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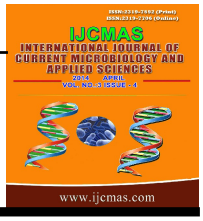

Original Research Article

A multiplex nanoparticles-based DNA electrochemical biosensor for the simultaneous detection of *Escherichia coli O157:H7* and *Staphylococcus aureus*

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Keywords

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A B S T R A C T

In this study, we evaluated the sensitivity of a multiplex nano particle-based DNA electrochemical biosensor for the simultaneous multiple detection of gene of *E. coli* O157:H7 and the nuc gene of *S. aureus.* The biosensor system was mainly composed of a sandwich structure: fixing probe (fDNA), target DNA (tDNA) and detective probe (dDNA). The fixing probe carries the sulfhydryl group strongly binded with the gold electrode and it was the complementary sequence of one end of the tDNA. The dDNA was complementary sequence of another end of the tDNA, which binds with the nanoparticle tracers (NTs), such as lead sulfite (PbS) and cadmium sulfite (CdS), that act as a signal reporter and amplifier. The sandwich structures were immersed in 1 M nitric acid, thereby dissolving the PbS and CdS. Another gold electrode was used to detect NTs ions by Square wave voltammetry (SWV). The results show that the detection limits of this multiplex DNA biosensor are 1×10^{-12} mol/L of the gene of *E. coli* O157:H7 using PbS, and 1×10^{-12} mol/L of the nuc gene of *S. aureus*, using CdS NTs respectively. The multiplex nanoparticles-based DNA electrochemical biosensor has potential application in rapid detection of multiple food-borne pathogens.

Introduction

E. coli O157:H7 has been associated with food related outbreaks in the United States, as well as in the European Union and others countries worldwide. More than 300 outbreaks have been reported in the United States and the United Kingdom since 1983. The first reported outbreak occurred in Michigan and Oregon, USA, in 1982 and it was associated with the consumption of undercooked hamburger (Fedio *et al.*, 2011; CDC 2007). *E. coli* O157:H7 causes several life-threatening diseases such as hemorrhagic colitis (HC) and Hemolytic Uremic Syndrome (HUS) and these can be fatal (Viazis *et al.*, 2010; Martinez-Perez and Blais 2010). The most

important virulence factor is the production of Shiga toxin. The primary source of infection is the consumption of contaminated food, particularly undercooked meat products (Dourou *et al.*, 2011; Delignette-Muller and Cornu 2008). Many outbreaks have been associated with the consumption of raw milk, yogurt, cheese, fermented sausage, lettuce and sprouts (Pennington, 2010). The infective dose is relatively low (1-100 cells), contributing to large scale outbreaks, thus the need to develop sensitive and rapid detection methods (Wang *et al.,* 2012; Fedio *et al.,* 2011).

S. aureus is the frequent causes of diseases in both humans and animals (Poli *et al.*, 2007). Intoxication by *S. aureus* toxins remains one of the most common causes of foodborne disease. Although small numbers of *S. aureus* are usually found in food, sufficient amounts of enterotoxins can be formed to the minimum toxic levels, causing symptoms of staphylococcal intoxication (Rajkovic 2012; Sospedra 2012). This pathogen is also one of the most known causes of nosocomial infections, ranging from middle skin infections to severe life-threatening infections. Studies showed presence of Methicillin-resistant *S. aureus* (MRSA) in retails cuts of pork, chicken, beef and others meats in the United States. Some studies have also reported contamination in lettuce, radish and seed sprouts (Graveland *et al.,* 2011; Hammad *et al.,* 2012; Seo *et al.,* 2010). Currently Polymerase Chain reaction (PCR) method is used for rapid detection of enterotoxigenic *S. aureus* and *E. coli* O157:H7, however this method is costly and time consuming (Chen *et al.,* 2012; Goto *et al.,* 2007; Mao *et al.,* 2005).

