JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Rapid and Sensitive Detection of Foodborne Pathogenic Bacteria (*Staphylococcus aureus*) Using an Electrochemical DNA Genomic Biosensor and Its Application in Fresh Beef

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ABSTRACT: Rapid early detection of food contamination is the main key in food safety and quality control. Biosensors are emerging as a vibrant area of research, and the use of DNA biosensor recognition detectors is relatively new. In this study a genomic DNA biosensor system with a fixing and capture probe was modified by a sulfhydryl and amino group, respectively, as complementary with target DNA. After immobilization and hybridization, the following sandwich structure fixing DNA−target DNA−capture DNA−PbS NPs was formed to detect pathogenic bacteria (*Staphylococuus aureus* EF529607.1) by using GCE modified with (multiwalled carbon nanotubes−chitosan−bismuth) to increase the sensitivity of the electrode. The modification procedure was characterized by cyclic voltammetry and electrochemical impedance spectroscopy. The sandwich structure was dissolved in 1 M nitric acid to become accessible to the electrode, and the PbS NPs was measured in solution by differential pulse voltammetry (DPV). The results showed that the detection limit of the DNA sensor was 3.17×10^{-14} M *S. aureus* using PbS NPs, whereas the result for beef samples was 1.23 ng/mL. Thus, according to the experimental results presented, the DNA biosensor exhibited high sensitivity and rapid response, and it will be useful for the food matrix.

KEYWORDS: *DNA electrochemistry biosensor, MWCNT-Chi-Bi, foodborne pathogenic, Staphylococcus aureus, fresh beef*

INTRODUCTION

Foodborne pathogens are a major public health concern because infection constitutes the major cause of death. In many developing countries, it continues to be the significant cause of infection illness and death worldwide. It is, therefore, essential to improve rapid detection tests for determining contaminated materials, especially microbial contamination, during all steps of food manufacturing, distributing, and trading. Many microorganisms in food have an effect on humans. Contamination comes from a variety of organisms, but among the most important ones are *Aeromonas hydrophila*, *Escherichia coli*, *Shigella* spp., *Salmonella* Typhimurium, *Pseudomonas*, *Campylobacter jejuni*, and *Staphylococcus aureus*. Investigation of new approaches and methodologies to detect these pathogens is not only important but necessary to protect health and fight disease.

S. aureus is one of the most common bacteria worldwide and is a major human pathogen related to numerous pathological processes, contributing significantly to morbidity. Humans are the main reservoir of *S. aureus*, and it naturally exists in the respiratory tract, mucous membrane, and pimples and boils of human beings and other animals. It is well adapted to colonize in human skin, and our bodies probably provide some major ecological niches for this species.[1−3] It can produce heat-stable enterotoxins capable of causing gastroenteritis. Wu and Su[4] mentioned that *S. aureus* is not always pathogenic, but some strains produce potent protein toxins and cause food poisoning. The symptoms of infection are nausea, vomiting, diarrhea, and dehydration.

In the late 19th century staphylococci were shown to be responsible for furunculosis, but the fact that some individuals were predisposed to the infection and that it could occur without any contact with other infected people remained unexplained for decades. One important prerequisite for infection is the ability to establish as a human commensal.[3] *A. hydrophila* is a Gram-negative bacteria and cause disease in both animals and humans including gastroenteritis, extra-intestinal infections, and diarrhea. It is found in fresh water, meat, fish, and some vegetables.

The detection of bacteria using traditional methods is time-consuming (5−6 days) and fails to differentiate species. Polymerase chain reaction (PCR) was, thus, created to amplify the target genes; however, this method was also time-consuming and required expensive instruments, skilled operators, and specialized reagents, which increased its cost.[4−7] In recent years, several techniques such as surface plasmon resonance, fluorescence, quartz crystal microbalance, fiber optic biosensors, piezoelectric immunosensors, and others techniques have been developed to decrease the time of detection.[8−12]

Electrochemical biosensors have been the subject of basic as well as applied research for many years and have become one of the important test methods because they are simple, rapid, and inexpensive with a high sensitivity.[13] Several inventive designs for DNA sensors based on an electrochemical readout have appeared,[14] as well as metal nanoparticles rendering sensitivities in the pico- and femtomolar ranges.[14] Accordingly, much attention has been directed to the development of rapid, sensitive, low-cost, portable biosensors for the biological

Received: August 13, 2014
Revised: November 13, 2014
Accepted: December 4, 2014

dx.doi.org/10.1021/jf503914f | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 1. Oligonucleotide Sequences Used in Amplification, Immobilization, and Hybridization Experiments Specific for *Staphylococcus aureus* (EF529607.1)

target	accession no.	oligonucleotide	sequence (5′−3′)	length (bp)	amplicon size (bp)
Aeromonas hydrophila	M84709	forward primer	5′-CCAATATGTCGGTGAAGA-3′	18	161
		reverse primer	5′-CATGTTTGAAGCTGTCAG-3′	18	
Staphylococcus aureus	EF529607.1	forward primer	5′-GCTATCAGTAATGTTTCG-3′	18	151
		reverse primer	5′-GCACTATATACTGTTGGA-3′		
Staphylococcus aureus	EF529607.1	capture probe	5′-AGTAGCTCAGCAAATGCA-SH-3′	18	
		detection probe	5′-NH_2-CACAAACAGATAACGGCG-3′		

detection of pathogens, such as those incorporating the use of nanoparticles for DNA detection.[15] They allow continuous, fast, sensitive, and selective detection of DNA hybridization, and they can also be reused.[16] These systems usually rely on the immobilization of a single-stranded DNA (ssDNA) probe onto a surface to recognize its complementary DNA target sequence by hybridization.[16] Transduction of hybridization of DNA can be measured electronically.[17] The electrochemistry relies on the use of different inorganic-colloid nanoparticles (zinc sulfide, cadmium sulfide, and lead sulfide) to differentiate the target DNA signals in connection with a sandwich hybridization and highly sensitive stripping voltammetry of the corresponding targets. DNA hybridization biosensors play an important role in pharmaceutical, clinical, and forensic applications. Some techniques have been successfully applied to the development of genosensors based upon the combination of a suitable transducer.[18]

The use of nanomaterials and nanotracers in biosensors allows new transduction technology and application of a bismuth layer on glass carbon electrodes to detect heavy metals in samples showing an alternative material to substitute toxic mercury.[5,19−21] The main advantages of bismuth are the high capacity to form fused alloys with metals, which are less affected by dissolved oxygen. However, it fails to detect low amounts of analyte due to its poor conductivity,[10,22] but applied multiwall carbon nanotubes−bismuth modified on glassy carbon electrodes showed increased sensitivity in the detection of trace metals.

