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Enzymatic amplification detection of peanut allergen Ara h1 using a stem-loop DNA biosensor modified with a chitosan-mutiwalled carbon nanotube nanocomposite and spongy gold film

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Abstract

In this paper, a highly sensitive biosensor was constructed for peanut allergen Ara h1 detection. The biosensor was constructed by coating a glassy carbon electrode with a chitosan-mutiwalled carbon nanotube nanocomposite and then adding a spongy gold film via electro-deposition to increase the effective area. The probe switched from an “on” to an “off” state in the presence of target DNA, which detached biotin from the electrode surface. This also detached streptavidin-horseradish peroxidase (HRP-SA), which was bound to the electrode via specific interaction with biotin. The HRP-SA catalyzed chemical oxidation of hydroquinone by H_2O_2 to form benzoquinone, and when it was detached, electrochemical reduction of the signal of benzoquinone could be used to monitor DNA hybridization via chronoamperometry. Under optimum conditions, a wide dynamic detection range (3.91×10^{-17}–1.25×10^{-15}mol L^{-1}) and a low detection limit (1.3×10^{-17}molL^{-1}) were achieved for the complementary sequence. Furthermore,

the DNA biosensor exhibited an excellent ability to discriminate between a complementary target and a one-base mismatch or non-complementary sequence. The sensor was successfully applied to Ara h 1 analysis in peanuts.

Keywords: electrochemical biosensor; stem-loop probe; chitosan-mutiwalled carbon nanotube nanocomposite; spongy gold film; peanut allergen; Arachishypogaea allergen 1 (Ara h1)

Highlights

- Spongy gold film/mutiwalled carbon nanotube modified DNA sensor has been fabricated.
- Streptavidin-horseradish peroxidase is utilized as signal amplification.
- The proposed strategy could detect the target DNA down to the level of 0.013 fmol.
- We applied the developed sensor for the analysis of real samples (peanut) with good results.

1. Introduction

Electrochemical DNA biosensors have been widely used for specific gene sequences detection because of their unique advantages such as low-cost, rapid response, simplicity, good selectivity and high sensitivity [1, 2]. Currently, the electrochemical DNA biosensor has played a major role in food safety [3], genetic disorders[4],biological research [5] and environmental monitoring [6]. Various sensing strategies have been proposed for detection of specific DNA sequences, with the aim of improving the sensitivity and selectivity of the biosensor.

The construction of an electrochemical DNA biosensor usually involves two critical steps: immobilization of probe DNA on the transducer and electrochemical signal amplification. To date, different kinds of strategies have been exploited to enhance the immobilization amount of probe DNA[7-9].Including which, nano-materials have received increasing attention in electrode surface modification for DNA biosensor, owing to their unique physical and chemical properties of high surface area, favorable electronic properties, electrocatalytic activity and good biocompatibility[10-14].The construction of nanostructure-modified electrode for DNA detection with high sensitivity is highly desirable. A new era in the field of nanotechnology has begun with the discovery of carbon nanotubes (CNTs) by Iijima in 1991 [15]. As one kind of CNTs, multi-wall carbon nanotubes (MWCNTs) show ultrahigh electrocatalysis capacity and surface area effects in biosensing analysis [16]. MWCNTs can be employed to bind with DNA through covalent bond or electrostatic attraction [17]. However, lack of stability and uniformity of MWCNTs directly immobilized onto the electrode surface without other reagents is the major challenge to their use as modifiers in the fabrication of biosensors. Then, chitosan which possesses distinct chemical and biological properties was employed to disperse MWCNTs [18, 19]. The advantages of using MWCNTs for electrode surface modification in the development of new designs of electrochemical sensors have been recently highlighted by many authors. Wang et al. [20] reported an electrochemical DNA (E-DNA) biosensor based on a CNT and polypyridyl copper complex for the detection of the PAT gene. Chung et al.[21]developed an E-DNA sensor modified with ionicliquid-multiwalled CNTs (IL-MWNTs) for ultrasensitive DNA sequence-specific detection of *Salmonella*typhi. Recently, gold nanoparticles have been widely applied in the fabrication of biosensors because these sensors are simple to prepare, relatively

inexpensive, and provide huge interfaces for biomolecule detection. To date, simple electro-deposition has been widely used for preparation of gold nanoparticles [22].Different morphologies of the gold nanostructures have been obtained by electro-deposition, including spherical [23], flower-like [24]and dendritic [25]. In DNA sequence detection, the MWCNTs and gold nanoparticles were introduced in this work can act as the immobilizing carrier to increase the immobilization amount of the DNA probe, and as the signal particle to magnify the detection signal and lower the detection limit.