Traditional methods to detect these pathogens are time-consuming (5-6 days) since it requires enrichment, selective culture, and biochemical identification and serotype confirmation. The detection limit is usually 105-106 cells/ml without pre-enrichment (Ivnitski *et al.,* 2000; Martinez-Perez and Blais 2010). The electrochemical method for DNA detection has received more attention due to its high sensitivity, selectivity, low cost and easy to operate. Several electrochemical DNA sensors which translated DNA hybridization into detectable signal have since then been developed. Nanomaterials and nanotracers in biosensors allow new transduction technology and different signal detection (Fan *et al.,* 2010; Liu *et al.,* 2012; Zhang *et al.,* 2010). This paper shows procedures of the multiplex nanoparticles-based DNA electrochemical biosensor for the simultaneous detection of the gene of *E. coli* O157:H7 and the nuc gene of *S. aureus* (both important foodborne pathogens). We also demonstrated the detection limit of this method. The biosensor utilizes NTs, such as CdS and PbS linked with dDNA. Hybridization between fDNA, dDNA and tDNA are detected after dissolving NTs into 0.1 M nitric acid. Finally, the signal of dissolved NTs is detected by Square wave voltammetry (SWV).

Materials and Methods

Reagents

Cadmium chloride, lead nitrate, and 3-mercaptoacetic acid were used for the synthesis of lead sulfide (PbS) and cadmium sulfide (CdS) NTs. Sodium hexametaphosphate was used as a stabilizer for the NTs. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-

hydroxysuccinimide (NHS) were used for the conjugation of carboxylic group on NTs and amine group on dDNA. All of the solutions were prepared using ultrapure water from a Milipore Mili-Q system. All the reagents were purchased from Sangon Biotechnology Co., Ltd (Shanghai, China) with HPLC purification.

Apparatus

A transmission electron microscope (TEM) was used to image and characterize the NTs. An incubator for the hybridization reaction and refrigerator for the preservation of NTs was used. After magnetic stirrer was used for synthesis of PbS and CdS NTs. The conjugation of the carboxylic group on NTs and the amine group on dDNA were prepared by vortex mixer. Electrochemical measurement was performed with a potentiostat/galvanostat (Shanghai CH Instruments Co.), which is connected to a personal computer. The gold electrodes (working electrode) and silver/silver chloride electrode (counter and reference electrode) were purchased from CHI660B. Workstation (Chenhua, Shanghai, China).

Synthesis of CdS and PbS nanoparticles

Cadmium and lead sulphide nanoparticles were prepared according to the literature (Deng Zhang et al., 2010 and Zhu et al., 2013) with slight modification. Briefly, 1.0 mL of 0.1 M $CdCl_2$ or $PbCl_2$ was diluted in 80 mL of ddwater and the volume was completed to 150 mL in beaker. Then, 1.5 mL of 0.1 M sodium hexametaphosphate was added into the solution as a stabilizer and stirred for 30 minutes. Then, 10 uL of 3-mercaptoacetic acid was added to solution under stirring and the pH was adjusted to 7 for lead nanoparticles and 11 for cadmium nanoparticles by adding 0.5 M NaOH. Then, the reaction was carried out under bubbled nitrogen for 30 min and stored at 4°C.

Functionalization of nanoparticles

1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were used to link the carboxylic group on NTs and amine group on dDNA (Vikesland and wigginton 2010; DeLong *et al.,* 2010; Zhang *et al.,* 2010). Briefly, EDC (5 mg) was added to 25 µL NTs, and then 50 µL of 9% NHS in dimethyl sulfoxide was added. The reaction was carried out on vortex at room temperature (25 ºC) for 20 min. The conjugation solution was shaken for 8 h at room temperature (25 ºC) and washed for 3 times at 13,000 rpm before usage. After conjugation, the two functionalized dDNA-NTs were mixed in a 1:1 ratio for detecting the gene of *E. coli* O157:H7 and the nuc gene of S. aureus.