In the present work, a sensitive and selective electrochemical DNA biosensor for the rapid detection of *S. aureus* was measured. Signalizing probe DNA was connected with lead sulfide nanoparticles coated with 5′-(NH_2) oligonucleotide using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) as a cross-linker. The fixing probe (fDNA) was another complementary sequence of DNA with thiol modification, which strongly adsorbed on the gold electrode surface (GE). After hybridization, the sandwich structure was dissolved in 1 M HNO_3 to release Pb^{2+}, which was electrodeposited on the glass carbon electrode (GCE) surface decorated with MWCNT−Chi−Bi. Finally, the signal was measured by differential pulse voltammetry (DPV).

EXPERIMENTAL PROCEDURES

Reagents and Materials. Lead nitrate $Pb(NO_3)_2$, sodium sulfide (Na_2S), sodium hydroxide (NaOH), and 3-mercaptoacetic acid $C_2H_4O_2S$ (used as a stabilizer for the NPs) were used for the synthesis of lead sulfide nanoparticles (PbS NPs). EDC, salmon sperm DNA, and buffers were used for the conjugation of the carboxylic group on NPs, and an amine group on the capture probe was obtained from Sigma Aldrich. 6-Mercapto-1-hexanol was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Hybridization buffer PBS (potassium phosphate buffer with NaCl, pH 7.4), washing buffer Tris-HCl (pH 8.0) with Tween-20, and sodium dodecyl sulfate (SDS) were used to remove the unhybridized DNA. Multiwalled carbon nanotubes were obtained from Nanjing Xianfeng Nanomaterials Technology Co. Ltd. (Nanjing, China). Chitosan was put on the surface of the electrode to increase the sensitivity. A PCR kit was set up to purify the DNA products after PCR amplification was purchased from Sangon Biotech Co. (Shanghai, China). Bismuth nitrate in nitric acid 0.5 mol/L obtained from Scharlau Chemie S.A. (Spain) was deposited on the GCE surface prior to electrochemical processing. All of the solutions were prepared using ultrapure water from a Millipore Milli-Q system. All oligonucleotides used in the sensors were purchased from Takara Co. (Dalian, China) and Sangon Biotechnology Co., Ltd. (Shanghai, China) with HPLC purification and working solutions prepared by dilution in PBS buffer.

Apparatus. The morphology and characterization of lead nanoparticles were studied by transmission electron microscopy (TEM) (JEM-2100, JEOL, Japan). An incubator for the hybridization reaction, magnetic stirrer, and vortex mixer were used to conjugate the carboxylic group on PbS NTs and the amine group on the capture probe. A centrifuge (5804 R Eppendorf, Hamburg, Germany) was used to separate the nanoparticles and extract the DNA. Nanodrop (Thermo Scientific, Ashville, NC, USA) was used to measure the concentration and purity of DNA. Gel electrophoresis was performed using a Bio-Rad electrophoresis instrument (Powerpac Universal, Bio-Rad, USA). The three electrode electrochemical detection system consisted of a GCE (working electrode), an Ag/AgCl reference electrode (saturated KCl), and platinum wire as counter electrode. The gold electrode, GCE (working electrode) and silver/silver chloride electrode (counter and reference electrodes) were purchased from (Chenhua, Shanghai, China). A gold electrode (diameter = 2 mm) was used to make sandwich structures and a GCE (diameter = 2.5 mm) was used for measuring target DNA. Electrochemical measurements were performed with an electrochemical station analyzer (Shanghai CH Instruments Co., China).

Bacteria Culture and Genomic Extraction. *S. aureus* (accession no. EF520720.1) was obtained from Zhangjiagang Entry-Exit Inspection and Quarantine Bureau (Suzhou, Jiangsu province, China), and *A. hydrophila* (accession no. M84709) was obtained from Fresh Water Fisheries Research Center (Wuxi, China). The nucleotide sequences submitted with these accession numbers were used in designing primers and probes for targeted gene amplification and detection. Other strains were collected and preserved at Jiangnan University laboratories. Pure cultures of *A. hydrophila* were grown in ampicilin starch agar phenol red, and *S. aureus* was grown in *Staphylococcus* selective agar (CM 310) and obtained from Beijing Land Bridge Technology Co., Ltd., China. Inoculum media were incubated at 37 °C for 24 h, in duplicate aerobiosis. Colonies were purified by streaking on the same media and incubating at 37 °C for 24 h. This treatment step was repeated several times to obtain a pure culture. Genomic DNA was extracted by slightly modified boiling methods[23,24] from tubes after incubation for 18 h in tryptic soy broth media (TSB). Briefly, the culture was heated in a water bath for 10 min to kill all surviving bacteria, and one portion of each broth was centrifuged at 13000 rpm for 5 min at 4 °C. The pellet was suspended in 500 μL of sterile distilled water (DD water) and vortexed