From the previous reports, enzyme amplification technology in electrochemical methods plays an important role in the sensitive determination of DNA [26-28]. Horseradish peroxidase (HRP), as an enzymatic label, is generally immobilized using streptavidin-biotin in a stem-loop probesystem [29-31]. To enhance the sensitivity, improve the selectivity, and reduce the detection limit and cost, electrochemical biosensors based on enzymatic signal amplification have been investigated to detect DNA. For example, Meng et al. [32]built an enzyme-based E-DNA sensor for sensitive detection of DNA with a low detection limit of 6.0 pmol L^{-1} (S/N = 3). Yin et al. [23]constructed a highly sensitive biosensor for sequence specific miRNA-21 detection based on dendritic gold nanostructure , graphene nano-sheets and a HRP labeling amplification strategy. The biosensor showed excellent selectivity and high sensitivity with a low detection limit of 0.06 pmol L^{-1}. Additionally, it has been reported that stem-loop DNA can amplify the electrochemical signal. Several biosensors based on stem-loop DNA have been developed with good performance, such as for *Pseudomonas aeruginosa* 16S rRNA detection in compost [33], sensitive and portable detection of RNA mutations [34],and adenosine-5'-triphosphate detection in *Escherichia coli* O157:H7 in water samples [35]. Significantly, stem-loop probes inherently possess high specificity because of their conformational constraints, and thus are superior to linear probes for DNA detection [36, 37].

In this work, a promising enzyme-based DNA biosensor was developed for detection of the peanut allergen Ara h1. To improve the sensitivity of the biosensors, chitosan (CS)–MWCNT nanocomposite (CS-MWCNT) and spongy gold film were used as the probe immobilization platform. The CS-MWCNT was coated on an electrode, followed by spongy gold film via simple electro-deposition to increase the effective area. A stem-loop probe dually labeled with 3'-thiol and 5'-biotin was assembled on the spongy gold film/CS-MWCNT/GCE via a thiol-Au interaction.

The results showed that the CS-MWCNT nanocomposite and spongy gold film could effectively immobilize the captured DNA probe and promote electron transfer kinetics of the foreign molecules. Next, HRP was immobilized through conjugation between biotin of the stem-loop probe and streptavidin for enzyme amplification. When the target DNA was captured by the probe, conformation of the stem-loop structure changed to trigger a specific interaction between biotin and streptavidin HRP on the electrode surface. Thus, the target was quantified via electrochemical detection of the enzymatic product in the presence of substrate. The electrochemical DNA biosensor based on gold film/CS-MWCN/GCE could be used for recognition of target DNA using the stem-loop DNA as the capture probe for hybridization with Ara h1 in food.

2. Experimental

2.1. Materials and Chemicals

All synthetic oligonucleotides were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of the oligonucleotides used in this work are given in **Table s1**. The stem-loop probes, dually labeled with 3'-thiol and 5'-biotin, contained a stem of complementary 6 bp segments and a loop recognition sequence complementary to a 24 bp segment of Ara h1. Gold chloride tetrahydrate ($HAuCl_4 \cdot 4H_2O$) and 6-mercaptohexanol (MCH) were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Mutiwalled carbon nanotubes were provided by XFNANOCo., Ltd(Nanjing, China).BSA and streptavidin-horseradish peroxidase (HRP-SA)were obtained from BOYAO Biotech Co., Ltd. (Shanghai, China).Chitosan (CS) and other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd(Beijing, China).The solutions used in the experiments were prepared with ultrapure water (Milli-Q 18.2 MΩcm, Millipore Co., Billerica, MA).

2.2 Apparatus.

Electrochemical measurements were performed at room temperature using a CHI760C electrochemical workstation (Shanghai Chenhua Instrument Co., Ltd., Yangpu, China). A conventional three-electrode cell was employed, and included a glassy carbon working electrode (ø2mm), a platinum wire counter electrode, and a saturated Ag/AgCl reference electrode. Potentials were determined with respect to the reference electrode. All spectra were measured in phosphate buffer (0.1 mol L^{-1} phosphate, pH 7.0) containing 0.1 mol L^{-1}KCl and 2.5mmol L^{-1}Fe $(CN)_6^{3-/4-}$ as a redox couple. All experiments were performed at least three times to ensure the consistency of the response. A S4800 type scanning electron microscope (Hitachi, Japan) and a

C1000 thermal PCR cycler (Bio-Rad, America) were used.

