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Electrochemical Detection of Peanut Allergen Ara h 1 Using a Sensitive DNA Biosensor Based on Stem−Loop Probe

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Supporting Information

ABSTRACT: A novel electrochemical DNA sensor was developed by using a stem−loop probe for peanut allergen Ara h 1 detection. The probe was modified with a thiol at its 5′ end and a biotin at its 3′ end. The biotin-tagged "molecular beacon"-like probe was attached to the surface of a gold electrode to form a stem−loop structure by self-assembly through facile gold−thiol affinity. 6-Mercaptohexanol (MCH) was used to cover the remnant bare region. The stem−-loop probe was "closed" when the target was absent, and then the hybridization of the target induced the conformational change to "open", along with the biotin at its 3′ end moved away from the electrode surface. The probe conformational change process was verified by circular dichroism (CD); meanwhile, electron-transfer efficiency changes between probe and electrode were proved by electrochemical impedance spectroscopy (EIS). The detection limit of this method was 0.35 fM with the linear response ranging from 10^{-15} to 10^{-10} M. Moreover, a complementary target could be discriminated from one-base mismatch and noncomplementarity. The proposed strategy has been successfully applied to detect Ara h 1 in the peanut DNA extracts of peanut milk beverage, and the concentration of it was 3.2×10^{-13} mol/L.

KEYWORDS: *peanut allergen, Ara h 1, stem−loop probe, electrochemical impedance spectroscopy (EIS), charge transfer resistance (Rct)*

INTRODUCTION

Food allergy is a significant worldwide public health problem. Estimates for the prevalence of peanut allergies are around 0.5−2% of the total population, and this trend appears to be increasing.[1] Peanut seed and its food derivatives belong to the "big eight" group of foods that account for the majority of food allergies worldwide, along with milk, eggs, fish, crustaceans, wheat, tree nuts, and soybean products. The symptoms of peanut allergy[2] range from mild oral allergy syndrome (OAS) to anaphylactic reactions and even death. As yet, avoidance of peanut allergens was the only choice for patients.

Up to now, there are 13 listed allergens that account for peanut allergy, including 11 allergens named Ara h 1 (*Arachis hypogaea* allergy 1) to Ara h 11, and two recently identified allergens, agglutinin and 18 kDa oleosin.[3] The major peanut allergens are Ara h 1−3. Ara h 1[4] is a 7S vicilin-like globulin also known as conarachin; 12−16% of the total peanut proteins are constituted by Ara h 1, which affects 35−95% of peanut-allergic patients in different populations.

At present, the main methods focus on testing allergic proteins[5] and genes of residual allergens.[6] Commercial production processes involve heat treatment, which often denatures food proteins, thereby altering protein tertiary structure and thus interfering with protein detection.[7] DNA remains are intact longer under heat and pressure processing and can therefore provide the basis of a robust assay for the detection of allergen residues in foods. Therefore, the methods of detecting DNA to reflect the existence of allergens, such as PCR[8] and real-time PCR,[8,9] have been developed. Although these methods offer good detection limits, they are time-consuming and need expensive reagents. Thus, development of a rapid and convenient detection method for food allergen analysis is extremely desirable.

Electrochemical DNA biosensors have been widely used for specific gene detection for their unique advantages such as low cost, simplicity, rapidity, high sensitivity, good selectivity, etc. As is well-known, the immobilization of a DNA probe onto the transducer surface is a crucial step during the fabrication of a DNA biosensor, which is the important link for improving the stability, reproducibility, regeneration properties, and sensitivity of the biosensor. Thus, some workers have developed a new class of reagentless, sensitive, and selective E-DNA sensors,[10] which used a single surface-confined stem−loop DNA structure as the capture probe. This design is in fact an electrochemical analogue of fluorescent "molecular beacons (MB)".[10c,11] The stem−loop structured DNA probes are superior to linear probes in several aspects for the detection of nucleic acid.[12] The greatest advantage is the superior mismatched discrimination ability. They also have great potential for real-time monitoring analysis.