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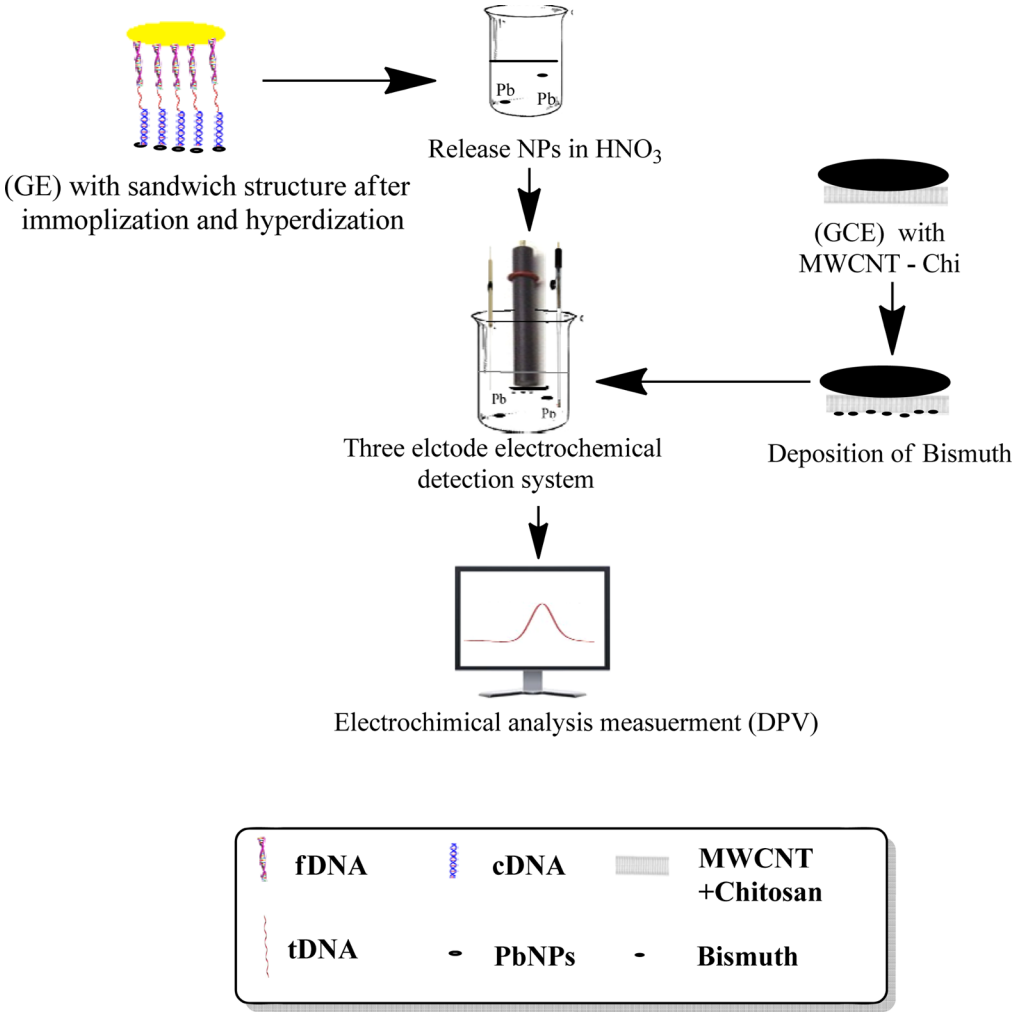
Scheme 1. Steps of Representation of Sandwich Structure and GCE with MWCNT−Chi



vigorously. The bacterial suspensions were boiled in a water bath for 15 min, then rapidly cooled at −20 °C for 10 min to shock the cells, and then centrifuged again at 12000 rpm for 3 min to precipitate the debris. The supernatant containing genomic DNA was harvested into a clean tube and used as the DNA template for PCR amplification.

Oligonucleotides. The primers were designed to provide PCR amplification, and the probe was complementary to part of the fragment, allowing it to hybridize with the PCR product. The probes were designed with modification (3′SH and 5′NH_2) and purification by HPLC to allow the detection of the PCR product using National Center for Biotechnology Information (NCBI) databases using Basic Local Alignment Tool (BLAST) software (http://www.ncbi.nlm.nih.gov/).[24] DNA calculators from Sigma-Aldrich to specify the primer sequences were further verified using software (http://www.sigmagenosys.com/calc/DNACalc.asp). All oligonucleotides used in the sensors were obtained from Takara Co. and Sangon Biotechnology Co., Ltd., and the reverse and forward primers were diluted to 0.4 μM. The sequences of the oligonucleotides are shown in Table 1.

PCR Conditioning. A fragment (151 bases) of the *S. aureus* (accession no. EF529607.1) gene was amplified using the forward and reverse primers. PCR was performed in a reaction solution with a total volume of 25 μL containing (2.5 μL) PCR buffer and (2 μL) deoxynucleoside triphosphate (dNTP) mixture obtained from Takara. Mixed together were 1 μL of each primer (0.4 μM), 1 μL of template DNA, 0.25 μL of Taq DNA polymerase, and 17.25 μL of sterile double-distilled water (DDW) to obtain the reaction mixture. Specificity of the primer amplifications was performed and verified using strains of *S. aureus* (EF529607) as target DNA and *A. hydrophila* (ATCC 7966) as the negative control. Amplification conditions were carried out using the following protocol: initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 46 °C for 30 s, and an extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min, and finally a hold at 4 °C. All amplification reactions were carried out in a thermal cycler (Takara, Gradient PCR). PCR products (5 μL) with a loading buffer were visualized by electrophoreses through 2% agarose gel in TAE buffer and stained with Andy Safe DNA gel stain (BioProbes, USA). A DNA molecular ladder (100 bp) obtained from (Takara) was included in each gel as a molecular weight marker. The gels were photographed under ultraviolet light using a Bio-Rad Gel doc 1000 molecular imager.

Genome Purification and Dilution. The PCR product was purified by using a PCR purification kit, from Shanghai Sangon Bioengineering Ltd. Co., according to the manufacturer's instructions, and then stored at −20 °C until use. Then, it was diluted serially with PBS buffer, and PCR product quantification and concentration were measured with a nanodrop spectrophotometer and used as DNA samples for the experiments.