2.3 Preparation of the electrode

Before use, the working GCE was etched with piranha solution (H_2SO_4/H_2O_2, 7/3 v/v) for 15 min and rinsed with ultrapure water. Next, the bare GCE was sequentially polished with 0.3 and 0.05 μm alumina powder on a microfiber cloth to obtain a mirror-like surface, and then by ultrasonic cleaning with ethanol and ultrapure water for 3 min. Subsequently, the electrode was dried thoroughly under a N_2 stream.

2.4 Construction of the E-DNA sensor

It is well known that carbon nanotubes are not easy to dissolve, which limits their application in biosensors. As a polysaccharide biopolymer, CS is widely used in sensors because of its numerous advantages, including excellent film forming ability, high permeability, and good dispersion of carbon nanotubes [38, 39].To enhance the electrical conductive properties and specific surface area of the biosensor, chitosan were doped into the MWNT to form a CS-MWNT nanocomposite. Purified MWCNTs (5 mg) was put into 5mL of 2% acetic acid containing 5mg of CS, and the mixture was treated with ultrasonication for 2 h. Then, 4mL of the CS-MWNT dispersion was dropped onto a prepared GCE and dried under an infrared lamp. Afterwards, the electrode was washed with PBS buffer and dried thoroughly under a N_2 stream. This produced a CS-MWCNT modified GCE (CS-MWCNT/GCE). Spongy gold film was electrodeposited on the CS–MWCNT/GCE surface via amperometry (–0.25 V for 600 s)in a 3mmol L^{-1} $HAuCl_4$solution containing 0.1 mol L^{-1} KNO_3. The obtained electrode was named a spongy gold film/CS–MWCNT/GCE.

Immobilization of the stem-loop probe was accomplished by dropping 4μL of 1μmolL^{-1}stem-loop probe solution onto the surface of the spongy gold film/CS-MWCNT/GCE and incubating at 4°C for 12h. The modified electrode was then thoroughly rinsed with PBS to remove any excess probe. Then, 10 μL of PBS buffer containing 10mmol L^{-1} MCH was dropped onto the electrode surface for 30 minutes to block any remaining bare regions. Finally, the electrode was washed with PBS to remove residual MCH. The electrode was stored at 4°C until use. The preparation of the electrochemical DNA biosensor is illustrated in **Scheme. 1.**

2.5 Hybridization and detection.

Hybridization experiments were performed by dropping 4 μL of different concentrations of

complementary target DNA onto the probe/spongy gold film/CS-MWCNT/GCE. This was followed by incubation at 37°C for 30 min to yield complementary target DNA-probe/sponge gold film/CS-MWCNT/GCE. Unhybridized target DNA on the electrode was removed by rinsing the electrode with PBS buffer (pH 7.4). Subsequently, the electrode was immersed in 1% BSA solution for 30min to cover the active site. The electrode was then washed and dried under a stream of N_2. After that, 8 μL of 10μgmL^{-1} streptavidin-HRP was added to the modified electrode and incubated at room temperature for 30 min. Before using the electrode, unspecific immobilized streptavidin-HRP was washed with PBS. Hybridization of the probe/spongy gold film/CS-MWCNT/GCE with single-base mismatch and non-complementary sequences followed the same protocol as above.

All the electrochemical measurements were performed at room temperature using a CHI760C electrochemical work station. Cyclic voltammetry (CV) was performed in PB buffer (pH 7.0) containing 0.1 mol L^{-1}KCl and 2.5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$.The CV experimental parameters were as follows: scan rate, 0.1V·s^{-1}; initial potential, 0.6 V; high potential,0.6 V; and low potential,–0.2 V. CV and chronoamperometry were performed in a working solution of 0.1 mol L^{-1} PBS (pH 7.4) containing1.0 mmol L^{-1} H_2O_2 and 0.2 mmol L^{-1} hydroquinone with an applied potential of –0.2V for chronoamperometry and a scan rate of 0.1V·s^{-1} in the potential range from –0.5 to 0.6V.

2.6. Detection of the peanut allergen Ara h1 gene in peanuts

The E-DNA sensor was tested in application to detect the Ara h1 gene in peanuts purchased from a local supermarket. The CTAB-based method was used for extracting DNA from the peanut[40]. After the DNA was extracted, PCR amplification was used to amplify the extracted DNA. A 7.5% PAGE was used to identify the Ara h1 gene in the PCR products. A 125bp region in the Ara h1 gene (GenBank No.AF432231) was the target segment for PCR amplification. The loop sequence of the probe (Oligo 1) used in this study was the conserved sequence of the 125bp region. The sequences of the upstream primer and the downstream primer are shown in **Table S1 (Supporting Information)**. The amplification protocol was as follows: 10 min at 95 °C, followed by 30 cycles at 95 °C for 30 s, 60 °C for 40 s, and 72 °C for 60 s. The reaction system was further incubated for 10 min at 72 °C to completely extend any incomplete products. After confirming the Ara h1 gene was extracted from the peanuts, the biosensor was used to extract genome DNA. Fluorescence real-time quantitative PCR was used to authenticate the test results from the DNA

biosensor. Then the extracted genome DNA was also detected using this fluorescence real-time quantitative PCR system.

