Talanta 141 (2015) 300–306

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A highly selective and sensitive electrochemical CS–MWCNTs/Au-NPs composite DNA biosensor for *Staphylococcus aureus* gene sequence detection

Yange Sun, Xingxing He, Jian Ji, Min Jia, Zhouping Wang, Xiulan Sun*

State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Synergetic Innovation Center of Food Safety and Nutrition, Jiangnan University, Wuxi, Jiangsu 214122, China

ARTICLE INFO

Article history:
Received 13 January 2015
Received in revised form
19 March 2015
Accepted 24 March 2015
Available online 1 April 2015

Keywords:
DNA biosensor
Staphylococcus aureus
MWCNTs
Gold nanoparticles
Methylene blue (MB)

ABSTRACT

This paper presents a new electrochemical DNA biosensor constructed using a substrate electrode composed of a novel nanocomposite material prepared using gold nanoparticles (Au-NPs) and multi-walled carbon nanotubes (MWCNTs) and further modified with an Au electrode (AuE), which was used as the substrate electrode. A single-stranded DNA (ssDNA) probe was immobilized on the Au-NPs/CS–MWCNTs/AuE electrode by means of facile gold–thiol affinity, which resulted in hybridization with the target ssDNA sequence. Hybridization reactions were assessed by using the reduction peak current of methylene blue (MB) as an electrochemical indicator. The advantages of the nanomaterials were found to include high surface area, favorable electronic properties, and strong electrocatalytic activity. The amount of ssDNA adsorbed on the electrode surface was increased and the electrochemical response of MB accelerated. The differential pulse voltammetric responses of MB were in line with the specific target ssDNA sequence within the concentration range 1.0×10^{-15}–1.0×10^{-8} M with the detection limit 3.3×10^{-16} M (3σ). In the colony forming unit (CFU) we were able to detect 10 CFU $mL^{-1}$of *Staphylococcus aureus* in the tap water, achieving good discrimination ability between one- and three-base mismatched ssDNA sequences. The polymerase chain reaction (PCR) amplification products of *S. aureus nuc* gene sequence were also detected with satisfactory results.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a dangerous bacterial pathogen that produces a variety of toxins. It is the root cause of a wide range of infections, including soft-tissue infections, Staphylococcal food poisoning, and a number of life-threatening diseases [1], such as pneumonia, endocarditis, osteomyelitis, arthritis, and sepsis. *S. aureus* is also one of the top five pathogens contributing to foodborne illnesses in America [2]. Therefore, it is of great importance to develop a simple, precise, and sensitive method for *S. aureus* detection.

Various methods have been used for the detection of pathogenic bacteria, including conventional culture methods, enzyme-linked immunosorbant assay (ELISA) and polymerase chain reaction (PCR) [3]. The high cost in preparing monoclonal antibodies and the long incubation time (at least 1 day) for ELISA restricted [4] and its sensitivity is insufficient to detect low levels of *S. aureus*. PCR method has distinct advantages in sensitivity, but it often encounters false positivity [5] and complicated pretreatment process [6]. These methods have been successfully applied in various fields, such as environmental test and medicine industry. However, the deficiencies in the current methods create an urgent demand for new, high-stability systems that detect the pathogen in faster and cheaper ways and at lower detection limit [7]. Therefore, the biosensor technologies for the *S. aureus* have attracted considerable interest for their intrinsic advantages such as high-throughput analysis, high sensitivity and specificity [8].

Due to the higher sensitivity and lower detection limit, electrochemical DNA sensor has been widely reported for the detection of target ssDNA sequences from different kinds of samples. Electrochemical analysis is a sensitive detection method with wider linear range and lower detection limit, which can be used for the detection of various kinds of electroactive samples [9–11]. Electrochemical detection of DNA hybridization is commonly based on the measurement of the changes of electrochemical response after the hybridization reaction took place on the surface of working electrode [12]. Therefore the design and preparation of probe ssDNA sequence modified electrode are of great importance

* Corresponding author. Tel./fax: +86 510 8532872.
E-mail address: sxlzzz@jiangnan.edu.cn (X. Sun).

http://dx.doi.org/10.1016/j.talanta.2015.03.052
0039-9140/

in developing the electrochemical DNA sensors [13,14]. Various kinds of transducer surfaces have been devised for the fixation of probe ssDNA sequence, which can result in the high immobilization amounts of ssDNA concentration on the electrode surface with suitable orientation. In recent years nanoparticle based electrodes have been devised for the immobilization of ssDNA sequence, which exhibit the advantages such as larger surface area and better biocompatibility with increased conductivity [15].

In particular, chitosan (CS), has been used extensively in the fields of agriculture, horticulture, industry, biomedicine, and chemical sensors [16,17]. The numerous advantages of CS include its nontoxic nature, excellent film forming ability, and cost effectiveness. However, its poor electrical conductivity often results in low sensitivity and hinders determination of analytes. In order to circumvent this problem, researchers have employed a number of strategies to improve the redox properties of CS. Popular methods include doping with nanoparticles [18] and/or chemical modification using a conducting polymer [19,20]. These methods fully consider the structural features of CS that contain large quantities of active amino and hydroxyl groups. However, in order to enhance the electrical conductive properties and specific surface area of the biosensor, a typical carbon material, multiwalled carbon nanotubes (MWCNTs), have been doped into CS film [21]. MWCNTs possess the characteristics of high electrical conductivity, chemical stability, and a high surface-to-volume ratio, resulting in biosensors that exhibit dramatically improved electron-transfer kinetics.