Synthesis of Lead Sulfide Nanoparticles. All glassware used in the following procedure was carefully cleaned by soaking in a bath of freshly prepared aqua regia solution 1:3 HCl/HNO_3 over 24 h followed by rinsing thoroughly in twice-distilled water and dried in an oven. The pH values of all nanoparticle solutions prepared were recorded using a pH meter (Mettler-Toledo instruments, Delta 320, Shanghai, China). All steps of the synthesis were performed at room temperature and ambient conditions. For the preparation of lead sulfide nanoparticles (PbS NPs), $Pb(NO_3)_2$ and Na_2S solutions were filtered through a nylon syringe filter (25 mm × 0.22 μm) (ANPEL Scientific Instrument, Shanghai, China) prior to use. PbS NPs were

dx.doi.org/10.1021/jf503914f | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

prepared according to the literature.[25,26] Briefly, 9.22 μL of thioglycolic acid ($C_2H_4O_2S$) used as a stabilizer was added to 50 mL of 0.4 mmol L^{-1} Pb $(NO_3)_2$ solution under vigorous stirring, and then the pH was adjusted to 7.0 with 0.5 mol L^{-1} NaOH. The solution was bubbled with nitrogen for 30 min, then 50 mL of 1.34 mmol L^{-1} Na_2S was added dropwise to the mixture solution. The reaction was carried out for 24 h under nitrogen bubbling, and gradually a brown colloid was formed. The colloid was stable for 3 weeks in a brown bottle at 4 °C.

Characterization of Nanoparticles. A JEOL JEM-2100 (HR) transmission electron microscope (TEM) from Electronic Inc. Co., Japan, was used to observe the morphology and particle size distribution in PbS NPs solution. Samples were prepared by dropping aliquots of the colloidal solution onto holey copper-coated grids, which were then dried at room temperature.

Preparation of Multiwalled Carbon Nanotubes−Chi. The MWCNT−Chi matrix was used to increase the sensitivity of GCE. Before mixing with chitosan, MWCNT was treated with concentrated HNO_3 for 2 h and dried in a vacuum at 30 °C. Then, 10 mg of treated MWCNT was mixed with 20 mL of (2%) acetic acid and 2.5 mg of chitosan, followed by ultrasonification for 2 h. The prepared MWCNT−Chi was stored at room temperature until further use.[22,27]

Immobilization of Probe DNA. Immobilization of capture probe DNA was performed according to the literature[26,28,29] with slight modifications. The sequences of the probe oligonucleotides used in this study are shown in Table 1. According to the literature,[26,29] the most critical step in the preparation of a DNA biosensor is the immobilization of the DNA probe on the surface of the sensing device such as an electrode. Prior to use, the gold electrode was first heated in a Piranha solution (3:7 H_2O_2/concentrated H_2SO_4) for about 10 min and then subsequently hand polished thoroughly with 0.03 μm and 0.05 nm alumina slurries, respectively, and a microfiber pad to a mirror-like finish. This was followed by cleaning for 5 min in ethanol and water, respectively, to remove all residual alumina powder and other possible contamination. The electrode was then electrochemically cleaned in 0.5 M of H_2SO_4 to ensure complete removal of contaminants from the electrode surface and finally dried in a stream of nitrogen gas.

The fixing probe was diluted by using 10 mM TCEP with a 1:1 ratio and incubated at room temperature for 1 h. The probe was then dropped in the electrode and incubated at 4 °C overnight to form chemisorption of the thiol groups onto the gold surface. The gold electrode was washed with 0.1 M, pH 8.0, Tris-HCl containing 0.05% Tween-20 to remove excess fixing probe and then immersed in a 1 mM MCH solution for 1 h to obtain a well-aligned DNA monolayer and for 30 min in 125 μg/mL salmon sperm DNA to block specific binding and 2% BSA in 0.1 M, pH 8.0, Tris-HCl to make DNA straight line.

Hybridization of Probes and PCR Amplified Target DNA. Prior to use, the purified PCR product (*S. aureus*) was denatured for 10 min at 95 °C and immediately put in −20 °C, and then target denatured PCR product was diluted to the desired concentration[30] to measure different concentrations in the sensor. A gold electrode content fixing probe was immersed into the denatured DNA and incubated for 1 h at 45 °C to make the first hybridization. The hybridized electrode was then immersed into 0.1% sodium dodecyl sulfate (SDS) for 10 min to remove the unhybridized DNA and any nonspecific adsorption of DNA. The second hybirdization was done by immersing the electrode into a solution of 200 μL (PBS buffer) containing the PbS NPs−capture DNA probe for 1 h at 45 °C to form a sandwich structure as fix DNA−target DNA−detective DNA−PbS NPs and then washed three times with a 0.1% SDS phosphate buffer (pH 7.3) to remove the unhybridized DNA probes.[28,29] After hybridization, the carrier containing nanoparticles was immersed into a cell containing 1.0 M nitric acid solution for 5 min to dissolute the sandwich structures. Scheme 1 shows the steps of immobilization and hybridization.

Electrochemical Analysis. The GCE and DNA detection procedures are illustrated in Figure 1. Prior to use, the GCE for the experiment was cleaned and dried according to the literature.[28,31] Then 10 μL of MWCNT−Chi was dropped onto the surface of the GCE and dried, then immersed into a solution containing 0.1 M acetate buffer solution (pH 4.5) and 100 μL of bismuth ions. The bismuth films were formed by deposition on the carbon electrode containing MWCNT−Chi, and the deposition was performed in a separate solution prior to measurement of lead and dried. After the hybridization reaction, the gold electrode with sandwich structure (fDNA−tDNA−cDNA−PbS NPs) was immersed in 150 μL of 1.0 mol L^{-1} HNO_3 solution for 5 min to dissolve the PbS NPs fixed on the surface of the electrode. All of the experiments were carried out at room temperature. Every test was replicated at least three times. All curves were the average of three test results.