3.Results and discussion

3.1 Fabrication of the sensor

The stem-loop probe specific to the Ara h1 gene had six complementary bases at its 5' and 3' ends (five of them are G-C pairs), and the DNA strand was closed by thermostable G-C pairs to form a stem-loop. The stepwise fabrication process of the DNA biosensor is shown in **Scheme 1.** The sensor was constructed by immobilizing CS-MWCNT on the GCE surface, and then the spongy gold film was electrodeposited on the CS-MWCNT surface. Through facile gold-thiol affinity, the probe labeled with 5'-SH was assembled on the surface of the spongy gold film/CS-MWCNT/GCE. Then, the electrode surface was blocked using MCH to decrease any non-specific adsorption of undesirable material to the modified electrode surface. In the absence of the target, the immobilized stem-loop probe was in the "closed" state, locating 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 a conformational change of the probe. This put the stem-loop probe in the "open" state, and favored formation of the more thermodynamically stable rigid target-probe duplex. Then, biotin was detached from the electrode surface. The BSA was added to block protein non-specific adsorption sites on the modified electrode surface. Because of the affinity interaction between biotin and streptavidin, the HRP-SA bonded with the detached biotin. Because of HRP catalysis, the redox signal of H_2O_2 can be measured from changes in the current and potential signal.

3.2 Characterization of the CS-MWCNT and spongy gold film

Transmission electron microscopy of the CS-MWCNTs (**Fig.1A**) showed the MWCNTs were consistent in length and thickness, and were uniformly dispersed in the chitosan solution. These properties would facilitate electron transfer. Scanning electron microscopy of the spongy gold film (**Fig.1B**) showed the gold nanoparticles had a dense structure like a porous sponge. The gold nanoparticles were distributed uniformly and orderly on the electrode surface, and provided fixed sites for the stem-loop probe.

3.3 Electrochemical behavior of the modified electrodes

The modified electrode was characterized in Fe (CN) $_6^{3-/4-}$ buffer. As shown in **Fig.2A**, compared with the bare GCE (curve a), the peak current of the CS-MWCNT/GCE (curve b) and

spongy gold film/CS-MWCNT/GCE (curve c) increased gradually, while the peak-to-peak separation decreased. These results indicate that because of the high electron transfer rate of the MWCNT for catalysis of the redox reaction, when the GCE was modified by CS-MWCNT, the peak current increased. When highly conductive gold nanoparticles were distributed densely on the surface of CS-MWCNT/GCE as a spongy gold film, the conductivity and electrocatalytic activity of the modified electrode improved. Consequently, the peak current increased further. After the stem-loop probes were assembled on the spongy gold film/CS-MWCNT/GCE, the peak current decreased (curve d), which indicates that the probes were successfully immobilized on the modified electrode surface. Because the stem-loop probe occupied a large area on the modified electrode surface, the electrode could not efficiently exchange electrons with the solution, and this reduced the electron-transfer efficiency of the electrode. After immobilization of MCH, the effective area of the modified electrode reduced and the peak current decreased further (curve e).

The hybridization was characterized in buffer containing H_2O_2 and hydroquinone. As shown in **Fig.2B**, the CVs of the MCH/stem-loop probe/spongy gold film/CS-MWCNT/GCE (curve a), target DNA/MCH/stem-loop probe/spongy gold film/CS-MWCNT/GCE (curve b), and BSA/target DNA/MCH/stem-loop probe/spongy gold film/CS-MWCNT/GCE (curve c) were almost the same, and the redox peak positions and peak currents showed no remarkable differences. However, when HRP-SA was attached to the surface of BSA/target DNA/MCH/stem-loop probe/spongy gold film/CS-MWCNT/GCE, the reductive peak current increased sharply, and redox peak-to-peak separation decreased. This indicated that the benzoquinone were produced by oxidation of hydroquinone by H_2O_2 via the catalytic action of HRP, and the benzoquinone was reduced back to hydroquinone which greatly increased the reduction peak current of the benzoquinone.