In this study, we used a stem–loop probe labeled with 5′-SH, which can be self-assembled through facile gold–thiol affinity on modified electrodes. The goals of this study were 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 accurately detect the *S. aureus* nuc gene sequence with the constructed biosensor.

2. Experimental

2.1. Apparatus and chemicals

All the electrochemical experiments were performed on a CHI 650C electrochemical station (Shanghai, China). A three-electrode system was employed for the electrochemical investigation: modified Au acted as the working electrode, a Pt wire as the auxiliary electrode, and a saturated calomel electrode (SCE) as the reference electrode. The PCR amplification was performed on an Eppendorf Mastercycler Gradient PCR system (Eppendorf, Germany).

Mutiwalled carbon nanotubes (MWCNTs) with a diameter of 10–20 nm were provided by the Shenzhen Nanotechnology Port Company (Shenzhen, China). Chitosan (M.W. 100,000– 300,000, deacetylation degree $\geq$ 95%) was provided by the Aladdin Reagent Company (Shanghai, China). Methylene blue (MB) and 6-mercapto-1-hexanol (MCH) were purchased from Sigma (USA). Solutions for the PCR experiments were prepared as usual. These included the final concentration of 0.8 μM primer mixture (10 μM each), (Sangon Biotech, Shanghai), 2 × PCR Master Mix (2 × Taq DNA Polymerase, 2 × PCR Buffer, 2 × dNTP) (Beyotime Biotech, Shanghai), 1 × TE buffer(10.0 mM Tris–HCl, 1.0 mM EDTA, pH = 8.0), and a 10.0 mM phosphate-buffered solution (PBS, pH = 7.4). All solutions were prepared with twice-distilled water and all the experiments were conducted at room temperature unless specifically indicated otherwise.

A 33-base oligonucleotides probe (probe ssDNA), target complementary sequence DNA (target ssDNA), 1-base mismatched ssDNA, 3-base mismatched ssDNA, and noncomplementary sequence DNA (ncDNA), which were related to the nuc gene sequence of *S. aureus,* were synthesized by Shanghai Sangon Biological Engineering Technological Co. Ltd. (China). Their base sequences are listed below:

Stem–loop probe ssDNA: 5′-HS-C6-GCG AGG GCG ATT GAT GGT GAT ACG GTT CCT CGC-3′

Target ssDNA: 5′-AAC CGT ATC ACC ATC AAT CGC-3′

1-base mismatched ssDNA: 5′-AAC CGT CTC ACC ATC AAT CGC-3′

3-base mismatched ssDNA: 5′-AAC CCT ATC ACG ATC AAT GGC-3′

Noncomplementary ssDNA: 5′-GCG ATT GAT GGT GAT ACG GTT-3′

The DNA sample used for PCR amplification was extracted from *S. aureus* strains. The PCR reaction was performed on an Eppendorf Mastercycler Gradient PCR system using oligonucleotide primers for the nuc gene of *S. aureus* with the following sequences:

Primer F: 5′-AAT TAA CGA AAT GGG CAG AAA CA-3′

Primer R: 5′-TGC GCA ACA CCC TGA ACT T-3′

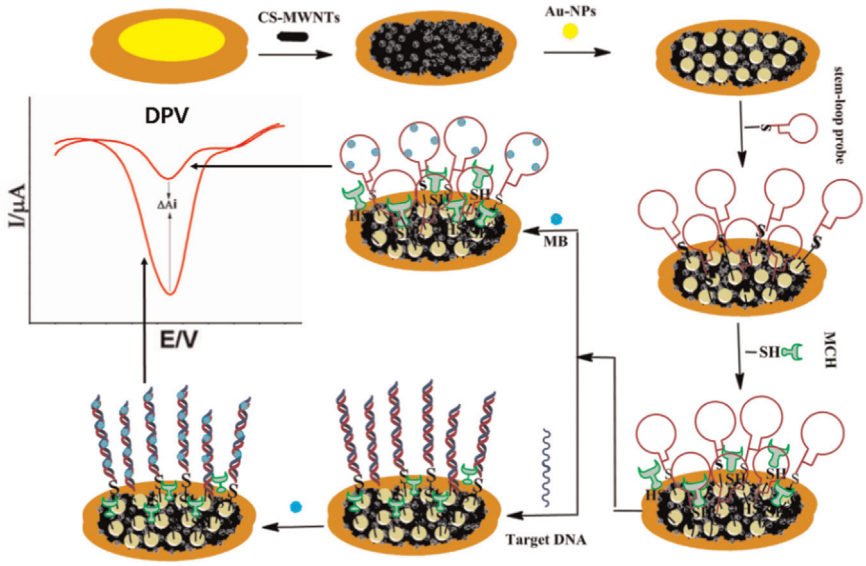
The process of constructing the electrochemical DNA biosensor is illustrated in Scheme 1. First, the dispersing solution for the CS–MWCNTs nanocomposite was prepared according to the literature. Then, 1 mg of MWCNTs was put into 2 mL of 2% acetic acid solution containing 0.5 mg CS and ultrasonicated for 2 h to obtain a uniform black solution, which was recorded as CS–MWCNTs. Before modification, the surface of the Au electrode was polished until mirrorlike with 1.0, 0.3, and 0.05 μm alumina consecutively using a microcloth pad, ultrasonicated in ethanol and water for 2 min, and finally dried under an N_2 flow. $HAuCl_4 \cdot 4H_2O$ was obtained from Guoyao Chemical Co.

