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Electrochemical DNA biosensor based on chitosan/nano-V_2O_5/MWCNTs composite film modified carbon ionic liquid electrode and its application to the LAMP product of *Yersinia enterocolitica* gene sequence

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ABSTRACT

A new electrochemical DNA biosensor was fabricated by using a V_2O_5 nanobelts (nano-V_2O_5), multi-walled carbon nanotubes (MWCNTs) and chitosan (CTS) nanocomposite materials modified carbon ionic liquid electrode (CILE) as the working electrode. The CILE was prepared by using *N*-hexylpyridinium hexafluorophosphate ($HPPF_6$) as the binder with the graphite powder. The CTS–V_2O_5–MWCNTs/CILE was used as the basal electrode for the immobilization of the single-stranded DNA (ssDNA) probe. After the hybridization with the target ssDNA sequence, the electrochemical indicator of methylene blue (MB) was used to monitor the hybridization reaction. Experimental data indicated that the synergistic effect of nano-V_2O_5 and MWCNTs increased the amounts of ssDNA adsorbed on the electrode surface and resulted in the corresponding increase of the electrochemical responses. This DNA biosensor combined the advantages such as the biocompatibility of V_2O_5 nanobelt, the excellent electron transfer ability of MWCNTs, the good film-forming ability of CTS and the high conductivity of CILE. Under the optimal conditions differential pulse voltammetry (DPV) was used to record the electrochemical response of MB and the specific ssDNA sequence could be detected in the concentration range from 1.0×10^{-11} to 1.0×10^{-6} mol L^{-1} with the detection limit as 1.76×10^{-12} mol L^{-1} (3σ). The DNA biosensor showed good stability and discrimination ability to the one-base and three-base mismatched ssDNA sequence. The loop-mediated isothermal amplification (LAMP) product of *Yersinia enterocolitica* gene sequence in pork meat was detected by the proposed method with satisfactory result, suggesting that the CTS–V_2O_5–MWCNTs/CILE had the potential for the sensitive detection of specific gene sequence.

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1. Introduction

Electrochemical DNA biosensor has been widely reported in recent years due to the advantages such as high sensitivity, simplicity, low cost, small dimension and cheap instruments (Teles and Fonseca, 2008). During the preparation procedure for the electrochemical DNA biosensor, the immobilization of the single-stranded DNA (ssDNA) probe on the surface of the substrate electrode shows great influence to the performances of the DNA electrochemical sensor such as accuracy, sensitivity, selectivity and lifetime. Different methods including covalent binding (Millan and Mikkelsen, 1993), adsorption (Wang et al., 1996), polymerization (Jiang et al., 2008), etc. have been used for the DNA immobilization. With the development of the nanoscience, various kinds of the nanoparticles are utilized for the ssDNA probe immobilization due to their unique characteristics such as high surface area, excellent biocompatibility and strong adsorption ability (Wang, 2003). For example, Selvaraju et al. (2008) fabricated a sandwich-type electrochemical DNA biosensor using streptavidin-coated magnetic beads and gold nanoparticles as the response magnifiers. Yang et al. prepared a DNA electrochemical biosensor with nanogold-modified poly-2,6-pyridinedicarboxylic acid film for the detection of specific gene sequences (Yang et al., 2007a). Ghanbari et al. (2008) prepared an electrochemical DNA biosensor based on the electrochemically fabricated polypyrrole nanofiber-modified electrode.

Yersinia enterocolitica is a common enteric pathogen in humans, which has a strong propensity to cause acute gastroenteritis, enterocolitis, and mesenteric adenitis, as well as a variety of extraintestinal disorders (Bottone, 1999). *Y. enterocolitica* strains have been isolated from a variety of domestic animals, especially from the tonsils and tongues of pig carcasses (Fredriksson-Ahomaa et al., 2001), and porcine products are considered to be the primary source of this pathogen in humans. In recent years many methods

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have been developed for the detection of the pathogenic *Y. enterocolitica* strain. Among them polymerase chain reaction (PCR) and real-time PCR method were developed as efficient tools for identifying the strains of *Y. enterocolitica* (Kechagia et al., 2007; Kot et al., 2007). Despite the high efficiency and enormous production amount from amplification, the requirement for a high precision thermal cycler in PCR method restricts this powerful tool to be widely used. Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method, which can amplify DNA with high specificity, efficiency and rapidity under isothermal conditions using a set of four specially designed primers (two inner and two outer primers) and a DNA polymerase with strand displacement activity (Notomi et al., 2000; Nagamine et al., 2002). The advantages of LAMP include: (1) an isothermal condition is required and it can be conveniently achieved in a water bath which is inexpensive and commonly use, but not the expensive PCR system; (2) only few target DNA molecules are needed for amplification assay and 10^9 copies can be gained within 1 h; (3) LAMP is highly specific for the target sequence which is attributed to four primers designed according to target DNA sequence. So LAMP method promises a novel pathway to achieve the qualitative and quantitative detection of specific gene sequence. By combining the advantages of electroanalytical method, such as inexpensive, rapid, simple and convenient, LAMP coupled electrochemical DNA biosensor shows great potential application in sensitive DNA detection (Ahmed et al., 2009).