In this study, we used the stem−loop probe dually labeled with 5′-SH and 3′-biotin. It can be self-assembled on gold electrodes by means of facile gold−thiol affinity. The focuses of this study are to design an appropriate stem−loop probe, to evaluate the sensitivity and selectivity of the electrochemical DNA sensor based on the modified hairpin probes, and to

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detect the peanut allergen Ara h 1 of a peanut milk beverage by this sensitive biosensor.

EXPERIMENTAL PROCEDURES

Materials and Apparatus. All synthetic oligonucleotides were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of oligonucleotides used in this work are given in Table 1.

Table 1. Base Sequences of Oligonucleotides Used in This Work

name	base sequence
oligo 1 (double-labeled stem–loop probe)	5′-HS-C6-GCG AGG TTC CGT GGC TGC TGA TGA CTT GGT CCT CGC-biotin-3′
oligo 2 (complementary target)	5′-ACC AAG TCA TCA GCA GCC ACG GAA-3′
oligo 3 (single mismatch)	5′-ACC AAG TAA TCA GCA GCC ACG GAA-3′
oligo 4 (noncomplementary)	5′-GTT CGA CTG CTG ATG ATT GTA AGG-3′
oligo 5 (single-labeled stem–loop probe)	5′-HS-C6-GCG AGG TTC CGT GGC TGC TGA TGA CTT GGT CCT CGC-3′
oligo 6 (upstream primer)	5′-AGA CTG GAG ACA ACC AAG AGA AG-3′
oligo 7 (downstream primer)	5′-TTT CTT CCC TCA CAT GGC TAC C-3′

Among them, the loop sequence of the probe is the conserved sequence of Ara h 1, the main allergen of peanut.[9b] The conserved sequence, the upstream primer, and the downstream primer were designed according to the GenBank AF432231 sequence.

In this experiment, 6-mercaptohexanol (MCH) was obtained from Sigma-Aldrich. All other chemicals were obtained from Sinopharm Chemical Reagent Co. Ltd. The solutions in the experiments were prepared with ultrapure water (Milli-Q 18.2 MΩcm, Millipore System Inc.).

Electrochemical measurements were performed at room temperature using a CHI760C electrochemical workstation (Shanghai Chenhua Instrument Corp., China). A conventional three-electrode cell was employed, which involved a gold working electrode of 2 mm diameter, a platinum wire counter electrode, and a saturated silver/silver chloride reference electrode. All of the potentials in this paper are with respect to reference electrode. All spectra were measured in PBS buffer (10 mM phosphate, pH 7.4, and 0.15 M NaCl) containing 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ as a redox couple. All experiments were performed at least three times to ensure the consistency of the response trend. The interfacial processes at the modified electrode–solution interface were observed as changes in impedance spectroscopy and charge transfer resistance. All of the experimental impedance curves were fitted to an equivalent circuit model that included a solution resistance in series with a parallel circuit containing a constant phase element, the charge transfer resistance,and Warburg impedance.

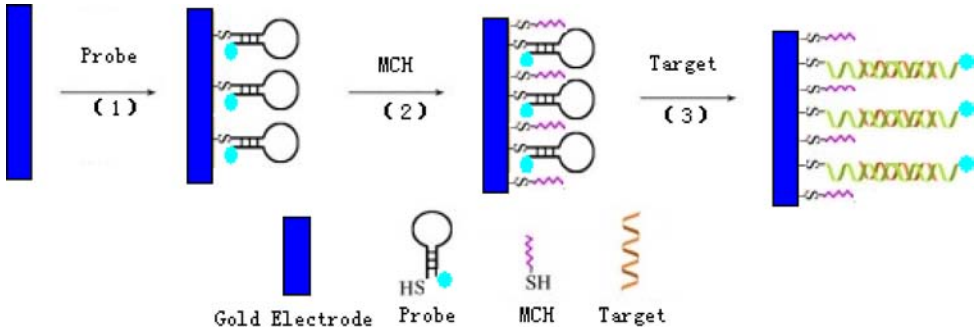
Pretreatment of Electrodes. The gold working electrode was treated with piranha solution ($H_2SO_4/_2O_2$ = 7:3) and rinsed with ultrapure water. Then it was polished to form a mirror sequentially with 0.3 and 0.05 μm alumina powder, followed by ultrasonic cleaning with ethanol and ultrapure water for 3 min each. The cleaned electrode was electrochemically pretreated in 0.5 M H_2SO_4 by potential scanning between −0.2 and 1.6 V (vs Ag/AgCl) until a cyclic voltammogram characteristic of a clean Au electrode was obtained. Finally, the electrode was then rinsed with ultrapure water and dried in air.