Then, 4 μL of the prepared CS–MWCNTs gel was dropped onto the surface of the cleaned electrode and dried under an infrared lamp to form a CS–MWCNTs nanocomposite film on the electrode surface (CS–MWCNTs/AuE). The electrode was washed with DDW and the fabricated CS–MWCNTs/AuE was then used for the electrodeposition of Au-NPs. The CS–MWCNTs/Au-NPs composite was modified from AuE by immersing the CS–MWCNTs/AuE into a 3.3 mM $HAuCl_4$ solution containing 1% $HAuCl_4$ (m/v) and 0.1 M HNO_3 solution and applying a constant potential of −0.2 V for 300 s. Fig. 1 shows a TEM image of the Au nanoparticles, which have an average diameter of 50 nm on the fabricated CS–MWCNTs. Finally, the fabricated CS–MWCNTs/Au-NPs composite modified from AuE was stored at 4 °C until needed for further experiments.

2.2. Fabrication of electrochemical DNA biosensor

Immobilization of the ssDNA probes was performed by dropping 8.0 μL of 1.0×10^{-5} M probe ssDNA (in 1 × TE buffer pH = 8.0) directly onto the surface of the Au-NPs/CS–MWCNTs/AuE electrode. The stem–loop probe labeled with 5′-SH was immobilized on the Au-NPs/CS–MWCNTs/AuE electrode by means of facile gold–thiol affinity and then kept at 4 °C overnight [22,23]. To avoid volatilization of the solution, the electrode solution was covered with a plastic cap after the probe was added. Then, the electrode was washed with 10.0 mM phosphate-buffered solution (PBS, pH = 7.4) and twice-distilled water 3 times successively to remove any unadsorbed probe ssDNA from the surface of electrode. This probe-captured electrode was denoted as ssDNA/Au-NPs/CS–MWCNTs/AuE. Then 8 μL of 1 mM MCH was dropped on the electrode surface and left to set for 1 h, to cover the remaining bare regions [24]. Subsequently, the electrode was rinsed thoroughly with a copious amount of PBS buffer to remove any unattached MCH.

An efficient drop hybridization procedure was then used to achieve hybridization of the target ssDNA sequence with the probe ssDNA-modified electrode [25,26]. A 4.0 μL droplet containing various concentrations of the target ssDNA sequence (ranging between 10^{-15} and 10^{-8} M) was deposited directly onto the electrode surface, which had been flipped upside down to hold the solution. The hybridization between the target sequence and



Scheme 1. Schematic diagram of Au–thiol affinity of stem–loop probe DNA on Au-NPs/CS–MWNTs/AuE DNA biosensor.

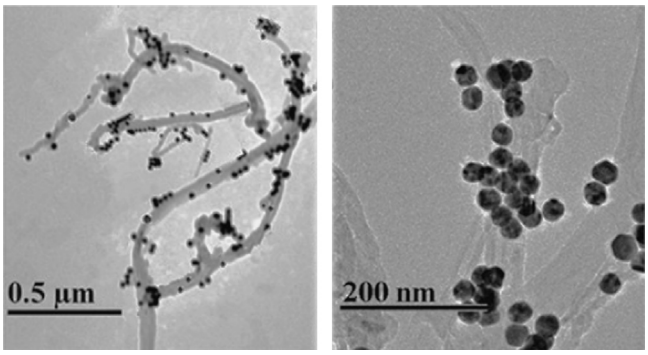


Fig. 1. TEM image of Au nanoparticles on the fabricated CS–MWNTs.

the probe sequence was allowed to proceed for 30 min at 37 °C. During this procedure, a small beaker was fit tightly over the electrode to prevent rapid evaporation of the ssDNA solution until the completion of hybridization. Then, the electrode was washed with 10.0 mM PBS buffer and twice-distilled water 3 times to remove the unhybridized target ssDNA sequence. This hybridized electrode was labeled dsDNA/Au-NPs/CS–MWCNTs/AuE.

2.3. Electrochemical detection

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were used to characterize the stepwise modification of the electrode. The supporting electrolytes investigated using CV and EIS were 2.5 mM $[Fe(CN)_6]^{3-/4-}$ with 0.1 M KCL. The scan rate of CV was 100 mV s^{-1}, and the potential window was between +0.6 and −0.2 V. Electrochemical impedance (EI) spectra were collected in the frequencies ranging from -10^4 to −0.1 Hz. The dsDNA/Au-NPs/CS–MWCNTs/AuE electrode was immersed in 5.0×10^{-5} M MB solution for 10 min to accumulate the electrochemical indicator (MB) and then washed with 10.0 mM PBS and twice-distilled water 3 times. The corresponding signal of MB accumulated on the electrode surface was measured using sensitive differential pulse voltammetry (DPV) in a 10.0 mM PBS buffer solution (pH=7.4) with the instrumental parameters pulse amplitude 0.008 V, pulse width 0.05 s, and pulse period 0.2 s.

2.4. Polymerase chain reaction of the S. aureus nuc gene sequence

Amplification of *nuc* gene fragments was carried out in a final volume of 20 μL in 0.2 mL tube containing 10.0 μM each of *nuc* primer F and *nuc* primer R, 2× PCR Master Mix (2× Taq DNA Polymerase, 2× PCR Buffer and 2× dNTP), and 1.0 μL DNA template purified from the samples. During the PCR procedure, the DNA was initially denatured at 94 °C for 3 min. PCR conditions were optimized as follows: 30 cycles of amplification (94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min), with a final extension conducted at 72 °C for 10 min. The PCR products without the DNA template were used for comparison. All of the PCR products were kept at 4 °C before use.

