed in Fig. 1. The EIS data were fitted to a Randles equivalent circuit (inset in Fig. 1A), which includes the solution resistance (R_s), R_{ct}, the constant phase element (CPE), and Warburg impedance (Z_W). As reported before [39], R_s is the resistance between the reference electrode and the films of DNA on the gold electrodes. For each measurement, the position of the two electrodes was kept the same. All measurements were carried out in the identical solution (0.1 M PBS) and at room temperature to minimize variations in R_s. The CPE accounts for the behavior of the 6-mercapto-1-hexanol-diluted films on the electrode surface, and it acts as a nonlinear capacitor accounting for the inhomogeneity of the films on the electrode surface with the exponential modifier $n = 0.8$ [42]. Diffusion of the redox probe from the solution to the DNA films is not important in this system. The most important parameter is the charge transfer resistance, R_{ct}, which is the polarization resistance at an equilibrium potential, and is utilized as a main indicator in the faradaic EIS detection. A redox pair $[Fe(CN)_6]^{3-/4-}$ is used as a redox indicator for the electrode kinetics at the interface, which is modified by a substrate layer as well as probes and target DNA on the electrode surface. The R_{ct} values indicate how crowded the electrode surface is when it is modified by a functional molecule, which is capable of selectively capturing a given analyte. Thus R_{ct} is determined by the concentration of analyte in the test solution and selective binding between a functional molecule modified on the electrode surface and the analyte [43].

In the Nyquist diagram, the diameter of the semicircle reflects the R_{ct} of redox conversion of $[Fe(CN)_6]^{3-/4-}$ on the electrode at certain applied potential. This process is strongly dependent upon any modification on the electrode surface. As can be seen in Fig. 1A, R_{ct} increases significantly after self-assembly of a mixed monolayer of capture probes, adjunct probes, MCH, and reporter probes on the electrode surface. The impedance of the bare gold electrode is primarily controlled by diffusion of the redox probe. It is presented in curve a that a nearly straight line indicates a very fast charge transfer of the redox probe $[Fe(CN)_6]^{3-/4-}$. After self-assembly of the negatively charged capture probes and adjunct probes, an obvious increase in R_{ct} is observed in curve b, which can be attributed to the interfacial charge transfer from the electrode modified with the capture probes and adjunct probes to the electroactive $[Fe(CN)_6]^{3-/4-}$ in solution. The negatively charged phosphate backbone of the probes immobilized on the electrode surface repelled the negatively charged $[Fe(CN)_6]^{3-/4-}$ to access the reactive centers on the electrode. Then the electrode is passivated with MCH to remove nonspecific probes absorbed on the electrode surface, obtaining well-aligned DNA monolayers on the electrode surface. Thus, a dramatic increase in R_{ct} is observed (curve c), implying remarkable increase in the charge transfer resistance. After hybridization with reporter probes and the formation of double-strand DNA, a further enlarged R_{ct} was found (curve d). The reason is that more negative charges and the steric hindrance prevent $[Fe(CN)_6]^{3-/4-}$ from approaching to the surface of electrode and cause increase in R_{ct}. These results were consistent with the expectation that the electrode was fabricated. When the electrode was incubated with target DNA solution in hybridization buffer (20 mM Tris–HCl, 1.0 M NaCl, 10 mM $MgCl_2$, pH 7.4), the impedance increased apparently (curve e). Such an increase in R_{ct} is mainly due to two facts. First, the hybridization between dissociative reporter probes and adjunct probes leads to the increment of steric hindrance and blocks charge transfer. Second, more negative charges electrostatically repel the negatively charged redox probe. These two effects thus result in a substantial increase in R_{ct}. The modified electrode was further characterized with cyclic voltammograms (CVs). CVs of the sensor at different stages corresponding to EIS measurements are presented in Fig. 1B, and the results were in accordance with those of the impedance test. After self-assembly of capture probes, adjunct probes, MCH, reporter probes, and target DNA in sequence, the difference of peak position was increased gradually, whereas the peak height of current was decreased.

