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

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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

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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