2.5. Regeneration of the biosensor

The regeneration of the biosensor was performed by immersing the hybridized electrode into 50 mM NaOH for 60 s and then washing it with TE buffer followed by DDW. The target sequence was consequently removed from the surface of the biosensor and the ssDNA/Au-NPs/CS–MWCNTs/AuE electrode was returned to its original state [27].

3. Results and discussion

3.1. Differential pulse voltammogram of MB at different modified electrodes

MB is a commonly used electrochemical indicator in DNA biosensors that can distinguish ssDNA from dsDNA sequences [28]. MB exhibits different behavior when interacting with ssDNA and dsDNA, including electrostatic binding and intercalation with the major or minor grooves of dsDNA helixes. How MB interacts with ssDNA and dsDNA is related to different experimental conditions. Its interaction is affected by the DNA sequences themselves, ion strength, MB concentration, and buffer pH, which results in the higher affinity of dsDNA with MB and can be used to distinguish between dsDNA and ssDNA [29]. Fig. 2 shows the differential pulse voltammograms of 5.0×10^{-5} M MB for Au-NPs/CS–MWCNTs/AuE (Curve a), ssDNA/Au-NPs/CS–MWCNTs/AuE (Curve b), MCH/ssDNA/Au-NPs/CS–MWCNTs/AuE (Curve c), and dsDNA/MCH/Au-NPs/CS–MWCNTs/AuE (Curve d)

in 10.0 mM PBS buffer solution. The smallest peak current of MB occurred with the Au-NPs/CS–MWCNTs/AuE electrode (Curve a). With the immobilization of ssDNA on the modified electrode, the reduction peak current increased a little (Curve b), indicating that a small amount of MB had been adsorbed by the electrode surface due to its interaction with ssDNA through electrostatic attraction. With the addition of MCH on the ssDNA modified electrode, the reduction peak current increased a little more (Curve c). Using the dsDNA modified electrode greatly increased the reduction peak current (Curve d), which can be attributed to the strong intercalation between MB and dsDNA. The reduction peak current of MB also increased gradually with the step-by-step incorporation of nanomaterials into the film, indicating that the amount of dsDNA on the electrode surface was also increased. These results were attributed to the incremental increase of the effective electrode area, which provides more sites for the immobilization of dsDNA on the electrode surface, which in turn leads to the accumulation of more MB molecules on the electrode surface, resulting in an increase in the reduction peak current of MB. The largest reduction peak current appeared with the dsDNA/MCH/Au-NPs/CS–MWCNTs/AuE electrode, illustrating that larger the amount of dsDNA present on the electrode surface, the more interaction there is with MB molecules.

3.2. Effect of MB concentration and accumulation time

The concentration and accumulation time of the hybridization indicator can influence the performance of the prepared DNA biosensor. Consequently, in order to maximize the DNA sensor's efficiency and sensitivity, experimental conditions were optimized according to the results shown in Fig. 3. As shown in Fig. 3A, current response increased with MB concentration from 1.0×10^{-5} to 7.0×10^{-5} M, stabilizing at a concentration of 5.0×10^{-5} M or above. Therefore, an MB concentration 5.0×10^{-5} M was employed as the indicator. MB accumulation time on the electrode surface was also investigated for optimization. The highest current signal of MB was obtained after 12 min of accumulation, and any accumulation time exceeding 12 min resulted in a signal plateau (Fig. 3B). Hence, 12 min was chosen as the optimal accumulation time.

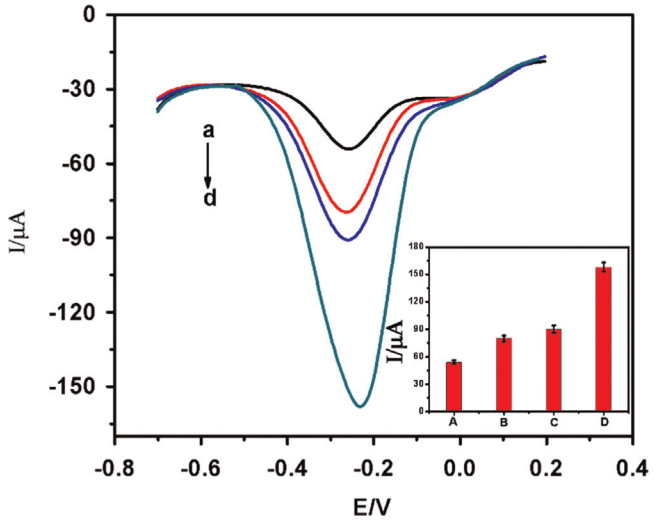


Fig. 2. DPV of 5.0×10^{-5} M MB at different modified electrodes including Au-NPs/CS–MWNTs/AuE (a), ssDNA/Au-NPs/CS–MWNTs/AuE (b), MCH/ssDNA/Au-NPs/CS–MWNTs/AuE (c), dsDNA/MCH/ssDNA/Au-NPs/CS–MWNTs/AuE, and (d) in 10.0 mM PBS buffer solution (pH 7.4).