Fabrication of Electrochemical DNA Sensor. Slf-assembly was carried out by adding 4 μL of stem–loop probe solution (1 μM) on the surface of the gold electrode, kept at 4 °C overnight.[13] To avoid volatilization of the solution, the electrode solution was covered with a plastic cap after dropped with probe. After that, the electrode was thoroughly rinsed with PBS buffer to remove those nonbonding materials. Then 10 μL of PBS buffer containing 1 mM MCH was dropped on the electrode surface for 1 h, to cover the remaining bare regions.[14] Subsequently, the electrode was rinsed thoroughly with a copious amount of PBS buffer to remove the unattached MCH. Then a 4 μL droplet containing various concentrations of complementary target (ranging between 10^{-15} and 10^{-7} M) was deposited on the electrode surface, and the electrode was kept at 37 °C for 30 min. After hybridization, the electrode was rinsed by PBS buffer to remove nonhybridized target DNA.

Circular Dichroism (CD) Spectroscopic Study of Interaction between Stem–Loop Probe and Complementary Target. The mixture of stem–loop probe and complementary target (10^{-6} M) was kept at 37 °C for 30 min. Then, probe, complementary target, and the mixture at the same concentration were respectively measured by CD (MOS-450 circular dichroism, Biologic) from 200 to 400 nm.[15]

DNA Extraction from Peanut Milk Beverage. The peanut milk beverage (Yinlu, Xiamen) was purchased from a nearby supermarket. The DNA extraction steps[16] were as follows: 35 mL of sample in a centrifuge tube was centrifuged for 10 min at 3000 rpm and 4 °C. After the supernatant had been discarded, lipids on the tube wall were rubbed out by absorbent cotton. Then 10 mL of PBS buffer was added to dissolve sediment. The last step was repeated. After the supernatant had been discarded and lipids rubbed out, an extraction buffer (0.2 M Tris/HCl, pH 7.5, 0.25 M NaCl, 25 mM EDTA, 0.5% SDS) was added to dissolve the pellet, followed by incubation for 20 min at 65 °C. Then 250 μL of cold phenol/chloroform/isoamyl alcohol (25:24:1) was added. After centrifugation for 10 min at 12000 rpm and 4 °C, 400 μL of precooling isopropyl alcohol was added to the supernatant, followed by incubation for 20 min at −20 °C. After centrifugation for 10 min at 12000 rpm and 4 °C, 250 μL of ethanol was added to wash the pellet. The supernatant was then discarded, and the pellet was resuspended by adding 50 μL of microfiltered water. At last, the concentration of the extracted DNA was determined by UV

Scheme 1. Schematic Illustration of the Fabrication of the Sensor[a]



[a](1) Immobilization of stem–loop probe to the gold electrode; (2) immobilization of 6-mercaptohexanol (MCH) to block the remaining bare region; (3) hybridization with complementary target DNA.

measurement (TU-1900 double-beam UV−vis spectrophotometer, Beijing).