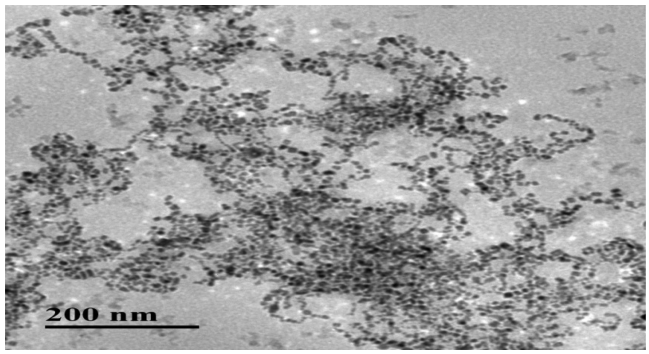


Figure 1. Transmission electron micrographs of the synthesized lead nanoparticles (PbS NPs).

Detection of Target DNA in Artificial Contaminated Beef Meat. Fresh beef was collected from a local supermarket (Wuxi, Jiangsu province, China), and the meat was removed from the carcasses and transported immediately to the laboratory in an ice box. Prior to inoculation, the samples were treated according to the literature[32] with slight modifications. Briefly, samples were divided into 10 g pieces using a knife that was sterilized under UV radiation. Then, each piece was transferred into sterilized Petri dishes with 100 μL of different concentrations of bacteria (10^5−10^1 CFU/mL) used as inocula. Aliquots of each dilution were spread onto the surface of the beef samples and stored at 4 °C for 4 h. A sterilized sample without inoculation was used as a negative control. Then, the samples were transferred into a sterilized 250 mL Erlenmeyer flask, and 90 mL of tryptic soy broth (TSB) medium was added immediately with homogenized shaking for 10 min at 200 rpm, inoculation of samples on Petri dishes, and enumeration after 24 h at 37 °C. The samples in the Erlenmeyer flask were incubated overnight at 37 ± 1 °C. A 1.5 mL rinse fluid was taken from the sample Erlenmeyer flask and placed in a sterile 2 mL microcentrifuge tube for DNA extraction. DNA of the pellet was extracted using the boiling methods (described above), and the PCR, purification kit, and electrochemicistry were done as above.

RESULTS AND DISCUSSION

Characterization of Nanoparticles. The colloid chemistry route offers a simple means to synthesize lead sulfide nanoparticles as it uses inexpensive inorganic starting materials and has a good control over the particle sizes and, more importantly, the size distributions. In this study, PbS NPs were chosen as an alternative to oligonucleotide labels. Lead is a good semiconductor due to its electronic and optical properties such as Pb^{2+} ion selective sensors, and lead sulfide nanoparticles are an important direct band gap semiconductor material with a small band gap.[26,33] The structure and morphology of the synthesized nanoparticle product PbS NPs were studied (TEM). Figure 1 shows a TEM image of the synthesized nanoparticles. TEM images of PbS NPs showed a spherical shape with a 4−6 nm diameter.[18] The difference in size depended on many different factors during synthesis but most importantly the pH, temperature, and time of nitrogen flow

dx.doi.org/10.1021/jf503914f | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

based on tests using different experimental conditions. The PbS NPs were stable and did not aggregate after 1 month.

PCR Amplification. Figures 2 and 3 show the PCR amplification of target DNA *S. aureus* and negative control *A. hydrophila* for the pure cultures, respectively. The length of primer used in this study was 18 bp, and the results showed that the primer pair was high specific for bacteria in the final product performance PCR reaction. The results indicated that each primer pair was specific for the corresponding target organisms, and it used the amplification reactions for PCR detection of the target genes of *S. aureus* at 151 bp, whereas the *A. hydrophila* band appeared at 161 bp,

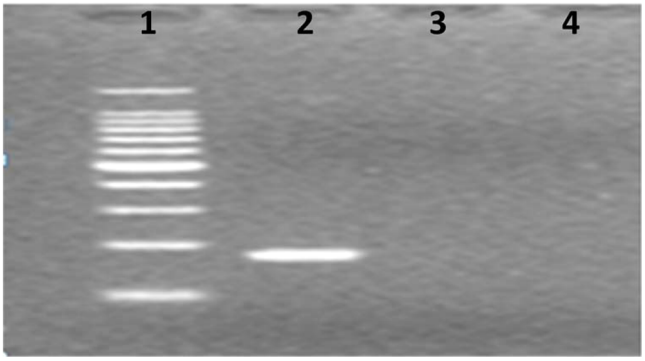


Figure 2. Agarose gel electrophoresis analysis (2%) of PCR amplicon. Lanes: 1, molecular marker (100 bp); 2, positive test sample *Staphylococcus aureus* (EF529607.1); 3, negative control containing PCR mixture with *Aeromonas hydrophila* (ATCC 7966); 4, negative control containing PCR mixture with *Escherichia coli* (JX206444.1).

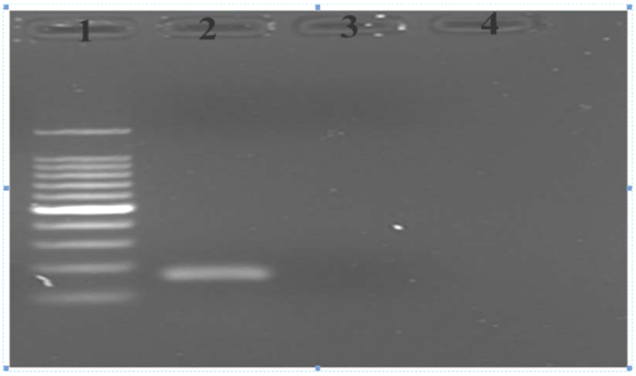


Figure 3. Agarose gel electrophoresis analysis (2%) of PCR amplicon. Lanes: 1, molecular marker (100 bp); 2, positive test sample *Aeromonas hydrophila* (ATCC 7966); 3, negative control containing PCR mixture with *Staphylococcus aureus* (EF529607.1).