Immobilization of probe DNA

The sequences of the oligonucleotides are shown in Table 1. GenBank numbers for the Staphylococcusaureus nue gene is 2827981.For the detection of the nuc gene of S. aureus, thiolated oligonucleotides: 5'-SH-GGTGTAGAGAAATAT-3' was fixed on gold electrode to form fDNA1 in which the fixed probe can hybridize to target DNA sequence 1 (tDNA1): 3'-CCACATCTCTTTATACCAGGA-CTTC GTTCACGT-5'. On the other hand, the sequence of dDNA1: 5'-GGTCCT-GAAGCAAGTGCA-NH2-3' connected with Cds can also hybridize to complementary sequence of tDNA1. GenBank numbers for *E. coli* O157:H7 eaeA gene is 2829861.For the detection of the gene of *E. coli* O157:H7, thiolated

oligonucleotides: 5'-SH-AACGCCGATA CCATT-3' was fixed on the gold electrode (fDNA2), which is able to hybridize to tDNA2: 3'-TTGCGGCTATGGTAATG AATATGGCGCTGC-5'. The sequence of dDNA 2: 5'-ACTTATACCGCGACG-NH2-3' connected with Pbs can also hybridize to complementary sequence of tDNA2. Thus sandwich structures fDNA1/tDNA1/dDNA1/CdS and fDNA2/ tDNA2/dDNA2/PbS were formed.

DNA hybridization

The hybridization reaction was maintained at 37 ºC for 1h with gentle agitation in NaCl-TE Buffer (pH=7.4) . After hybridization two sandwich structures (fDNA/tDNA/dDNA-NTs) were formed. The hybridized electrode was then immersed into 0.1% sodium dodecyl sulfate (SDS) for 10 min to remove the unhybridized DNA. The gold electrode which carries the sandwich structures was then submerged in 1M HNO3 to release the NT ions (Pb^{2+} and Cd^{2+}), washed in ultrapure water, and 0.1 M acetate (pH 4.5) was immediately added (Hansen *et al.,* 2006; Spain *et al.,* 2012). Fig. 1 illustrates the main approach to probing DNA immobilization and hybridization with tDNA. The released NT ions were ready for electrochemical measurement.

2.6 Electrochemical analysis

To remove the impurities, the glassy carbon disk electrode and gold electrode were polished using 0.05 µ alumina slurry on a polishing pad before used. The electrodes were then ultrasonically washed in ethanol and ultrapure water for approximately 3 min. Stripping voltammetric measurements were performed with an in situ deposition of the bismuth film and NTs ions. After 100 uL of the sample solution was dropped in the well, the deposition potential of -1.2 V vs. Ag/AgCl was applied to the carbon working electrode. The voltammogram was recorded by applying a positive-going square-wave voltammetric potential scan with a potential step of 5 mV, frequency of 20 Hz and amplitude of 25 mV. The scan was from -1.2 V to -0.2V and 100 seconds of deposition time were used for SVW measurements (Mirceski et al., 2012; Zhang et al., 2010; Wang et al., 2009). All experiments were carried out at room temperature (25 ºC). Every measurement was replicated three times. All curves were the average of three test results.

Results and Discussion

Characterization of nanoparticles

The dimensions of the two nanoparticles were characterized by using a TEM. Fig. 2 shows a TEM image of synthesized CdS nanoparticles with an average diameter of 10±5 nm, and PbS nanoparticles with an average diameter of 5±3 nm. Some studies showed the similar results (Jie *et al.,* 2007; Zhang *et al.,* 2010). After 3 weeks of storage at 4 ◦C, the NTs were stable and did not aggregate.

Functionalization of nanoparticles

NTs linked with detective probe was characterized by Fourier Transform Infrared Spectroscopy (FT-IR). 1-Ethyl-3-[3-dimethylaminopropyl] and carbodiimide hydrochloride (EDC) were used to crosslink the carboxylic group on NTs and the amine group on dDNA. Fig. 3 shows an Infrared Spectroscopy that confirm the conjugation of NTs and dDNA. The carboxyl group of NTs and the amino group of detective probe formed