RESULTS AND DISCUSSION

Schematic Procedure of the Fabrication of the Sensor. The stem−loop probe we used, dually labeled with 5′-SH and 3′-biotin, has been designed such that it has six complementary bases at its 5′ and 3′ ends (five of them are G−C pairs), so that the DNA strand will be closed by the thermostable G−C pairs to form a stem−loop. The stepwise fabricating process is shown in Scheme 1. The sensor was constructed by assembling the probe at a bioelectronic interface to form the self-assembled monolayer (SAM) through probe 5′-SH's strong tendency to diffuse from diluted aqueous solutions to clean gold surfaces. Then the electrode was blocked by using MCH to decrease unspecific adsorption.[17] Previously, the immobilized stem−loop probe was in the "closed" state in the absence of the target, which localized the biotin unit at the 3′ end in proximity to the electrode surface. Then the added target hybridized with the stem−loop probe, bringing about the conformational change of probe. The stem−loop probe was in the "opening" state in favor of the formation of the thermodynamically more stable, rigid target−probe duplex. Then the biotin was detached from the electrode surface, which changed the electron-transfer efficiency. The stem−loop probe has a great advantage in mismatched discrimination.

Electrochemical Characterization of the Modified Electrode. As shown in Figure 1A, compared with the bare gold electrode (curve a), the peak current of the stem−loop probe modified electrode (curve b) decreased obviously and the peak-to-peak separation increased, which indicated that the probes were immobilized on the gold electrode surface successfully. As the probe occupied a large area on the electrode surface, the electrode could not efficiently exchange electrons with the solution,[18] and the electron-transfer efficiency reduced. After immobilization of MCH, the peak current further decreased (curve c). After hybridization with complementary target DNA, the stem−loop structure opened, and the target−probe duplex formed, moving the attached biotin group away from the electrode surface. Early study showed that the addition of target made an increase of conductivity for the sensor,[18] but in our work, the peak current decreased again after hybridization with target nucleic acid (curve d). As shown in Figure 1B, the results of electrochemical impedance spectroscopy (EIS) experiments were in correspondence with that of CV, also confirming the successful modification and showing the effect of each modification step on electron-transfer kinetics.

To prove the effect of the 3′-end biotin, the single-labeled stem−loop probe modified with only a thiol at its 5′ end was designed, and the electron transfer coefficients of it and the double-labeled probe were determined, respectively.[19] As Figure 2 shows, the CV curve was scanned by different scan rates from 10 to 100 mV/s, after the two kinds of probes were immobilized on the electrode surface, respectively. According to the position of the anodic peak and cathodic peak, the electron-transfer coefficients of single-labeled probe and doubled-labeled probe were calculated, the former being 0.55 and the latter, 0.28. Therefore, the electron-transfer rate of the single-labeled probe was faster than that of the doubled-labeled probe. The result indicated that the 3′-end biotin hindered the