Cyclic Voltammetry (CV) and Impedance Spectroscopy. DNA immobilization and hybridization on the films were studied by CV. CV on a ferricyanide (K_3 Fe $(CN)_6$ solution) is a valuable tool to monitor the barrier of the modified electrode. CV is recorded by a three-electrode system, which consists of a working electrode (WE), a reference electrode (RE), and a counter electrode (CE). These electrodes are inside the analyte and connected to an electrochemical station analyzer.[34] The current between the working and the counter electrodes is measured and plotted versus the applied potential between the working and reference electrodes. CV measurements look at redox processes of an analyte in solution Fe^{3+} that converts to Fe^{2+} and then converts again back to Fe^{3+}. Figure 4 shows the evolution of the cyclic voltammogram. First the bare electrode was measured, and then the surface was covered with MWCNT−Chi. After that, the deposition of bismuth was optimal after 150 s at −1.4 V. The results showed that signal increased significantly. When the electrode was first treated with fixing probe, a small increase in peak current was observed as the target DNA was deposited on the electrode.

Impedance Spectroscopy (EIS). Impedance techniques are useful to monitor changes in electrical properties arising from biorecognition events at the surfaces of modified electrodes. EIS is a powerful tool to monitor the whole procedure in preparing modified electrodes and can provide useful information on various properties including electrode impedance.[35] EIS can also give further information on the impedance changes of the electrode surface during the modification process. EIS measurements were carried out using ferricyanide (1 mM $K_3Fe(CN)_6$ solution) in three electrodes. In EIS, the semicircle diameter equals the electron-transfer resistance. This resistance controls the electron-transfer kinetics of the redox probe at the electrode interface. This is also a powerful and sensitive tool for studying the charge-transfer processes occurring in electrode solutions or modified electrodes.[9,36] Figure 5 shows the EIS at different stages, which was used to test the immobilization of the probe DNA on the electrode.When the immobilized probe DNA was hybridized with its complementary target DNA, the peak increased further. The bare Au electrode exhibited an almost straight line, which was characteristic of a diffusion-limited electron-transfer process, after the electrode was treated by the immobilization of fixing probe peak and remarkably increased. This can be attributed to physical coverage by the oligonucleotides and repulsive electrostatic interaction between the negatively charged phosphate backbone of the single-strand nucleic acid and ferricyanide anion.[35,36] The function of MCH was employed to force the tethered DNA strands to "stand up" on the electrode surface and reduce its nonspecific adsorption on the surface through hydrophobic and electrostatic interactions.[37] The impedance initially increased due to interaction of the fixing probe and immobilization target DNA. The reason behind this observation was the increase in conductivity, followed by an impedance decrease in sandwich structure step. The nanoparticles were conjugated to form a complete sandwich structure onto the modified electrode surface. EIS possesses the ability to study any intrinsic material property or specific processes that could influence the conductivity/resistivity or capacity of an electrochemical system.

Electrochemical Analysis. In this study MWCNT was mixed with chitosan and then the bismuth was deposited in the GCE surface. They were used to increase the sensitivity of GCE and to help to improve the stability and sensitivity of the modified electrode. The fixing and capture DNA probes were modified by thiol and amino groups, respectively. The thiol group binds strongly with the gold electrode, and it was complementary with specific target sequence (*S. aureus*). Thiol−metal interactions are frequently used to bind biomolecules covalently onto gold surface biosensors.[16] Amino groups were bound with the PbS NPs and possessed specific target sequences to build the sandwich structure. The probe oligonucleotides (probe DNA) had a thiol modification at the 5′ end to covalently bond to the gold surface. The anchor group (SH unit) was responsible for the chemical link with the gold surface. After immobilization and hybridization, the

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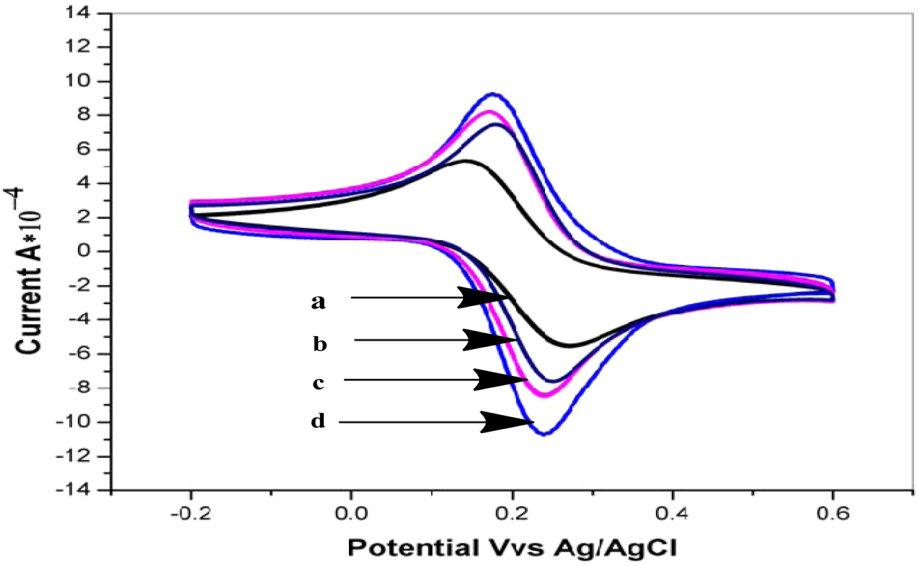

Figure 4. Cyclic voltammogram (CV) of the electrode at different stages, responses of 1 mM $K_3Fe(CN)_6$ on (a) clean GCE, (b) GCE with carbon nanotube, (c) GCE with carbon nanotube and bismuth, and (d) GCE with carbon nanotube, bismuth, and NPs.