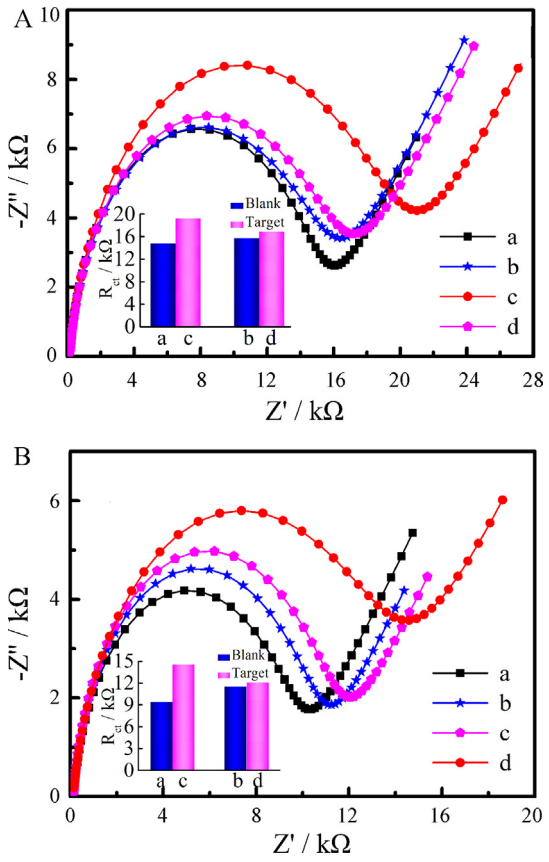


Fig. 2. Nyquist diagrams for sensors in the presence of 0.4 μM (A) and 0.5 μM target DNA (B): (a) Tris–HCl buffer (pH 7.4) containing 0.08 μM adjunct probes, (b) Tris–HCl buffer (pH 7.4) without adjunct probe, (c) complementary target DNA containing 0.08 μM adjunct probes, and (d) complementary target DNA without adjunct probe. Inset: column charts for comparison of R_{ct} with adjunct probe and R_{ct} without adjunct probe.

3.4. Improved sensitivity by the adjunct probe

To improve the detection sensitivity, we introduced an adjunct probe nearby the capture probe. The adjunct probe was a thiol-terminated 14-base ssDNA which could hybridize with part of the reporter probe. This adjunct probe might function as a fixer to immobilize the dissociative reporter probe onto the electrode surface. From the Nyquist plots in Fig. 2, we can see that in the presence of 0.4 and 0.5 μM target DNA, R_{ct} without adjunct probe only increased by 0.63% and 0.75%, respectively (curve d vs. curve b), which is due to the increment in a little adsorption of dissociative reporter probes, blocking the charge transfer. However, under the same concentration of target DNA, R_{ct} with 0.08 μM adjunct probes respectively increased by 10.6% and 15.4% (curve c vs. curve a). The reason is that the adjunct probe can hybridize with the dissociative reporter probe and immobilize the reporter probe onto the electrode surface to form a loop. This structure can increase the steric hindrance of electrode surface, and effectively block charge transfer, leading to the increase in R_{ct} and improved sensitivity. These results clearly suggest that the combination of the adjunct probe improves the sensitivity of EIS detection of DNA sequence.

3.5. Detection of target DNA with electrochemical impedance biosensor

Under the optimum conditions (see Supplementary Material for details), EIS measurements of the target DNA at various concentrations were used to determine the detection limit and dynamic

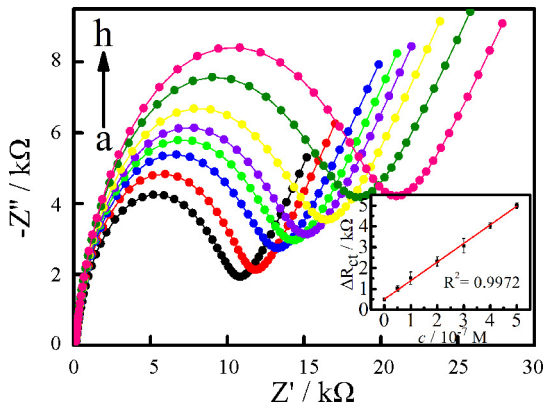


Fig. 3. Nyquist diagrams for the sensor incubated with different concentrations of target DNA (a–h): (a) blank (0 nM target), (b) 0.1 nM, (c) 50 nM, (d) 0.1 μM, (e) 0.2 μM, (f) 0.3 μM, (g) 0.4 μM, and (h) 0.5 μM. Inset: the resulting calibration curve of c vs. ΔR_{ct} for the target DNA over the range of 0.1 nM–0.5 μM. Error bars represent the standard deviation of three replicates.