amide group by dehydration/condensation reaction. 3-mercaptoacetic acid was linked with S2- ions of NTs by electrostatic adsorption (Sperling and Parak, 2010). The medium and broad absorption peak centered at around 3384 cm-1 is attributed to the stretching vibration of N-H. This characteristic absorption band suggests the presence of secondary amines. The absorption at 3184 cm-1 suggests carboxyl group. Additionally, the weak absorption band around 3000 cm-1 suggests CH_2 stretching vibrations. Several intense bands were observed at lower frequency. The weak absorption band around 1607cm-1 suggests N-H bending. The weak absorption band near 1667 cm-1 and 1485 cm-1 suggest C=O stretching and C-N stretching respectively. However these bands were not as strong as predicted, this must be due to DNA disturburbance and influence of impurities. The S-H vibration band was observed around 2565 cm-1 (Cascant *et al.,* 2011; Zhang 2012).

Electrochemical analysis

Before hybridization, two functionalized dDNA-NTs were mixed in a 1:1 ratio. ddwater was used as negative control. After immobilization of both oligonucleotides on the surface of gold electrode and connection of NTs with respective dDNA a structure of the specificity of pDNA formed. Thus, one was specific to gene of *E. coli* O157:H7 and the other was specific to nuc gene of *S. aureus*. CdS and PbS nanoparticles after dissolution in 1 M HNO_3 were centrifuged and washed in ultrapure water. The NTs were then dissolved in detective solution. When only CdS was present in solution at different concentration, the stripping voltammogram showed only current peaks at -0.79 V as shown in Fig.4.

When the sample contains both tDNAs there are striping signals at -0.41 V (Pb) and -0.79 V (Cd). Similar studies showed striping signals near -0,8 V and -0,4 V for Cd and Pb respectivelly (Hansen *et al.,* 2006; Mirceski *et al.,* 2012; Meepun *et al.,* 2012). The average intensity for gene of *E. coli* O157:H7 detection was higher than nuc *S. aureus.* This is attributed to highest current generated by Pb compared with Cd (Fig. 5). For detection of the nuc gene of *S. aureus*, the peaks currents at -0.79 V (vs.Ag/AgCl) of various concentrations (1×10^{-7}, 1×10^{8}, 1×10^{-9}, 1×10^{-10}, 1×10^{-11} and 1×10^{-12} M) are 26.72 μA, 23.93 μA, 20.95 μA, 18.91 μA 17.25 μA and 15.86 μA respectively. For the detection of *E. coli* O157:H7, peak currents at -0.41 V at various tDNA concentrations (1×10^{-7}, 1×10^{8}, 1×10^{-9}, 1×10^{-10}, 1×10^{-11}, 1×10^{-12} M) are 35.47 μA, 32.68 μA, 29.71 μA, 27.76 μA, 25.45 μA and 20.08 μA respectively. It was observed that the signals obtained were different when both tDNAs were present in sample (Fig. 5).

Sensibility and specificity of biosensor

The two dDNAs connected with respective nanotracers successfully hybridized with specific complementary tDNA. High sensibility can be attributed to the method applied (SWV) which allows simultaneously detention of low levels of nanotracers such as Cd and Pb (down to 10-10 M). The deposits of bismuth film also facilitated the peaks separation (Mirceski *et al.,* 2012; Kimmel *et al.,* 2011).