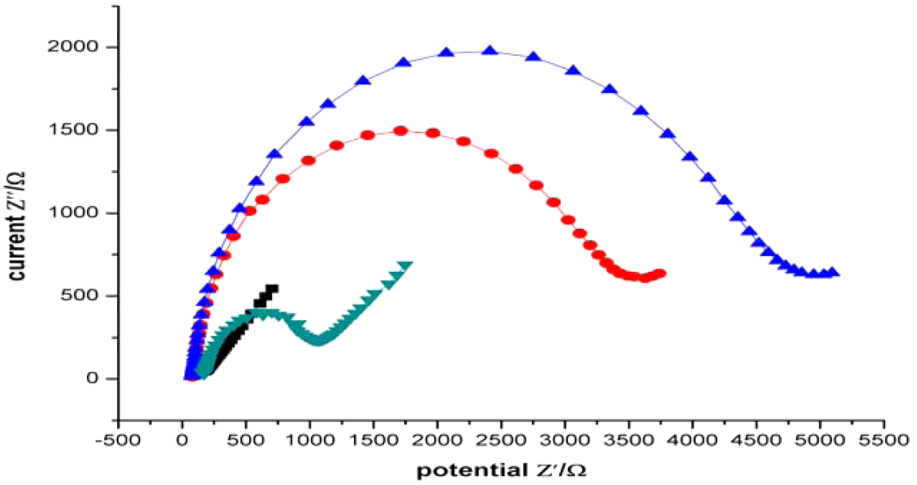

Figure 5. EIS of the electrode at different stages (different film-modified electrodes) in otassium ferricyanide $[K_3OFe(CN)_6]$ on (Black) the bare gold electrode surface, (Green) gold electrode and fixing probe, (Red) gold electrode, fixing probe, and tDNA, (Blue) gold electrode, fixing probe, tDNA, and capture probe.

sandwich structure (fDNA−tDNA−cDNA−PbS NPs) was formed. The immobilization step for the DNA probe was essential to develop a range of biosensors,[16] after which it was released in 1 M nitric acid and the PbS NPs ions were measured. A potentiostat controlled the voltage, according to a preselected voltage−time program between the working electrode and the counter electrode to maintain the potential difference between the working and the reference electrodes.

Figure 6 shows the DPV measurements performed with target DNA from pure culture bacteria obtained for the detection of DNA using different concentrations of target DNA. As the concentration of the complementary target DNA sequence continuously increased, the concentration of dissolved Pb^{2+} was quantified by DPV. A significant amplification for the detection of target DNA was observed. The DPV signal of the target DNA concentration was 3.87×10^{-14}−1.22×10^{-15} M, and the limit detection was 3.17×10^{-14} M. The voltammetric response increased with target concentration. The peak current of various tDNA concen-

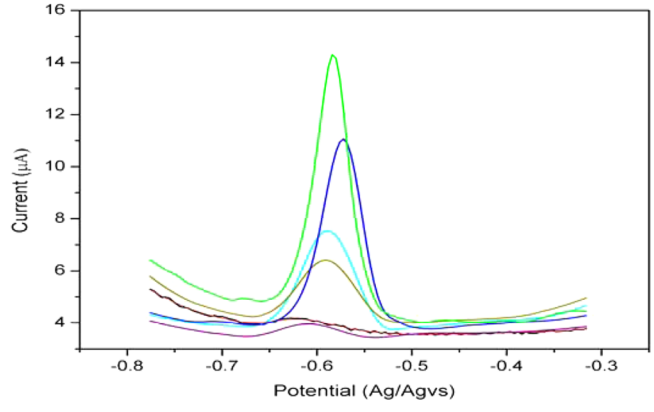

Figure 6. DPV analysis of electrochemical DNA sensors to detect tDNA from pure culture with different concentrations using PbS NPs in sandwich structure fDNA−tDNA−cDNA−PbS NPs for *Staphylococcus aureus* genomic DNA detection.

dx.doi.org/10.1021/jf503914f | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

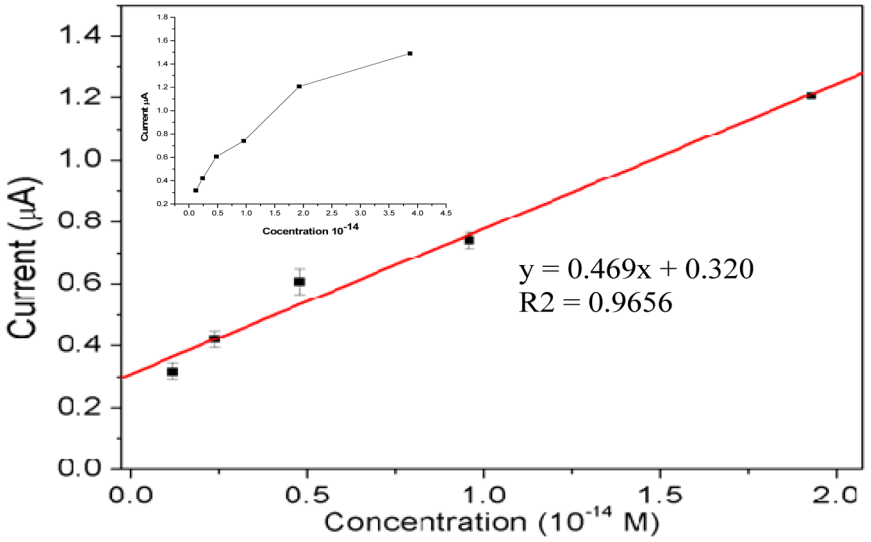

Figure 7. Corresponding calibration plots of peak current versus the concentration of *Staphylococcus aureus* genomic DNA detection. The presented values are an average of triplicates with standard deviation.