3.3. Selectivity of the electrochemical DNA biosensor

Selectivity was investigated by using ssDNA/Au-NPs/CS–MWCNTs/AuE to hybridize four different ssDNA sequences related to the *nuc* gene of *S. aureus* and ssDNA sequences of four different pathogenic bacteria. Fig. 4A shows the reductive peaks of MB using the different DNA-modified electrodes. The biggest reduction peak current (130.6 μA) was obtained after hybridization with the complementary ssDNA sequence, indicating that the formation of hybridized dsDNA molecules on the electrode surface led to the greatest amount of MB accumulation among the four sequences. The reduction peak current of MB was 54.2 μA after hybridization with the non-complementary ssDNA, which may be attributed to the non-specific adsorption of ssDNA on the electrode surface. After the probe ssDNA was hybridized with the 3-base mismatched ssDNA and the 1-base mismatched ssDNA, the reductive peak currents increased to 79.7 and 96.9 μA respectively. As shown in Fig. 4B, the current intensities from *S. aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophytics*, and *Bacillus cereus* were approximately 135 μA, 62 μA, 58 μA and 52 μA respectively. The difference could be attributed to the hybridization efficiency of the four types of ssDNA with the specific immobilized capture probes, as the probes could specifically and robustly bind to the complementary ssDNA while the others could not. Overall, the results demonstrated that the fabricated DNA biosensor exhibited high selectivity and a fairly strong ability to distinguish different hybridized ssDNA sequences.

3.4. Calibration curve

As previously described, under the given optimal conditions, the electrochemical DNA biosensor was hybridized with different concentrations of the target ssDNA sequence related to *nuc* gene sequence. Then, the reductive peak current of MB was measured for each sequence hybridized with the biosensor. The MB peak current increased gradually with the increase of the target ssDNA concentration: the typical differential pulse voltammograms are shown in Fig. 5. The differences between reductive peak currents of MB before and after hybridization were linear with the logarithmic value of the *nuc* gene ssDNA sequence concentration in the range from 1.0×10^{-15} M to 1.0×10^{-8} M with a correlation coefficient of 0.9945. The linear regression equation is given by ΔI (μA) = 11.7238 log[c/(M)] + 215.9663, where c is the concentration of *S. aureus nuc* gene sequence and ΔI is the difference between the MB peak current before and after hybridization. The detection limit of the *S. aureus nuc* gene sequences was estimated to be 3.3×10^{-16} M (3σ), where σ is the relative standard deviation of the blank solution, $n=11$. The relative standard deviation (RSD) for the detection of 1.0×10^{-9} M target ssDNA sequences was 4.7%, indicating the stronger plicability of this measurement.

3.5. Detection of the PCR product of the S. aureus nuc gene

The electrochemical DNA biosensor was then used to detect PCR-amplified product of the *S. aureus nuc* gene under the selected conditions. The template DNA used in PCR amplification was extracted from *S. aureus* strains, and the amplification process was based on the previously mentioned PCR conditions. The PCR-amplified samples were analyzed via agarose gel electrophoresis separation and visualized by first straining the DNA with ethidium bromide and then viewed using a UV transilluminator. The visualization process revealed a final gene fragment length of 166 bp, as shown in Fig. 6A. The obtained product was subsequently diluted with 1 × TE buffer solution (PH 8.0), denatured in a boiling water bath for 10 min and cooled in an ice bath for 2 min. Then 4.0 μL of the denatured PCR-amplified product was dropped

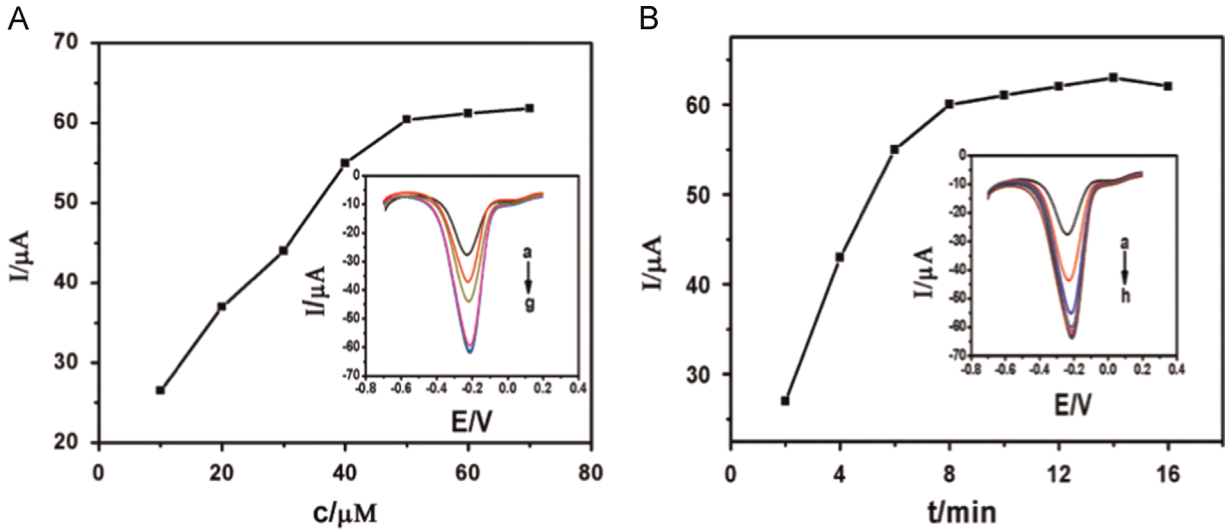


Fig. 3. Optimization of concentration (A) and accumulation time (B) of MB.