Room temperature ionic liquids (RTILs), which are composed of organic cations and various anions, have been widely used in the fields of chemistry due to the unique advantages such as high chemical and thermal stability, negligible vapor pressure, high ionic conductivity, wide electrochemical windows, low toxicity and the ability to dissolve a wide range of organic and inorganic compounds (Liu et al., 2005a; Pandey, 2006). Wei and Ivaska (2008) reviewed the recent application of RTILs in the electrochemical sensor. ILs can be used as not only the supporting electrolyte but also the modifier in chemically modified electrode. Safavi et al. (2008) utilized octylpyridinium hexafluorophosphate (OPFP) as the binder to make a carbon ionic liquid electrode (CILE), which showed some specific characteristics including better reversibility, higher sensitivity and the ability to lower the overpotential of electroactive compounds. Lu et al. used chitosan/RTILs composite film for the hemoglobin immobilization and investigated its electrocatalysis (Lu et al., 2006). Sun et al. applied the CILE as the basal electrode for the redox protein electrochemistry with different nanoparticles such as $CaCO_3$ nanoparticles (Sun et al., 2007) and CdS nanorods (Sun et al., 2008).

In this paper a CILE, which was fabricated by the addition of IL into the carbon paste electrode as binder and modifier, was constructed and used as the basal electrode for the electrochemical DNA biosensor. The CILE showed the advantages including high electron conductivity, remarkable electrocatalytic activity, inexpensive and easily fabrication. V_2O_5 nanobelts and multi-walled carbon nanotubes (MWCNTs) were mixed with chitosan (CTS) to form a novel nanocomposite material and further cast on the surface of the home-made CILE. Chitosan (CTS) is an abundant natural cationic biopolymer with excellent characteristics such as film-forming ability, biocompatibility, nontoxicity, good water permeability, high mechanical strength and adhesion, which had been used in the chemically modified electrodes (Liu et al., 2005b; Tkac et al., 2007). CTS has also been widely used in the electrochemical DNA biosensor as the matrix for the ssDNA immobilization, which can easily interact with DNA and other polyanions through electrostatic attraction (Xu et al., 2001). Carbon nanotubes (CNTs) are consisted of cylindrical graphite sheets with many unique electronic and mechanical properties, and CNTs have been widely used in the fields of electroanalysis including protein electrochemistry, electrochemical biosensor or electrocatalysis. Liu et al. constructed a glucose biosensor based on glucose oxidase entrapped carbon nanotubes/chitosan composite (Liu et al., 2005b). Sun et al. investigated the direct electrochemistry of myoglobin on the MWCNTs composite film modified CILE and its electrocatalytic behaviors (Sun et al., 2009). Electrochemical DNA biosensors based on the usage of CNTs had also been reported due to their specific properties (Baughman et al., 2002; Zhu et al., 2005). Cai et al. (2003) applied CNT-enhanced electrochemical DNA biosensor for the DNA hybridization detection. Vanadium pentoxide (V_2O_5) is an inorganic "n-type" semiconductor oxide with an electronic conductivity in the order of $0.5\,S\,cm^{-1}$ at room temperature. It has aroused considerable interests over the years due to the excellent properties such as multiple valency, wide optical band gap, good chemical and thermal stability, excellent thermoelectric property, etc., which make it have the potential application in microelectronic, electrochemical, gas sensing and optoelectronic devices (Jin et al., 2008; Khudaish and Al-Hinai, 2008; Dhayal Raj et al., 2009). It had also shown great interest in the field of chemistry due to the redox-activity and layered structures (Lee et al., 2005). In recent years, the fabrication of vanadium oxide one-dimension nanostructures has been studied intensively due to its potential applications in lithium batteries (Ding et al., 2009), electric field-effect transistors (Guerra et al., 2009), chemical sensors or actuators (Tian et al., 2006) and nanodevices. In the present work CTS, V_2O_5 nanobelt and MWCNTs were mixed together to form a nanocomposite matrix for ssDNA immobilization. Fang investigated the electrochemical characterization of V_2O_5/CNT composite for supercapacitors (Fang, 2008). V_2O_5–CNT mixture also was prepared by other methods such as hydrothermal synthesis or electrochemistry to investigate its application in pseudocapacitor (Kim et al., 2006; Jayalakshmi et al., 2007). Huguenin et al. used layer-by-layer technique to prepare a nanoarchitecture of V_2O_5/CTS/poly(ethylene oxide) and studied its electrochemical and electrochromic properties (Huguenin et al., 2004). Yao et al. also fabricated an electrochemical DNA biosensor based on CTS doped with CNT for the detection of salmon sperm DNA (Li et al., 2005). However the usage of CTS–V_2O_5–CNT nanocomposite matrix for the enlargement of the electrochemical signal of the DNA indicator and the increase of the sensitivity for DNA detection has not been reported previously. The use of CTS in the composite material can increase the stability of the modified film on the electrode surface. At the same time the V_2O_5–CNT nanomaterials in the CTS film can form a porous structure to provide an enhanced loading interface for the ssDNA probe immobilization. By combining the advantages of the materials used and with the fabricated CTS–V_2O_5–MWCNTs/CILE as the basal electrode, ssDNA probe was successfully adsorbed on the modified electrode to get a new electrochemical DNA biosensor, which was applied to the *Y. enterocolitica* ssDNA sequence fragment and further used to the *Y. enterocolitica* LAMP product detection. The proposed DNA biosensor showed the advantages such as the simple preparation procedure, high selectivity and broad linear range.