Fig.1 Schematic of the immobilization and hybridization detection of probe DNA

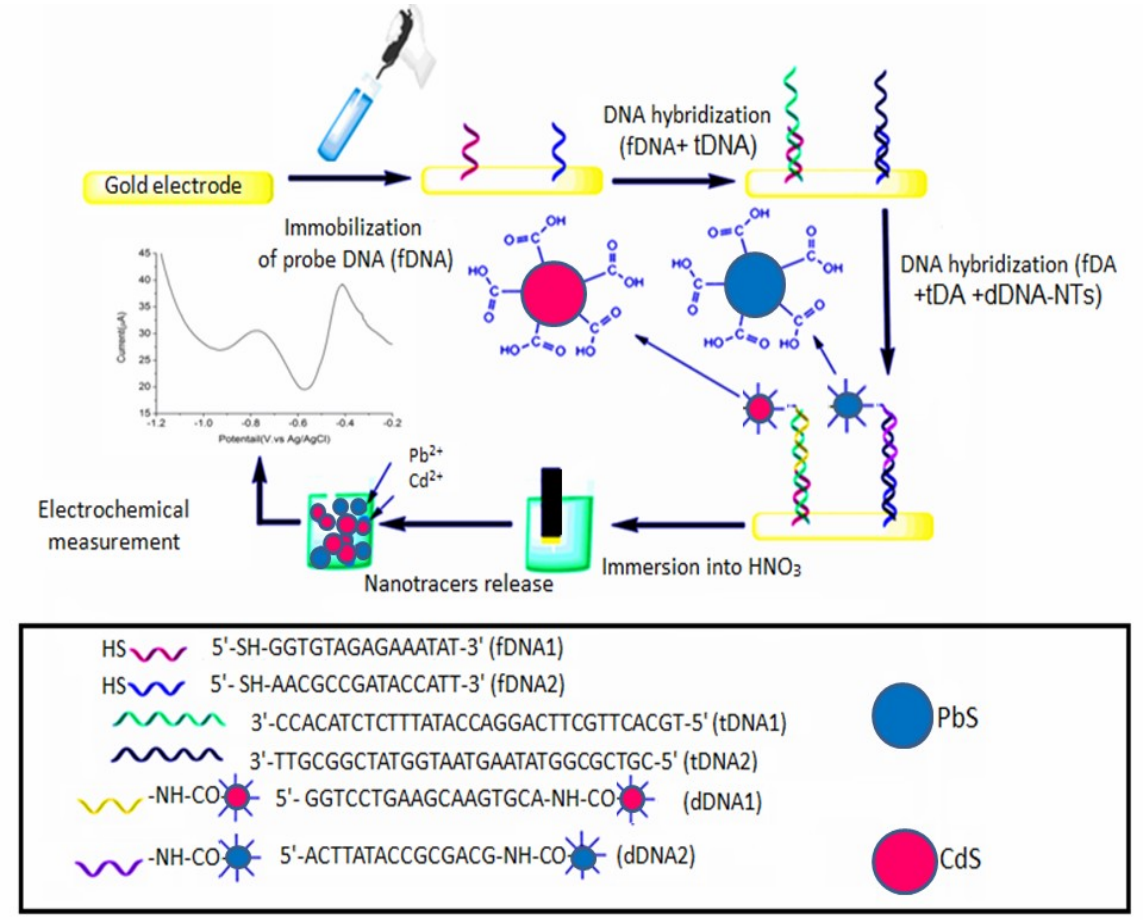


Fig.2 TEM Images of nanotracers PbS (a) and CdS (b).

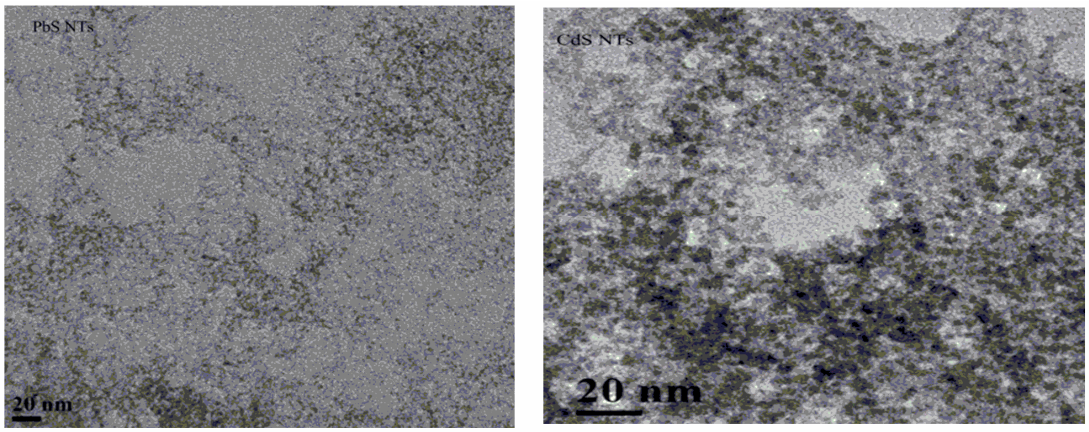


Fig.3 Infrared absorptions of functional groups of NTs-detective DNA.