3.4 Quantification of target DNA

In this study, chronoamperometry was used to examine the sensitivity of the prepared electrochemical DNA biosensor. The chronoamperometry current was measured in 0.1 mol L^{-1} PB (pH 7.0) containing 1 mmol L^{-1} H_2O_2 and 0.2mmol L^{-1} hydroquinone. As shown in **Fig.3**, the current increased gradually as the target DNA concentration increased from 3.91×10^{-17}mol L^{-1} to 1.25×10^{-15}mol L^{-1}. The inset of **Fig.3** shows the change in the measured current (I) as a function of the target concentration (C).There was a linear relationship between the current and the

logarithm of target concentration, with a regression equation of I=0.6756logC+12.953 and a correlation factor of 0.9943. The detection limit calculated using three times the standard deviation of the background signal was 1.3×10^{-17}mol L^{-1}.Theperformance of this biosensor was compared with that published in similar work (**Table 1**). The present DNA sensor had good analytical performance for DNA detection with the target sequences.

3.5 Selectivity of the DNA biosensor

The selectivity of the DNA biosensor was investigated, and the results are shown in **Fig. 4**. The prepared sensor was hybridized with various DNA sequences (complementary oligonucleotides, one base mismatch oligonucleotides and non-complementary oligonucleotides), and then the chronoamperometry current was measured. As Fig. 4 shows, compared with the current of MCH/stem-loop probe/spongy gold film/CS-MWCNT/GCE (curve a), the current obtained following hybridization with the non-complementary oligonucleotides (curve b) was almost the same. The current obtained following hybridization with the one-base mismatch oligonucleotides (curve c) was slightly higher. After hybridization with the complementary oligonucleotides (curve d), the current increased obviously. This result indicates that the immobilized stem-loop probes react only slightly with the one base mismatch and non-complementary oligonucleotides, and complete hybridization does not occur because of the base mismatch. In other words, the prepared DNA biosensor has excellent selectivity for detection of target DNA sequences, which means the ability of the DNA biosensor to discriminate between non-complementary or single-base mismatch oligonucleotides and complementary oligonucleotides are remarkable.

3.6 Optimization of experimental conditions.

Analytical performance by this new electrode was optimized by investigating the effects of some important detection parameters, including deposition time of gold nanoparticles, probe concentration during immobilization and hybridization time and temperature.In order to improve determination sensitivity for A rah1, the effect of deposition time on peak current were investigated by the differential pulse voltammogram (DPV). It was observed that the peak current increased obviously as deposition time increases from 200s to 600s (**Fig. s1**). When the time increased to 800s and 1000s the current value increased slightly or not at all. Thus, we chose 600s as the optimal deposition time for this work.

The effect of probe concentration, hybridization time and temperature was estimated by

chronoamperometry. The reduction peak current of benzoquinone was investigated. It showed that the signal increased as the probe concentration increased from 10^{-9} to 10^{-5} M, with the maximum value at10^{-6} M (**Fig. s2**). As the DNA probe concentration increased, more probe material was immobilized on the electrode until saturation adsorption. Many studies have shown that hybridization temperature is related to the Tm of the probe and the optimal hybridization temperature considered being at 25 °C lower than the Tm. In the present study, the DNA probe Tm was 74.61°C and the theoretical optimal hybridization temperature thus under 50°C. Therefore, the temperature was analyzed at three levels(27,37,47℃). **Fig. s3 shows** the effects of hybridization time and temperature on the reduction current of benzoquinone. It was observed the hybridization of DNA reached to the highest within 20 min at 47℃, with time extending the current signal increasingly only slightly or not at all. It may be explained higher temperatures significantly affect the hairpin dynamics, which is reflected in increased sensor sensitivity. when hybridization temperature were 27 and 37℃, the current value increased to the highest at 30 min. Then the current changed slightly during longer times. Moreover, compared to 27℃ the reduction current is higher at 37℃. Thus, we choose 37℃, 30 min as the optimal hybridization conditions for the following experiment.

3.7 Stability of the DNA biosensor

Because of the activity of streptavidin and HRP, the stability of the DNA biosensor was evaluated over a 10 day period. The HRP-SA/BSA/target DNA/MCH/stem-loop probe/spongy gold film/CS-MWCNT/GCE were stored in 10 mmol L^{-1} PBS buffer (pH 7.4) at 4 °C for 10 days. After this time, the chronoamperometry current of the sensor decreased to 80.62% of its initial response. This indicated that the stability of the developed DNA biosensor would be greatly affected by the activity of streptavidin and HRP.

3.8 Detection of the peanut allergen Ara h1 in peanuts

With the high sensitivity and selectivity, the biosensor could detect Ara h1 residues directly in foods. In this study, a PCR study confirmed the successful extraction of the Ara h1 gene from peanuts. After the extracted genome DNA from the peanuts was amplified by PCR, the products were detected by gel electrophoresis with 7.5% PAGE. In **Fig. s4**, the bands in the rightmost lane were markers, and the bands in the left three lanes were amplified DNA. The 125 bp band was the

amplified target strip.