Table 2. Limit of Detection of Different Studies Including This Study

target	method	detection limit	ref
nopaline synthase (NOS) terminator gene sequences of GMOs	DNA genosensor electrochemical DNA detection with CdS nanoparticle labels	2.75×10^{-12} mol L^{-1}	28
synthesized oligonucleotides	DNA sandwich electrochemical biosensor based on the amplification of magnetic microbeads and Au nanoparticles	5.0×10^{-15} M	30
Mycobacterium sp.	DNA electrochemical biosensor	0.25 ng/mL	29
Salmonella invA gene	electrochemical DNA sensor	0.5 pM	35
Staphylococcus aureus nuc gene	chitosan−Co_3O_4 nanorod−graphene	4.3×10^{-13} M	38
Staphylococcus aureus	electrochemical DNA biosensor based on MWCNT−Chi and bismuth	2.44×10^{-14} M	this study

trations increased with increasing concentration. The equation for the resulting calibration plot was $Y = 0.469x + 0.320$ ($R^2 = 0.965$) as shown in Figure 7. To test the analytical performance of the assay, a calibration experiment was conducted using target DNA. All results shown in this study are the means of a minimum of triplicate measurements. The system was highly sensitive, selective, and efficient in comparison with other DNA biosensors. Table 2 shows the DNA biosensor for this study compared with other studies.

Detection in Artificially Contaminated Samples. The present system tested for enumeration of bacteria, and in the six dilutions, the counts ranged from $(4.31 \pm 2.15) \times 10^2$ to $(5.86 \pm 0.74) \times 10^4$ CFU/mL, for *S. aureus*. There were significant growth increases for the different dilutions. Table 3 shows the enrichment for bacteria inoculated into the beef samples, followed by extract and amplification of the DNA. Figure 8 shows the amplification of six different dilutions where the reliability of the PCR amplification was satisfactory. Figure 9 shows the curves of target DNA from beef samples after incubation for 24 h. DPV measurements were performed at −0.2 V versus Ag/AgCl. The calibration curve of DPV peak current increased with different concentrations of target DNA. The results showed that the efficiency was influential in the detection of different concentrations (10^1 −10^5) of target pathogenic bacteria. The results also showed a gradual increase

Table 3. Enumeration of *Staphylococcus aureus* on Fresh Beef and after Samples Had Been Inoculated[a]

dilution factor	*S. aureus* ± SD (CFU/mL)
10^1	$(5.86 \pm 0.74) \times 10^4$
10^2	$(1.82 \pm 0.67) \times 10^4$
10^3	$(1.65 \pm 0.26) \times 10^4$
10^4	$(5.50 \pm 0.54) \times 10^3$
10^5	$(4.31 \pm 2.15) \times 10^2$

[a]The number was counted after incubation for 24 h at 37 °C. Data are expressed as the mean ± standard deviation (average of three replications for *S. aureus*). PCR analysis was carried out after overnight culture.

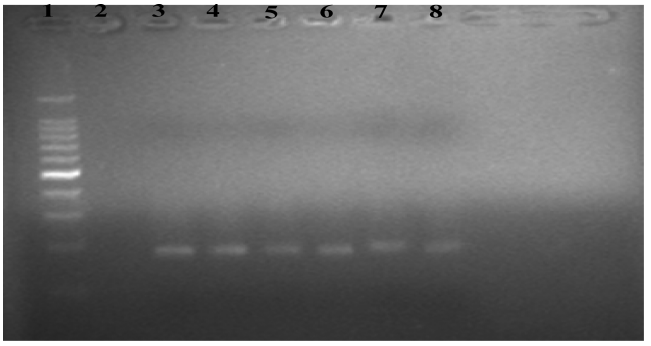

Figure 8. Agarose gel electrophoresis analysis (2%) of PCR amplicon extraction from fresh beef. Lanes: 1, molecular marker (100 bp); 2, negative control containing PCR mixture with *Aeromonas hydrophila* (ATCC 7966); 3−6, positive test sample *Staphylococcus aureus* (EF529607.1) in different concentrations.

dx.doi.org/10.1021/jf503914f | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

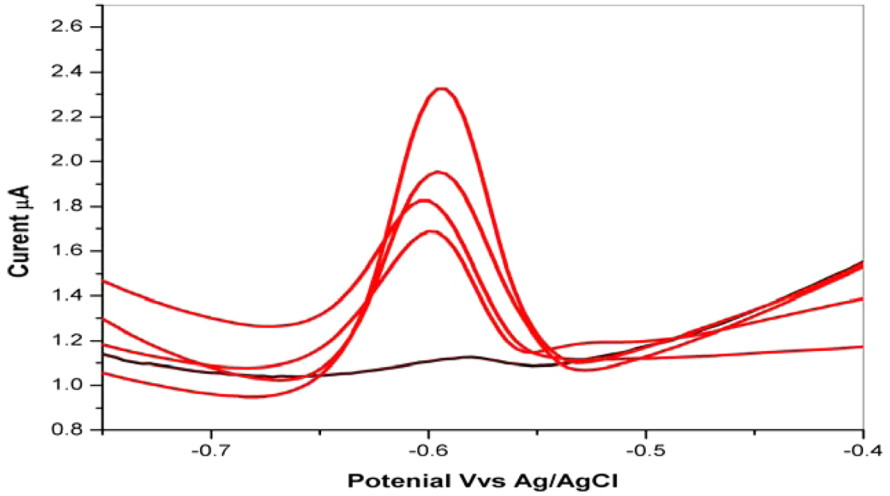

Figure 9. DPV analysis of electrochemical DNA sensors to detect tDNA from fresh beef with different concentrations using PbS NPs in sandwich structure.

in the yield of each band with increases in the dilute factor (serial dilutions).

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Funding

This work has been supported by the National Natural Science Foundation of China (No. 31371768), the National Research Program (No. 201203069-1), the Program for New Century Excellent Talents in Jiangnan University, Qinglan Project, Synergetic Innovation Center of Food Safety and Quality Control, and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Notes

The authors declare no competing financial interest.

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