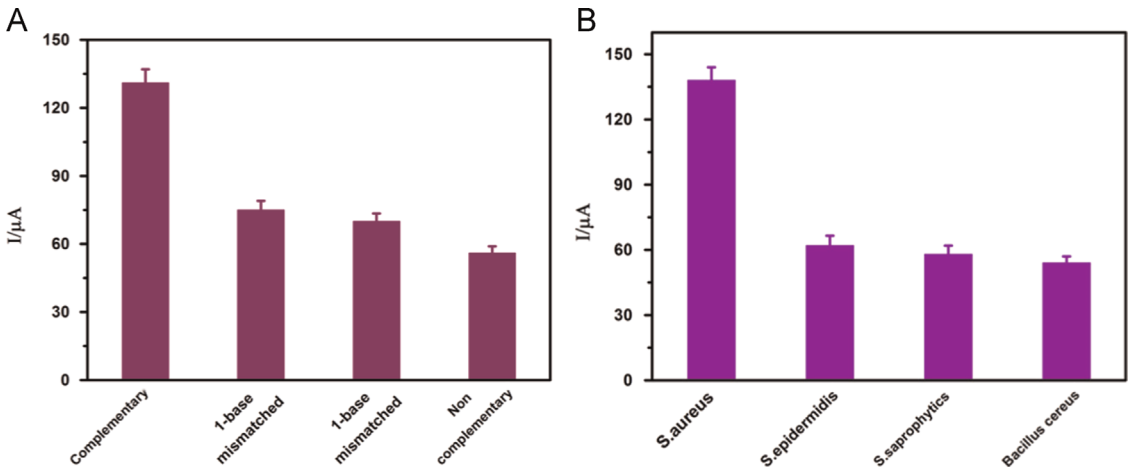


Fig. 4. The histogram for the reduction peak current of 5.0×10^{-5} M MB after hybridization with four different synthetic ssDNA (A) and four ssDNA from different types of pathogenic bacteria (B).

directly onto ssDNA/Au-NPs/CS–MWCNTs/AuE for hybridization. After hybridization, the electrode was immersed into MB solution for detection using the experimental procedure, the results of which are given in Fig. 6B. As shown in Fig. 6B, an increase in the reduction peak current appeared after hybridization with the denatured PCR-amplified sample of the *nuc* gene (Curve b). This increase was much larger than that of the hybridization performed using the PCR solution without the template DNA (Curve a), indicating that the PCR experiment had proceeded smoothly and that the target ssDNA sequences had been successfully amplified. The slight reduction peak current on Curve a may have been caused by the non-specific adsorption effect. The obvious increase in the reduction peak current shown by Curve b indicates that the hybridization reaction was successfully accomplished on the electrode surface, resulting in the formation of dsDNA and the accumulation of more MB molecules. The significant differences between the MB signals of the control sample and the hybridized dsDNA modified electrode confirm the strong selectivity of the constructed DNA biosensor. Given the strength of its selectivity, the sensor can be further applied to effectively detect the *S. aureus nuc* gene sequence in PCR-amplified samples.

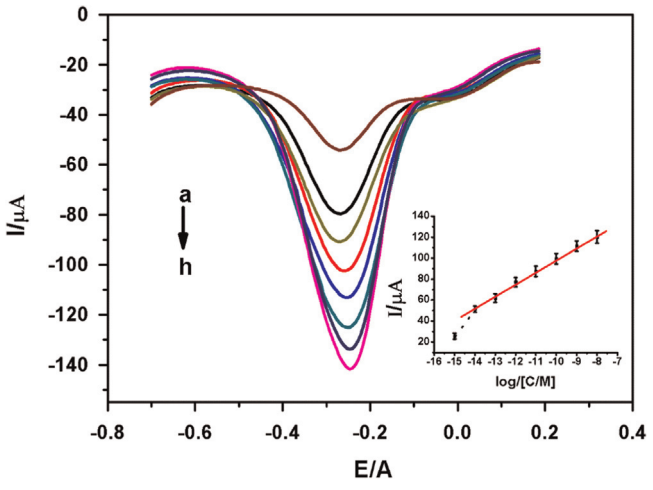


Fig. 5. DPV of MB on ssDNA/ Au-NPs/CS–MWNTs/AuE after hybridization with different concentrations of target ssDNA sequences (from a to h were 1.0×10^{-15}, 1.0×10^{-14}, 1.0×10^{-13}, 1.0×10^{-12}, 1.0×10^{-11}, 1.0×10^{-10}, 1.0×10^{-9} and 1.0×10^{-8} M respectively). Inset: plots of *I* versus logarithm of target ssDNA concentration.

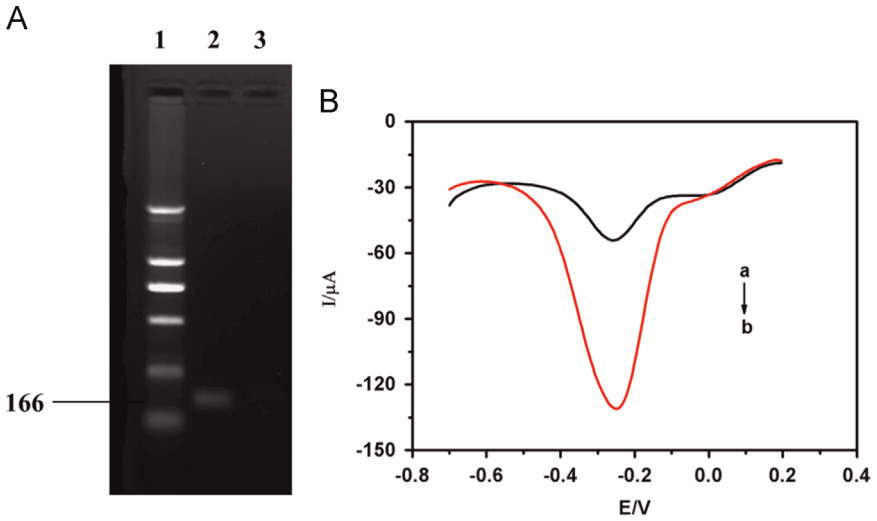


Fig. 6. Gel electrophoresis and DPV peak currents. A: Lanes 2 and 3 show each 10 μL PCR product with (2) and without (3) DNA template of *Staphylococcus aureus nuc* gene. B: Curves a and b were DPV of MB after hybridized with the PCR product without (a) and with (b) DNA template of *S. aureus nuc* gene.