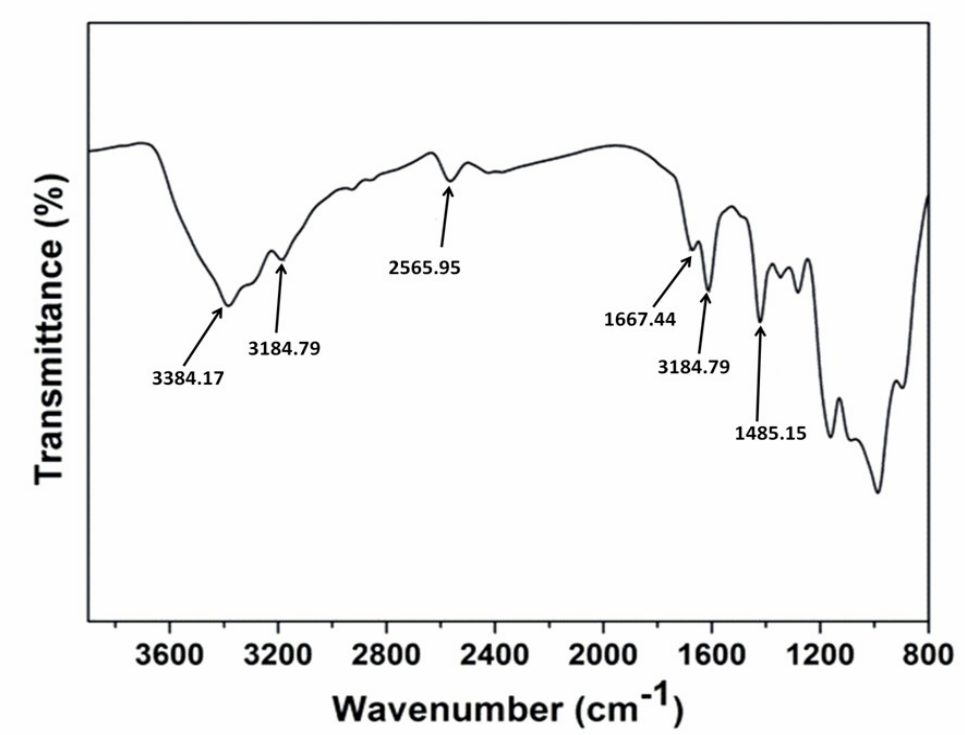


Fig.4 Single detection of the CdS NTs.