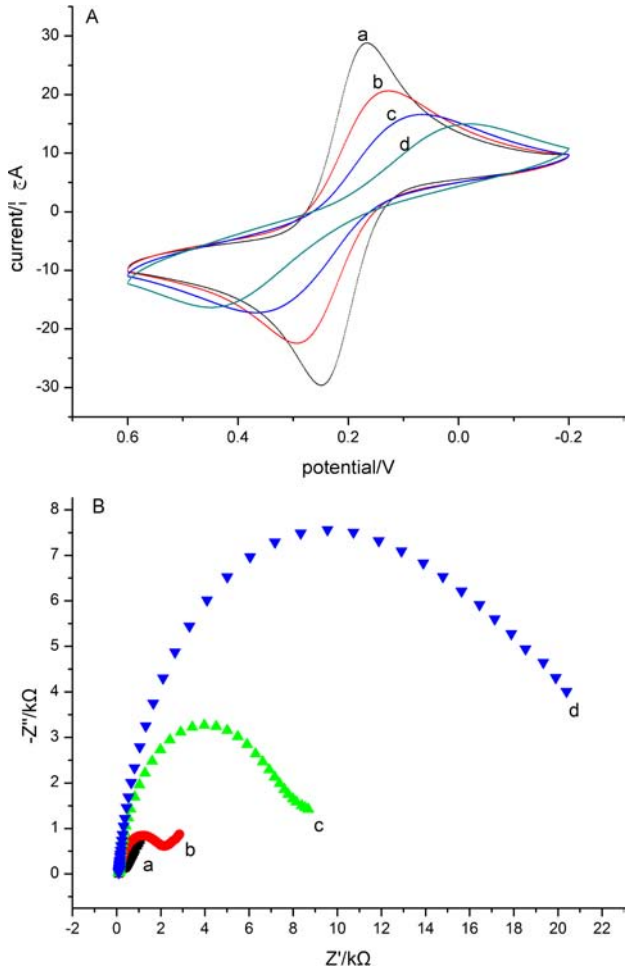


Figure 1. Characterization of forming self-assembled monolayers (SAMs) by cyclic voltammogram (A) and electrochemical impedance spectroscopy (B): (a) bare Au electrode; (b) after immobilization of the hairpin probe; (c) after immobilization of 6-mercaptohexanol (MCH); (d) after hybridization at 37 °C with 10^{-8} M complementary target DNA.

electron transfer between the electrode surface and the test solution.

CD Characterization of the Interaction of Probe and Target. As shown in Figure 3, the spectra contained the negative peak with minimum around 239 nm attributed to the type B structure conformation of DNA double helix and positive peak at 278 nm caused by base stacking.[20] Because of the double-helix structure of the probe stem, the intensity of the probe (curve a) negative peak was stronger than that of the target (curve b). When the target−probe duplex formed, base stacking force increased and the intensity of the positive peak (curve c) was enhanced. This proved that the conformation of the probe has changed after hybridization with target.

Quantification Detection of Target DNA. To investigate the sensitivity of the prepared electrochemical DNA biosensor for detection of the target DNA, the sensor was hybridized with different concentrations of the target, and then the impedance response of the electrode surface was measured in 10 mM PBS (pH 7.4) containing 2.5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$. The electrochemical impedance response to different target concentrations is depicted in Figure 4A. As can be seen, with the increase of the target concentration, the impedance signals increased obviously. Figure s1 of the Supporting Information

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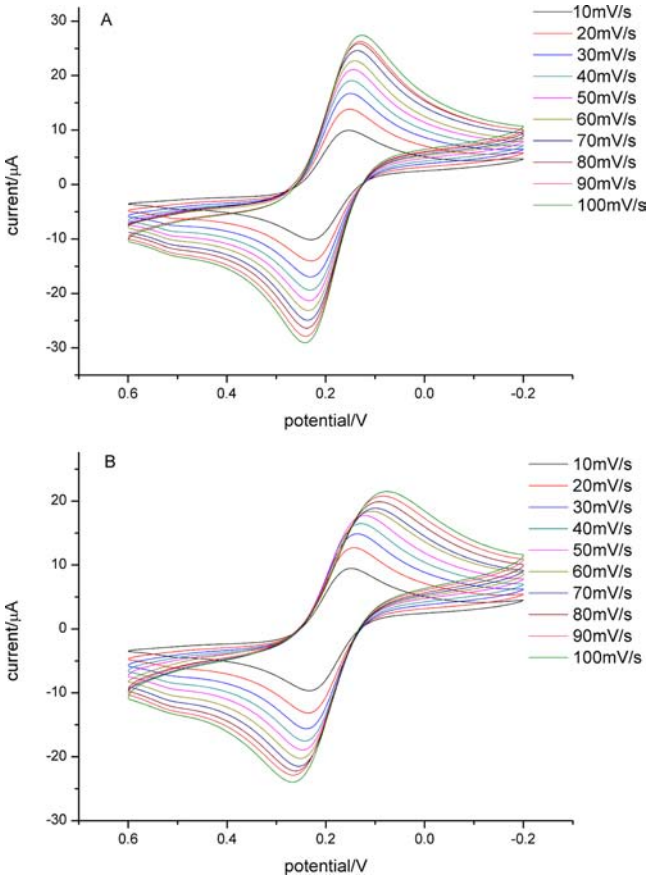

Figure 2. Cyclic voltammogram of single-labeled stem−loop probe (A) and double-labeled stem−loop probe (B) by different scan rates: 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mV/s.