range of the proposed sensor. Typical Nyquist plots of the sensor before and after incubation with different concentrations of target DNA are shown in Fig. 3. It is apparent that the R_{ct} value shows a dependence upon the concentration of the target DNA. As the concentration of the target DNA increases, the R_{ct} value increases correspondingly. The corresponding calibration plot (Fig. 3, inset) of concentration (c) vs. ΔR_{ct} (the difference of impedance before and after incubation with the target DNA) exhibits a dynamic range from 0.1 nM to 0.5 μM with an R^2 of 0.9972. After that, a plateau was reached and further increase in the target concentration did not cause an obvious change in signal. The regression equation is $\Delta R_{ct}\ (\Omega) = 8.735 \times 10^9 c + 569.3$ (c is the concentration of the target DNA, M). A detection limit of 6.3 pM of the complementary oligonucleotides could be estimated using 3σ (where σ is the standard deviation of the blank solution, $\sigma = 1.8\%$, $n = 11$). The reproducibility of the proposed design was also evaluated by performing a series of three repetitive experiments for the target sequence at the concentration of 0.1 μM, which provided a relative standard deviation of 7.5%. This result suggests that our assay protocol is endowed with good reproducibility. In addition, we have compared the detection performance of this method with those of other methods reported to detect DNA sequence (Table S1). The sensitivity of the proposed method was comparable to or better than those of other methods.

3.6. Selectivity of the biosensor

The selectivity of the sensor was examined by incubation with complementary, three-base mismatched, and non-complementary DNAs, and the EIS results are displayed in Fig. 4. For each data point three sets of parallel samples were performed. Although the presence of 0.25 μM three-base mismatched DNA leads to increment in R_{ct} (Fig. 4c), it is not comparable with that of the presence of 0.25 μM target DNA (Fig. 4d). Compared with the blank test (Fig. 4a), the addition of 0.25 μM non-complementary DNA to the sensor causes unconspicuous increment in R_{ct} (Fig. 4b). These results indicate that the label-free impedimetric sensor could effectively distinguish target DNA from base-mismatched and non-complementary DNA. Such identification between target and non-target DNA suggests the function of the adjunct probe as well as the minimized non-specific adsorption by the surface blocking [44,45] and washing.

3.7. Analytical application of the biosensor

The feasibility of the biosensor for analytical applications was investigated by the test using a mimic real sample. Because the

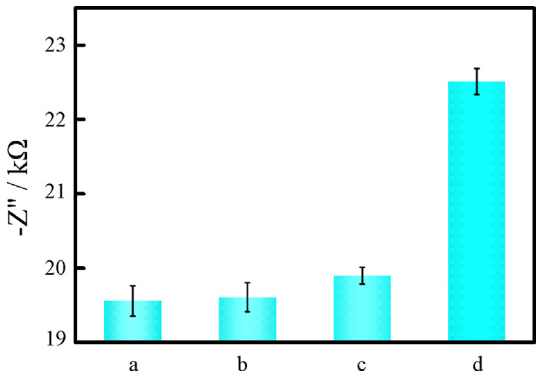


Fig. 4. Selectivity investigations against non-target DNA molecules: (a) blank, (b) non-complementary DNA (0.25 μM), (c) three-base mismatched DNA (0.25 μM), and (d) complementary DNA (0.25 μM). Error bars represent the standard deviation of three replicates.

type and concentration of various ions in human body extracellular fluid are similar to those in blood plasma, a human serum sample was used as a mimic real sample. Clinical human serum samples were from the Southwest University Hospital. The human serum samples were treated with deproteination in order to minimize protein interferences. In addition, the accuracy of the method was verified by spiking different concentrations of target DNA sequence into the human serum samples, and each sample was analyzed for three times. The results are shown in Table S2 (see Supplementary Material). The results indicate that the proposed biosensor has a promising path toward the determination of DNA sequence in real biological samples.

4. Conclusions

In summary, we have developed a sensitive and label-free electrochemical biosensor for EIS detection of sequence-specific target DNA by simply introducing the adjunct probe. This adjunct probe is able to immobilize the element of reporter probe which is displaced by the target DNA sequences, making the reporter probe bend to collide with the electrode surface and effectively blocking the charge transfer. Compared with other modified biosensors, we achieve a label-free detection of target DNA in a large dynamic range with high sensitivity and selectivity. The very low detection limit of 6.3 pM was obtained. This biosensor could potentially be applied to the detection of other DNA sequences and mRNAs, which serves as a technical platform for DNA and mRNA sequence detection.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2013.03.030.

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