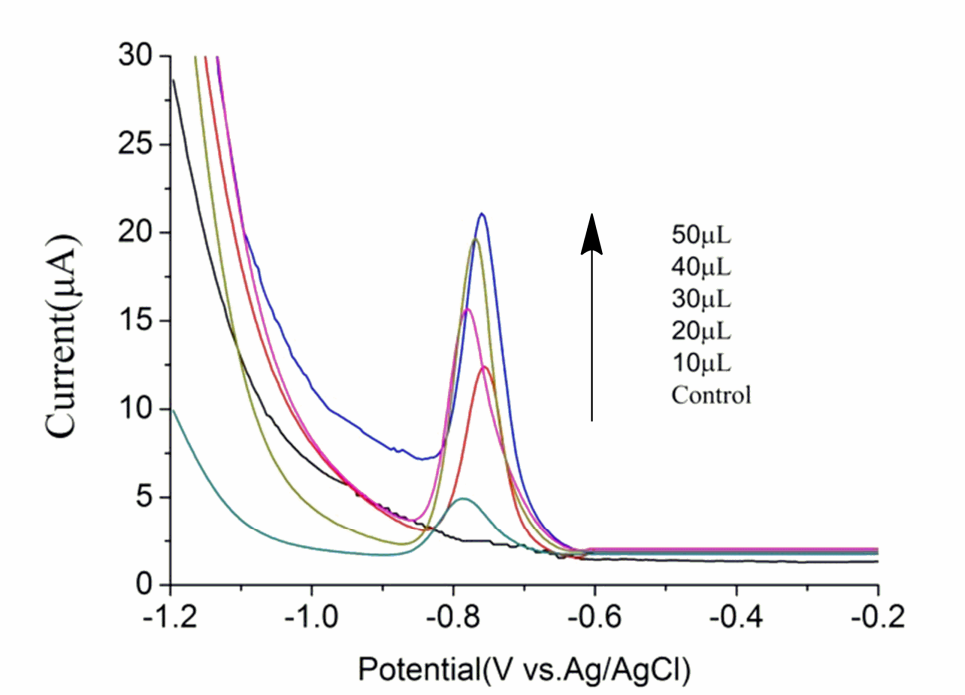


Fig.5 The signals of CdS and PbS NTs linked with different concentrations of tDNAs

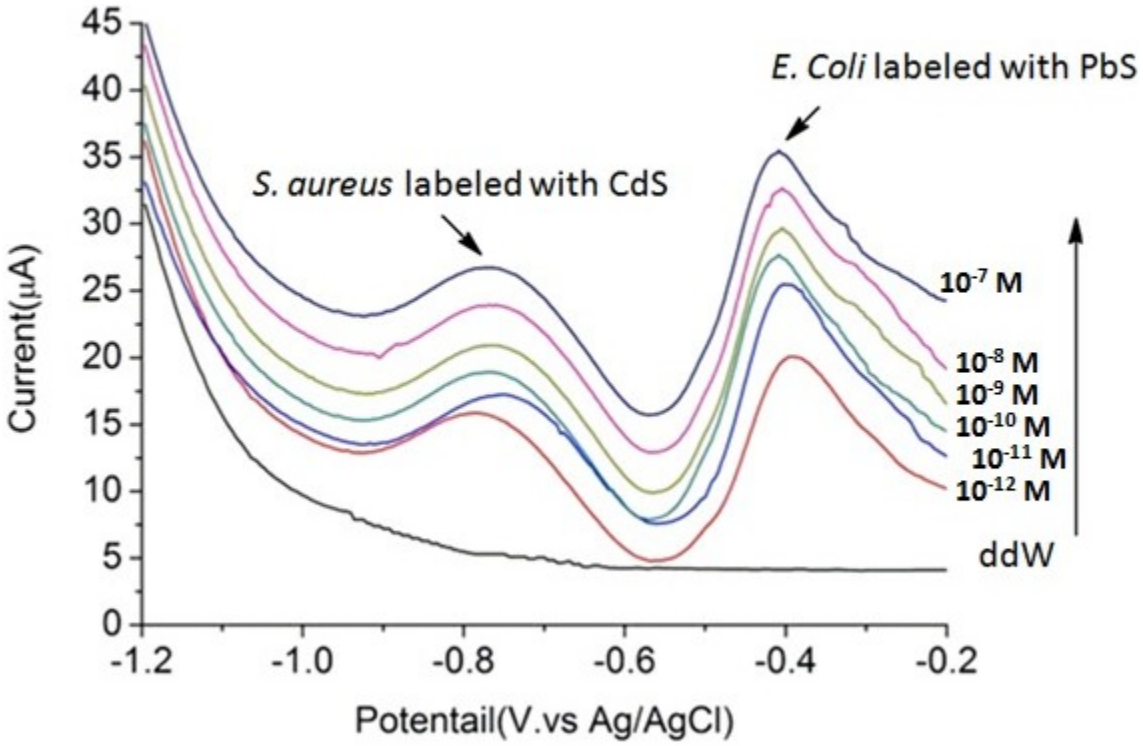
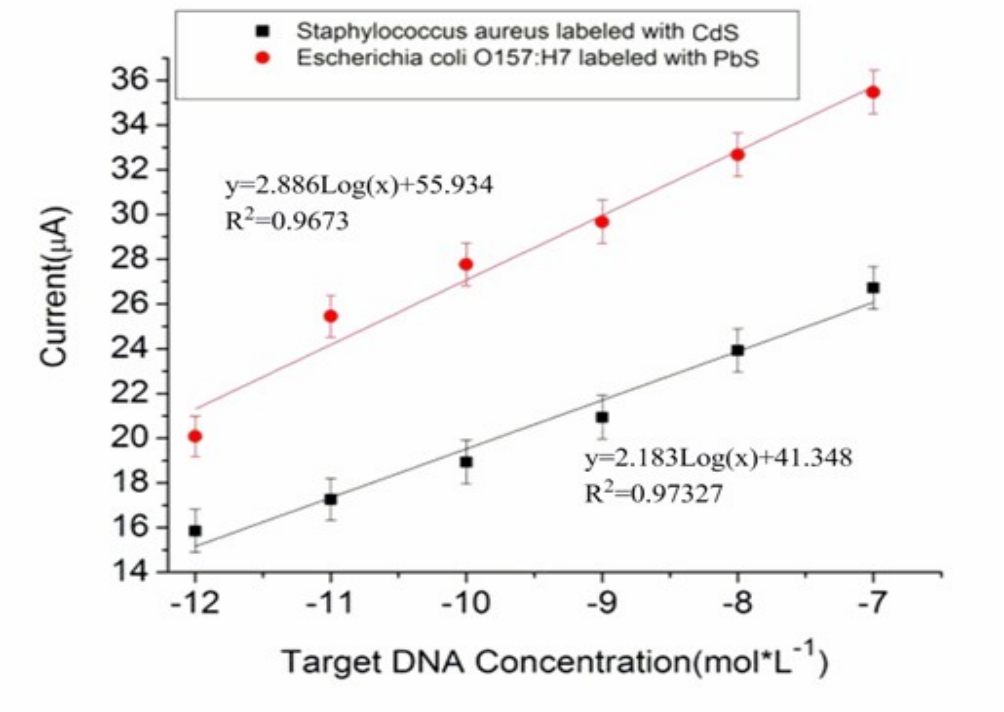