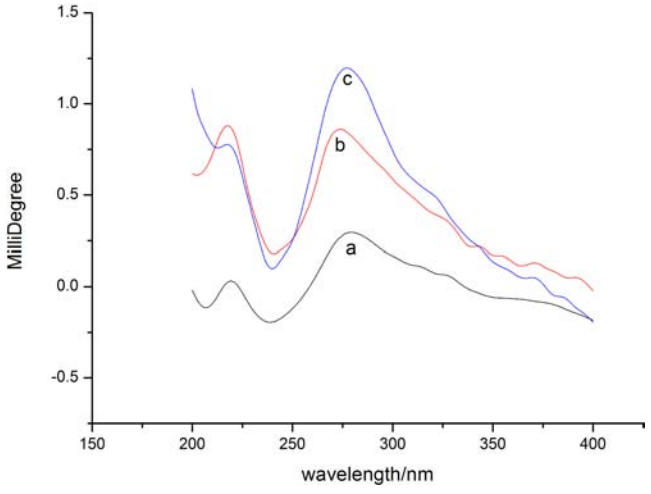

Figure 3. CD spectra of stem−loop probe (a), complementary target (b), and probe−target hybridized mixture (c) in 10 mmol/L PBS buffer (pH 7.4).

shows the tendency chart of the ΔRct as a function of the target concentration. A series of target solutions with concentrations from 1 fM to 0.1 μM were investigated, and a linear relationship between ΔRct and the logarithm of the complementary target concentration in the range of 1 fM to 0.1 μM can be found from the regression equation ΔRct = 1.9635 log$C$ + 32.01 and a correlation factor of 0.9623 (Supporting Information Figure s1,