After the extracted genome DNA was incubated in a 95 °C water bath for 10 min, it was diluted to different concentrations for hybridization with the MCH/stem-loop probe/spongy gold film/CS-MWCNT/GCE. Then after the hybridized electrode was blocked with BSA, and bonded to HRP-SA, the chronoamperometry current was measured. The concentration of Ara h1 gene in the peanuts was calculated as $(2.74\pm0.11)\times10^{-11}$ molL^{-1}.

We also used a fluorescence real-time quantitative PCR method to authenticate the test results obtained with the DNA biosensor. As **Fig.5** shows, a linear relationship existed between the Ct value and the logarithm of the standard concentration from 5×10^{-17} to 5×10^{-12} mol L^{-1} with a regression equation of Ct=–1.8156logC–4.3691 and a correlation factor of 0.9955.Then the extracted genome DNA was also detected using this fluorescence real-time quantitative PCR system. The concentration of Ara h1 gene in the peanuts was $(2.88\pm0.05)\times10^{-11}$ molL^{-1}. The two results were consistent, which indicates that the developed DNA biosensor is reliable for sample detection. However, the relative standard deviation shows the electrochemical sensing method is less stable than fluorescence quantitative PCR method.

4. Conclusions

We developed a novel electrochemical DNA biosensor using CS-MWNTs, spongy gold film, and an immobilized stem-loop probe that was dually labeled with 5'-SH and 3'-biotin.The DNA biosensor showed high sensitivity, with a detection limit of 0.013 fmol L^{-1}. The DNA biosensor had excellent selectivity for the detection of target DNA sequences. In addition, the proposed DNA biosensor was also beneficial for the analysis of peanut DNA extracts. The concentration of the Ara h1 gene in the peanuts tested was $(2.74\pm0.11)\times10^{-11}$ molL^{-1}, which is consistent with fluorescence real-time quantitative PCR results. It is expected that the proposed electrochemical assay using the newly developed electrochemical DNA biosensor will be widely applied in the clinical diagnosis of peanut allergens and in food safety.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.10.041

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Figure captions

Fig. 1 The TEM photo of CS-MWCNT (A) and SEM photo of spongy gold film (B)

Fig.2 The characterization of CV in the progress of electrode's modification in the $Fe(CN)_6^{3-/4-}$ buffer (A) and characterization of CV in the progress of hybridization in the buffer containing H_2O_2 and hydroquinone (B). In figure A, a: GCE, b: CS-MWCNT/GCE, c: spongy gold film/CS-MWCNT/GCE, d: probe/spongy gold film/CS-MWCNT/GCE, e: MCH/probe/spongy gold film/CS-MWCNT/GCE. In figure B, a: MCH/ probe/spongy gold film/CS-MWCNT/GCE, b: target DNA/MCH/ probe/spongy gold film/CS-MWCNT/GCE, c: BSA/target DNA/MCH/ probe/spongy gold film/CS-MWCNT/GCE, d: HRP-SA/BSA/target DNA/MCH/ probe/spongy gold film/CS-MWCNT/GCE.

Fig. 3 The chronoamperometry curve and standard curve of hybridization of DNA biosensor with target DNA with different concentrations (a-f: 3.91×10^{-17}, 7.81×10^{-17}, 1.56×10^{-16}, 3.13×10^{-16}, 6.25×10^{-16}, 1.25×10^{-15} molL^{-1})

Fig. 4 The chronoamperometry curve of MCH/stem-loop probe/spongy gold film/CS-MWCNT/GCE (a) hybridization with non-complementary sequence (b), single mismatched sequence (c) and complementary target sequence (d), then reaction with SA-HRP

Fig. 5 The standard curve of real-time quantitative PCR

Scheme1. The schematic diagram of fabricating the stem-loop DNA biosensor of amplifying signal by enzyme catalysis

Table 1 Comparison of linear ranges and detection limits of several electrochemical DNA sensors

Layers for DNA immobilization	Detection limit($nmolL^{-1}$)	Linear range ($molL^{-1}$)	Reference
AuNPs/pThion/graphene/GCE[a]	0.035	1.0×10^{-8}–1.0×10^{-13}	[41]
PANI/AuNPs/GSPE[b]	0.1	1.0×10^{-8}–2.0×10^{-8}	[42]
AuNPs/PANI/CS-GS/GCE[c]	2.11×10^{-3}	1.0×10^{-9}–1.0×10^{-11}	[43]
PANIw/graphene/GCE[d]	3.25×10^{-8}	2.12×10^{-6}-2.12×10^{-12}	[44]
Spongygoldfilm/CS-MWCNT/GCE[e]	4.1×10^{-8}	3.91×10^{-17}–1.25×10^{-15}	This work