2. Experimental

2.1. Apparatus and chemicals

A LK 2005 electrochemical analyzer (Tianjing Lanlike Chemistry & Electron High Technology Co., Ltd., China) was used for cyclic voltammetric and differential pulse voltammetric measurements. Electrochemical impedance spectroscopy (EIS) was performed on a CHI 750B electrochemical analyzer (Shanghai CH Instrument, China). Three-electrode system was used throughout, which was consisted of a modified electrode as working electrode, a Pt wire as auxiliary electrode and an Ag/AgCl electrode as reference electrode. Scanning electron microscopy (SEM) was obtained by a

JSM-6700F scanning electron microscope (Japan Electron Company, Japan).

N-Hexylpyridinium hexafluorophosphate ($HPPF_6$) was purchased from Hangzhou Chemer Chemical Co. Ltd. (China). Multi-walled carbon nanotubes (MWCNTs, purity >95%, main range of diameter 10–20 nm, length of 5–15 μm, Shenzhen Nanotech. Port Company, China) were purified and carboxylated through a well-established method with a slight modification (Zhang et al., 2003). V_2O_5 nanobelts were synthesized in a hydrogen peroxide aqueous solution by an environmentally friendly chemical route (Li et al., 2006). Chitosan (CTS, minimum 92% deacetylated, Dalian Xindie Co. Ltd., China), graphite powder (average particle size 30 μm, Shanghai Colloid Chemical Company, China), methylene blue (MB, Shanghai Chemicals Plant, China) and DNA from herring sperm (Sigma, USA) were used as received. All the solutions were prepared with doubly distilled water.

The 20-base oligonucleotides probe (probe ssDNA), target complementary sequence DNA (target ssDNA), one-base mismatched ssDNA, three-base mismatched ssDNA and noncomplementary sequence DNA (ncDNA) were synthesized by Shanghai Sangon Biological Engineering Technoloical Co. Ltd. (China), which were related to the gene sequence of *Y. enterocolitica*. Their base sequences were as below:

probe ssDNA: 5′-CCGGCAAAACGTCTGCGTGA-3′,
target ssDNA: 5′-TCACGCAGACGTTTTGCCGG-3′,
one-base mismatched ssDNA: 5′-TCACGCAGACGTATTGCCGG-3′,
three-base mismatched ssDNA: 5′-TCAGGCACACGTATTGCCGG-3′,
ncDNA: 5′-ATTGACGTATAGCGCTA-3′.

LAMP assay was performed on an Eppendorf Mastercycler Gradient PCR system (Germany). The sequences of the LAMP primers used in the current study were designed by the Primer Explorer V4 software program (http://primerexplorer.jp/elamp4.0.0/index.html) according to the sequences present in the gyrB gene (GenBank access number, DQ386883.1, EF175580.1, AB084027.1). The primers used in LAMP amplification included:

BIP primer: 5′-CCGGTTTGATCGGTTTCGCCCACTTACAAGATGGGT-GTGCC-3′;
FIP primer: 5′-GTGCGTTTCTGGCCGAGCTT-GCAGACGTTTTGCCAG-GATT-3′;
B3 primer: 5′-CGCCGTGAAGGTAAAGTTCA-3′;
F3 primer: 5′-CAGAGTTCAGGAACGACAGC-3′.

2.2. Fabrication of CTS–V_2O_5–MWCNTs/CILE

3.2 g of graphite powder and 1.0 g of $HPPF_6$ were mixed thoroughly in a mortar to form a homogeneous carbon paste and further heated at 70 °C for 1 h. A portion of the carbon paste was filled into one end of a glass tube (Φ = 4 mm) and a copper wire was inserted through the opposite end to establish an electrical contact. The CILE surface was smoothed on a piece of weighing paper just before use.