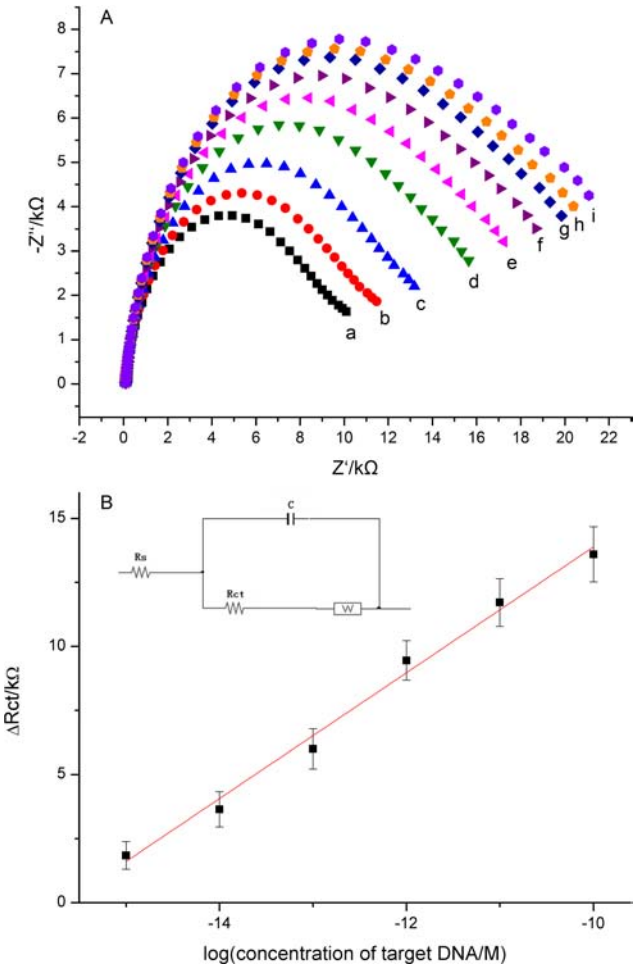

Figure 4. (A) Nyquist plots, −Zim versus Zre for a Au electrode in PBS buffer solution containing 2.5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$: after immobilization of the hairpin probe and MCH, then hybridization at 37 °C with (a) 10^{-15} M, (b) 10^{-14} M, (c) 10^{-13} M, (d) 10^{-12} M, (e) 10^{-11} M, (f) 10^{-10} M, (g) 10^{-9} M, (h) 10^{-8} M, (i) 10^{-7} M of complementary target DNA. (B) Linear regression of ΔRct versus the logarithm of target concentration (10^{-15}−10^{-10} M) representing charge transfer resistance change, ΔRct (kΩ) taken as the sensor response, before and after hybridization at 37 °C with different concentrations of complementary target DNA in PBS buffer solution containing 2.5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$. The vertical bars designate the standard deviations for the means of three replicative tests. (Inset) Equivalent circuit model, to which all of the experimental impedance curves were fitted.

inset), but from 1 fM to 0.1 nM with the regression equation ΔRct = 2.4689 log$C$ + 38.57 and a correlation factor of 0.9918 (Figure 4B). Meanwhile, the detection limit was 0.35 fM as calculated according to the rule of 3 times the standard deviation over the background signal. The result was comparable with other methods reported in Table s1 of the Supporting Information.

In detail, Liu et al.[21a] reported an enzyme-based E-DNA sensor, which employed a stem−loop DNA probe dually labeled with biotin and digoxigenin (DIG). Through DIG combining with the horseradish peroxidase-linked anti-DIG antibody, the electrochemical signal changed. The detection limit of this method was 10 fM. Liu et al.[21b] designed a biosensor based on immobilized stem−loop structured probe to detect *Pseudomonas aeruginosa* 16S rRNA, with the detection

limit of 0.012 pg/μL. Yang et al.[21c] applied β-cyclodextrin derivative functionalized aligned carbon nanotubes for electrochemical DNA sensing via host−guest recognition, with the detection limit of 0.5 pM. Fan et al.[21d] developed an E-DNA based on the ferrocene-tagged DNA stem−loop probe with the detection limit of 10 pM. Liu et al.[21e] reported a DNA biosensor based on nicking endonuclease assisted electrochemistry signal amplification, with the detection limit of 0.167 pM.

Selectivity of the DNA Biosensor. The selectivity of the DNA biosensor was investigated, and the results are shown in Figure 5. The prepared sensor was hybridized with various