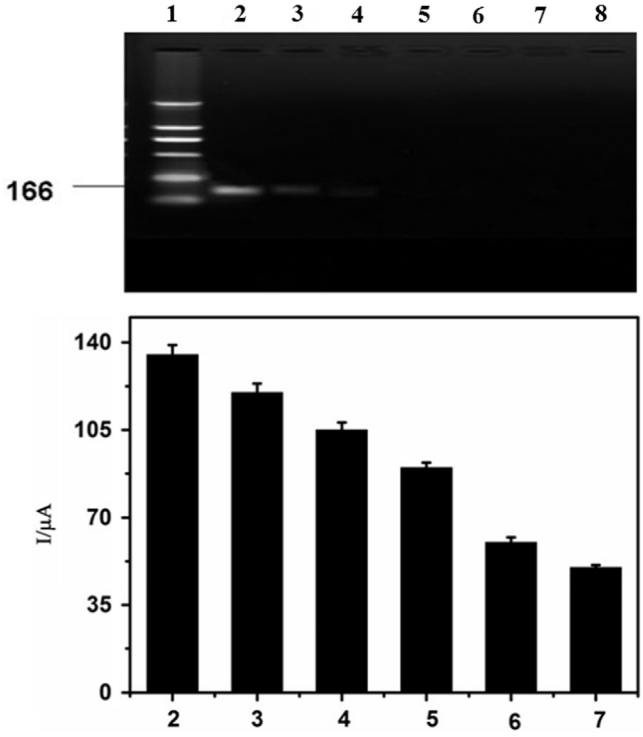


Fig. 7. The experimental results of different concentrations of *S. aureus* by DNA biosensor and gel electrophoresis images. (2–7: serial diluition of *Staphylococcus aureus* in tape water, 10^6–10^1 CFU mL^{-1}, 1 and 8 represent marker and control, respectively).

3.6. Detection of S. aureus in tap water

In order to test the capacity of the biosensor to detect the target bacteria in samples, tap water was filtered through a 22 μL membrane to eliminate any contaminants. Then, 10 mL water was inoculated with different concentrations of *S. aureus* (10^6–10^1 CFU mL^{-1}) and centrifuged (10,000 rpm for 5 min) to concentrate the inoculum. This was followed by DNA extraction using the rapid boiling method. Then we used electrochemical DNA biosensor and gel electrophoresis to detect PCR-amplified product of DNA extracted. It is hard to see a stripe in gel electrophoresis images under low concentrations, but there were strong response signals by electrochemical DNA biosensor in this work. The results, shown in Fig. 7, demonstrate that the sensor could detect *S. aureus* in concentrations from 10^6 to 10^1 CFU mL^{-1}. Conversely, the gel electrophoresis resulted in a low resolution image when the concentrations were amplified.

Table 1
Recovery experiment results of *S. aureus* gene sequence by PCR in synthetic milk samples.

Spiked milk samples	Added gene sequence (M)	Response value (μA)	Measured gene sequence (M)	Recovery (%)
1	1.0×10^{-15}	39.9012	0.96×10^{-15}	96
2	1.0×10^{-14}	52.0325	1.04×10^{-14}	104
3	1.0×10^{-13}	63.7072	1.03×10^{-13}	103
4	1.0×10^{-12}	75.1776	0.98×10^{-12}	98
5	1.0×10^{-11}	87.0550	1.01×10^{-11}	101
6	1.0×10^{-10}	98.4670	0.95×10^{-10}	95
7	1.0×10^{-9}	110.5528	1.02×10^{-9}	102
8	1.0×10^{-8}	122.3263	1.03×10^{-8}	103

3.7. Analysis of synthetic milk samples

Recovery experiments were performed to evaluate the performance of the prepared *S. aureus* biosensor using standard addition methods. Different concentrations of *S. aureus* gene sequence by PCR were added to milk and then detected according to the methods used in this study. As shown by Table 1, some results obtained using the proposed biosensor in spiked milk sample test are acceptable, with recoveries between 95% and 104%, indicating that the proposed biosensor is a viable means of *S. aureus* detection and is capable of satisfying practical analyses needs. Furthermore, the results of the developed method were compared to those of the previous methods for the detection of *S. aureus* (Table 2). As shown in Table 2, the developed method is both more sensitive and has a lower detection limit than the others.

4. Conclusions

A CS–MWCNTs nanocomposite film modified with AuE was fabricated and characterized using a transmission electron microscope and electrochemical impedance spectroscopy. The presence of CS–MWCNTs on the electrode surface greatly increased effective surface area and electron conductivity. Using the Au-NPs/CS–MWCNTs/AuE modified electrode, the ssDNA probe was immobilized on the

Table 2
Figures of merits of comparable methods for determination of *Staphylococcus aureus*.

Method used	LOD (CFU mL^{-1})	Application	Ref.
The sensitive fluorescent detection method using nanogold linked CdTe nanocrystals	50	Spiked milk samples	[23]
The fluorescence detection method by using water-soluble CdSe quantum dots	10^2	N.I	[24]
Gold based immune sensors	10^5	N.I.	[25]
Label-free detection method using real-time potentiometric biosensors based on carbon nanotubes and aptamers	8×10^2	Freshly excised dorsal pig skin	[26]
Electrochemical DNA biosensor with CS–MWNTs/Au-NPs composite	20	Spiked milk samples	This work

electrode surface through sulfur–Au interaction, producing a new variant of electrochemical DNA sensor, which was then employed in the detection of the *S. aureus nuc* gene and its PCR products.