[a]AuNPs/pThion/graphene/GCE: GCE modified with gold nanoparticles, polythionine, and graphene

[b]PANI/AuNPs/GSPE: graphite screen-printed electrode modified with polyaniline film and gold nanoparticles

[c]AuNPs/PANI/CS-GS/GCE: gold nanoparticles, PANI layer (polymerization of aniline) , chitosan- graphene modifided GCE

[d]PANIw/graphene/GCE: graphene and polyaniline nanowires modified GCE

[e]Spongy gold film/CS-MWCNT/GCE: chitosan-mutiwalled carbon nanotubes nanocomposite and spongy gold film modified GCE

Fig.1

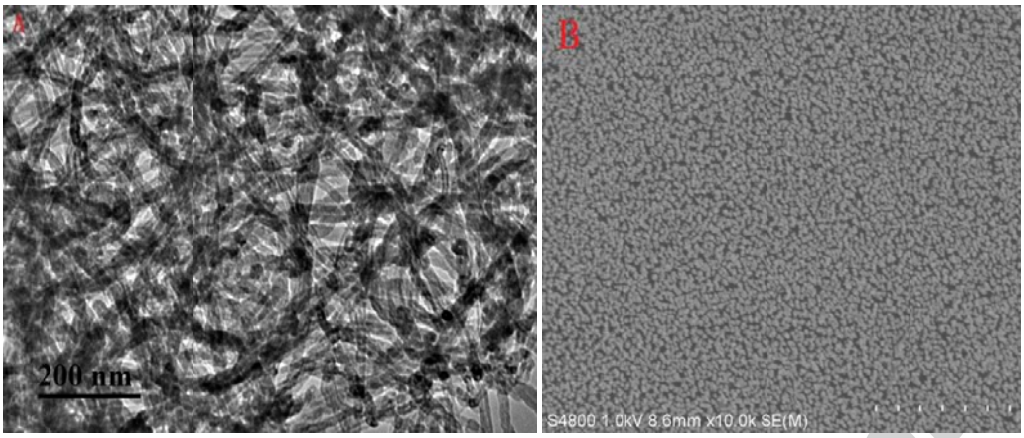


Fig.2

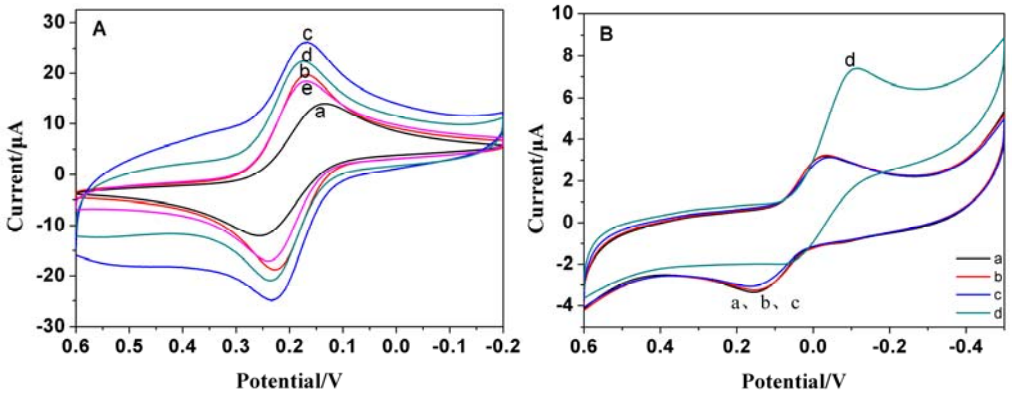


Fig.3

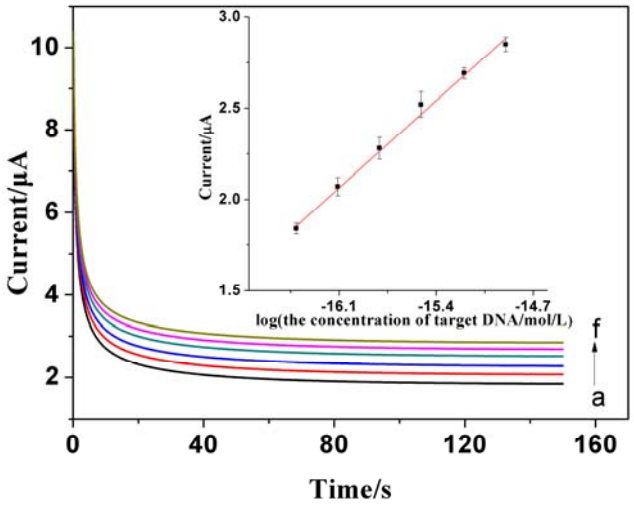


Fig.4

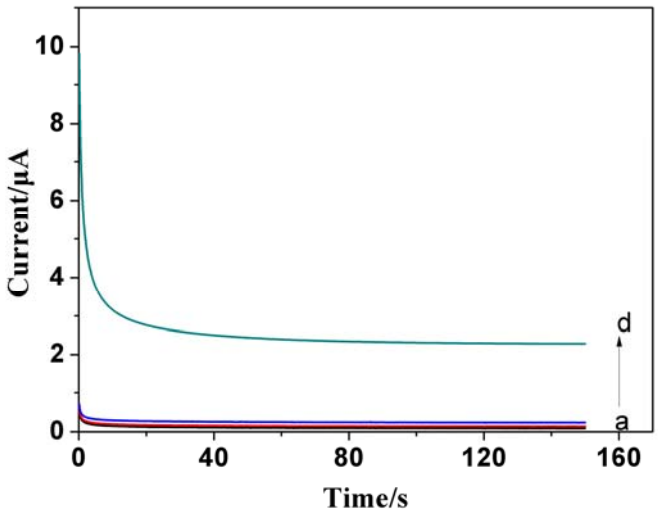
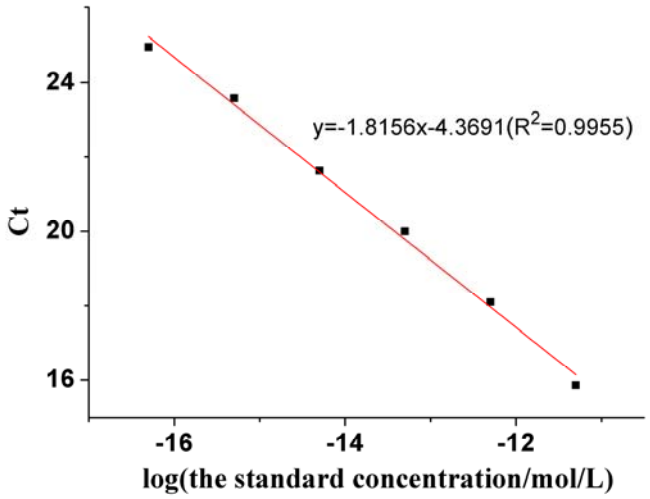
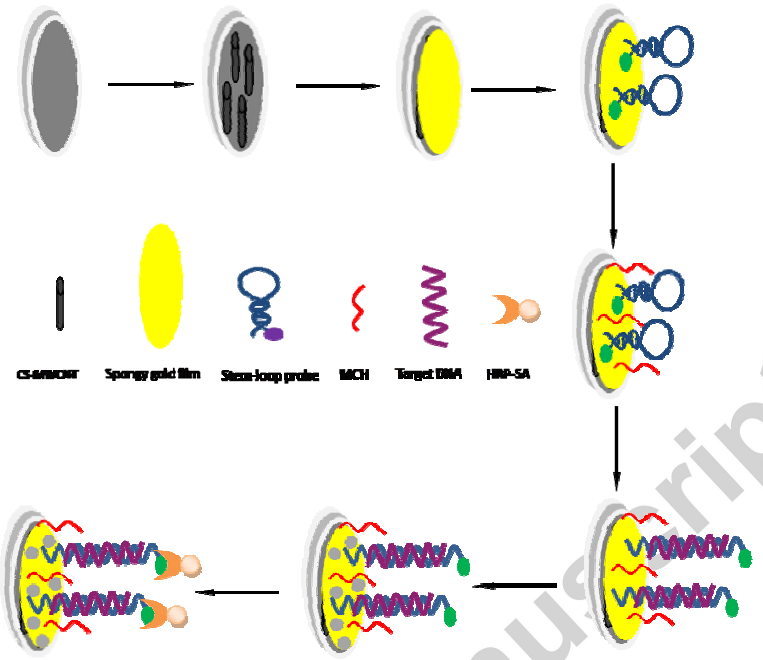


Fig.5



Scheme.1



Enzymatic amplification detection of peanut allergen Ara h1 using a stem-loop DNA biosensor modified with a chitosan-mutiwalled carbon nanotube nanocomposite and spongy gold film

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Graphical abstract