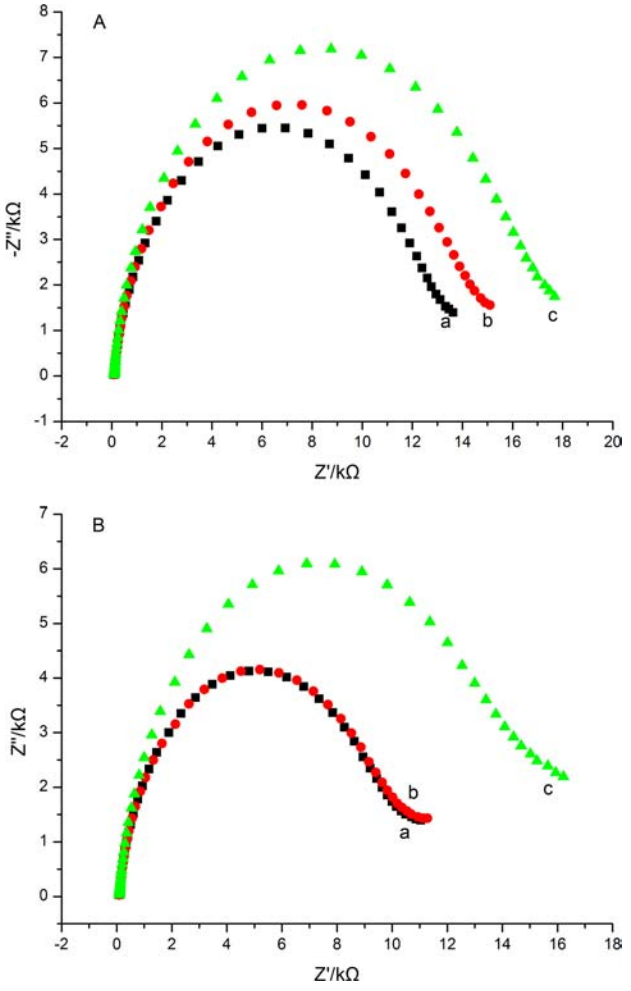

Figure 5. Nyquist plots, −Zim versus Zre for a Au electrode in PBS buffer solution containing 2.5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$: A(a) and B(a) after immobilization of the hairpin probe and MCH, A(b) after hybridization with 10^{-8} M single-base mismatch target sequence; B(b) after hybridization with 10^{-8} M noncomplementary sequence; A(c) and B(c) after hybridization with 10^{-8} M complementary target DNA.

DNA sequences (complementary DNA, one base mismatch DNA, and noncomplementary DNA), and then EIS was measured, respectively. After hybridization with complementary target (10^{-8} M) (curve c), a visible curve was generated; meanwhile, low signals emerged after hybridization with a single mismatch (10^{-8} M) and noncomplementarity (10^{-8} M) (curve b). However, the sensor selectivity to noncomplementary target was superior compared to one-base mismatch. As shown in Figure 5A, the single mismatch (curve b) slightly responded to the prepared DNA biosensor, compared to immobilization probe and MCH (curve a). This fact clearly demonstrated that the prepared DNA biosensor had an excellent selectivity for the detection of target DNA sequence, and the discrimination ability for a noncomplementary was better than that for a single-base mismatch.

Optimization of Hybridization Time. The hybridization time between the probe and target was analyzed by monitoring electrochemical impedance spectroscopy. Figure s2 of the Supporting Information displays the effect of hybridization time on charge transfer resistance. As the hybridization time increased, the Rct response rose and tended to a maximum at 40 min. The response signal did not enhance with the longer hybridization time, which indicated that the reaction achieved the dynamic balance. Therefore, we chose 30 min for the hybridization time as a time-saving consideration.

Reproducibility and Stability of the DNA Biosensor. To demonstrate the reproducibility of this electrochemical assay, the electrode was immersed in 10 mM PBS (pH 7.4) at 95 °C and rechallenged with the target sequence. After five rounds of regeneration, it also retained its 82% original value, which well illustrated the reproducible characteristic of the prepared sensing platform.

The stability of the DNA biosensor was evaluated over a 21 day period. When the sensor was stored in a refrigerator at 4 °C, the Rct of the sensor retained 87% of its initial response. This indicated that the developed DNA biosensor had good stability.

Measurement of Ara h 1 in Peanut Milk Beverage. To test the practicality of the developed biosensor, we applied this method to detect Ara h 1 in extracts of a peanut milk beverage. The concentration of the extracting DNA was 0.24 mg/mL. The PCR result (Suppporting Information, Figure s3A) showed the extracting DNA contained the target DNA of Ara h 1. The 125 bp sequence was the amplified target, and the 20 bp sequence was the primer dimer. As shown in Figure s3B of the Supporting Information, the peanut allergen DNA extracts of the sample could be determined. The Rct for the logarithm of sample DNA was linear from 0.24×10^{-6} to 0.24×10^{-2} mg/mL, and the concentration of the Ara h 1 gene in the peanut milk beverage was 3.2×10^{-13} mol/L through calculation, which implied that the method could be used to detect the peanut allergen in actual samples.

Conclusions. In this study, we demonstrated a new electrochemical method for peanut allergen Ara h 1 detection that combined the stem−loop probe dually labeled with 5′-SH and 3′-biotin and electrochemical impedance spectroscopy detection. The method showed high sensitivity as the detection limit reached 0.35 fM. In addition, the proposed method was also beneficial for the determination of peanut DNA extracts. The concentration of the Ara h 1 gene in peanut milk beverage was 3.2×10^{-13} mol/L. It was expected that the proposed electrochemical assay would be widely applied in the clinical diagnosis of peanut allergen as well as food safety control.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures and table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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