Fig.5 The calibration plot between peak current and the tDNA concentration



The stripping signals of Pb and Cd have a linear relationship with the logarithmic concentrations of tDNAs. The signal increases with increasing logarithmical concentration of tDNA (Fig. 6). In all cases, the reproducibility was excellent, even in low oligonucleotides concentration. Considering the ratio of signal-to-noise (S/N) > 3, the results shows that the detection limits of this DNA sensor are as low as 1×10^{-12} M of the nuc gene of *S. aureus* using CdS, and 1×10^{-12} M of the gene of *E. coli* O157:H7 using PbS NTs. The results show that the sandwich-type hybridization in this study provides high specificity in both the capture and detection probes selectively bound to the tDNA. The possible reason for high specificity would be that any unstable duplex formed between the probe and unintended single-stranded was removed during the washing steps, resulting in a negative reading during electroanalysis that avoided unreacted NTs to generate the noise (Low *et al.,* 2011). Thus it can differentiate the gene of *E. coli* O157:H7 (labeled with CdS) and the nuc gene of *S. aureus* (labeled with PbS).

A highly sensitive, nanoparticle-based DNA electrochemical biosensor was developed for the simultaneous multiple detection of the gene of *E. coli* O157:H7 and the nuc gene of *S. aureus* in this study. The biosensor system is mainly composed by nanotracers (such as PbS and CdS), fDNA, dDNA and tDNA. PbS and CdS nanoparticles were synthesized by chemical methods and characterized by TEM. The conjugation of carboxylic group on NTs and amine group on detective probe were efficient. After fDNA and dDNA hybridized to the tDNA, two sandwich structures (fDNA/tDNA/ dDNA) were formed. The detection limits of the biosensor were as low as 1×10^{-12} M of the nuc gene of *S. aureus* using CdS and low as 1×10^{-12} M of the gene of *E. coli* O157:H7 using PbS NTs. The results show that this biosensor has a good specificity and sensitivity for the simultaneous multiple detection of foodborne pathogens.

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