The loading amount of the probe ssDNA sequences was improved by the synergistic effects of the nanomaterials, which improved the detection sensitivity of the sensor in target ssDNA hybridization. This method was capable of detecting 3.3×10^{-16} M of the target *nuc* gene and 10^1 CFU mL^{-1} of *S. aureus* in real water samples. Based on its capacity, the proposed technique has potential applications in biomedical diagnosis, food safety, and environmental monitoring. Additionally, the optimal study conditions given here may be helpful for further studies.

Acknowledgment

This work has been supported by National Natural Science Foundation of China (No. 31371768), Key Laboratory of Industrial Biotechnology Ministry of Education (KLIB-KF201109), Program for New Century Excellent Talents in Jiangnan University (NCET-12-0877), and Priority Academic Program Development of Jiangsu Higher Education Institutions.

References

[1] P. Francois, A. Scherl, D. Hochstrasser, J. Schrenzel, J. Proteomics 73 (2010) 701–708.
[2] S.P. Chakraborty, S.K. Mahapatra, S.K. Sahu, S. Chattopadhyay, P. Pramanik, S. Roy, Asian Pac. J. Trop. Biomed. 1 (2011) 102–109.
[3] H.-M. So, D.-W. Park, E.-K. Jeon, Y.-H. Kim, B.S. Kim, C.-K. Lee, et al., Small 4 (2008) 197–201.
[4] F. Jia, N. Duan, S. Wu, X. Ma, Y. Xia, Z. Wang, et al., Microchim. Acta 181 (2014) 967–974.
[5] J.R. Johnson, J. Microbiol. Methods 41 (2000) 201–209.
[6] G.A. Zelada-Guillen, J. Riu, A. Duezguen, F.X. Rius, Angew. Chem. – Int. Ed. 48 (2009) 7334–7337.
[7] Z. Wang, J. Zhang, P. Chen, X. Zhou, Y. Yang, S. Wu, et al., Biosens. Bioelectron. 26 (2011) 3881–3886.
[8] F. Jia, N. Duan, S.J. Wu, X.Y. Ma, Y. Xia, Z.P. Wang, et al., Microchim. Acta 181 (2014) 967–974.
[9] M.K. Bojdi, M. Behbahani, A. Sahragard, B.G. Amin, A. Fakhari, A. Bagheri, Electrochim. Acta 149 (2014) 108–116.
[10] M.K. Bojdi, M.H. Mashhadizadeh, M. Behbahani, A. Farahani, S.S.H. Davarani, A. Bagheri, Electrochim. Acta 136 (2014) 59–65.
[11] H. Hosseini, M. Behbahani, M. Mahyari, H. Kazerooni, A. Bagheri, A. Shaabani, Biosens. Bioelectron. 59 (2014) 412–417.
[12] J.P. Tosar, G. Branas, J. Laiz, Biosens. Bioelectron. 26 (2010) 1205–1217.
[13] G.-j. Li, L.-h. Liu, X.w. Qi, Y.-q. Guo, W. Sun, X.-l. Li, Electrochim. Acta 63 (2012) 312–317.
[14] J. Wang, Anal. Chim. Acta 500 (2003) 247–257.
[15] K. Xu, J. Huang, Z. Ye, Y. Ying, Y. Li, Sensors 9 (2009) 5534–5557.
[16] M. Friedman, V.K. Juneja, J. Food Prot. 73 (2010) 1737–1761.
[17] A. Tiwari, J. Polym. Res. 15 (2008) 337–342.
[18] R. Singh, R. Verma, A. Kaushik, G. Sumana, S. Sood, R.K. Gupta, et al., Biosens. Bioelectron. 26 (2011) 2967–2974.
[19] C. Mao, A. Zhu, Q. Wu, X. Chen, J. Kim, J. Shen, Colloids Surf. B: Biointerfaces 67 (2008) 41–45.
[20] W.L. Meyer, Y. Liu, X.-W. Shi, X. Yang, W.E. Bentley, G.F. Payne, Biomacromolecules 10 (2009) 858–864.
[21] A. Tiwari, S.P. Singh, J. Appl. Polym. Sci. 108 (2008) 1169–1177.
[22] A.A. Lubin, B.V.S. Hunt, R.J. White, K.W. Plaxco, Anal. Chem. 81 (2009) 2150–2158.
[23] F. Ricci, R.Y. Lai, A.J. Heeger, K.W. Plaxco, J.J. Sumner, Langmuir 23 (2007) 6827–6834.
[24] W. Yang, R.Y. Lai, Analyst 136 (2011) 134–139.
[25] M.S. Hejazi, M.H. Pournaghi-Azar, E. Alipour, F. Karimi, Biosens. Bioelectron. 23 (2008) 1588–1594.
[26] M.H. Pournaghi-Azar, E. Alipour, S. Zununi, H. Froohandeh, M.S. Hejazi, Biosens. Bioelectron. 24 (2008) 524–530.
[27] Q. Wang, B. Zhang, X. Lin, W. Weng, Sens. Actuators B: Chem. 156 (2011) 599–605.
[28] M.J. Wang, C.Y. Sun, L.Y. Wang, X.H. Ji, Y.B. Bai, T.J. Li, et al., J. Pharm. Biomed. Anal. 33 (2003) 1117–1125.
[29] J.Y. Gu, X.J. Lu, H.X. Ju, Electroanalysis 14 (2002) 949–954.