0.2 mg of V_2O_5 nanobelts and 0.4 mg of MWCNTs were dispersed into 1.0 mL of 1.0 mg mL^{-1} CTS (1.0% HAc solution) and sonicated for 0.5 h to get a CTS–V_2O_5–MWCNTs mixture solution. Then 10 μL of the prepared solution was coated onto the surface of CILE and dried in air at room temperature to get a modified electrode denoted as CTS–V_2O_5–MWCNTs/CILE.

2.3. Fabrication of electrochemical DNA biosensor

Immobilization of probe ssDNA was achieved by immersing CTS–V_2O_5–MWCNTs/CILE in 1.0 mL of 50.0 mmol L^{-1} Tris–HCl buffer (pH 7.4) containing 1.0×10^{-6} mol L^{-1} probe ssDNA for 1 h at room temperature, then the electrode surface was washed with 0.5% SDS solution and doubly distilled water for 3 min successively, to remove unadsorbed probe ssDNA on the surface of electrode.

The hybridization assay was carried out by immersing probe ssDNA/CTS–V_2O_5–MWCNTs/CILE in 1.0 mL of 50.0 mmol L^{-1} Tris–HCl buffer containing target ssDNA for 40 min at 40 °C with stirring. Then the electrode was washed with 0.5% SDS solution and Tris–HCl buffer for three times successively to remove the unhybridized target ssDNA.

2.4. Electrochemical detection

After hybridization the modified electrode was immersed into 1.0 mL of 2.0×10^{-5} mol L^{-1} MB solution (containing 20.0 mmol L^{-1} NaCl) for 10 min, then washed with 50 mmol L^{-1} Tris–HCl buffer for 3 min. The response of MB was measured by differential pulse voltammetry (DPV) in a 50 mmol L^{-1} Tris–HCl buffer solution (pH 7.4) with pulse amplitude as 0.001 V, pulse width as 0.05 s and pulse period as 0.2 s. Cyclic voltammetric experiments were performed in a mixture solution containing 50.0 mmol L^{-1} $K_3[Fe(CN)_6]$ and 0.1 mol L^{-1} KCl with the scan rate as 100 mV s^{-1}. Electrochemical impedance spectroscopy (EIS) was carried out in 1.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 mol L^{-1} KCl with the frequencies swept from 10^5 to 0.1 Hz.

2.5. Loop-mediated isothermal amplification of Y. enterocolitica gene sequence in pork meat

The *Y. enterocolitica* samples obtained in the form of unfrozen meat from the slaughtered pig were minced into tiny pieces, placed in 10 mL of enrichment of peptone sorbit bile broth, and then incubated at 25 °C for 48 h. The 48-h-old culture (10 mL) was then centrifuged at 12,000 × *g* for 4 min. The pellet obtained on centrifugation was resuspended in 100 μL of sterile distilled water and DNA was subsequently extracted using the DNA extraction kit (Beijing Tiangen Biotech. Co. Ltd., China). The LAMP assay was performed with a total amount of 25 μL of the reaction mixture containing 30 pmol of the primers FIP and BIP, 4 pmol of the primers F3 and B3, 2.5 μL of a 10× reaction mixture (New England Biolabs), 12.0 mmol L^{-1} $MgSO_4$, 1.0 mol L^{-1} betaine, 1.0 mmol L^{-1} of deoxynucleotide triphosphates (dGTP, dATP, dCTP and dTTP), 2 μL of the template DNA, and 8 U of Bst DNA polymerase (New England Biolabs). The reaction mixture was incubated at 65 °C for 60 min and then at 80 °C for 4 min in order to terminate the reaction.

3. Results and discussion

3.1. Morphology of the CTS–V_2O_5–MWCNTs composite

CTS–V_2O_5–MWCNTs nanocomposite can form a film on the surface of used electrode for the ssDNA immobilization. So the SEM images of materials used were recorded and the results were shown in Fig. 1. In Fig. 1A belt-like nanostructures V_2O_5 appeared with random distribution on the surface of CILE. The width and thickness of the nanobelt can be observed in the range of 100–300 nm in width and 30–40 nm in thickness, respectively. While the SEM images of MWCNTs (Fig. 1B) indicated that MWCNTs were interlocked together to form net-like highly porous nanostructure with the typical length of MWCNTs in the range of several micrometers. By mixing V_2O_5 nanobelts and MWCNTs together in water and sonicated for 0.5 h, the image of V_2O_5–MWCNTs nanocomposite on the surface of CILE was shown in Fig. 1C. It can be seen that the V_2O_5 nanobelts were well-distributed and surrounded by a lot of smaller MWCNTs. After they were mixed with CTS (Fig. 1D), a uniform composite film appeared with rough appearance and increased surface

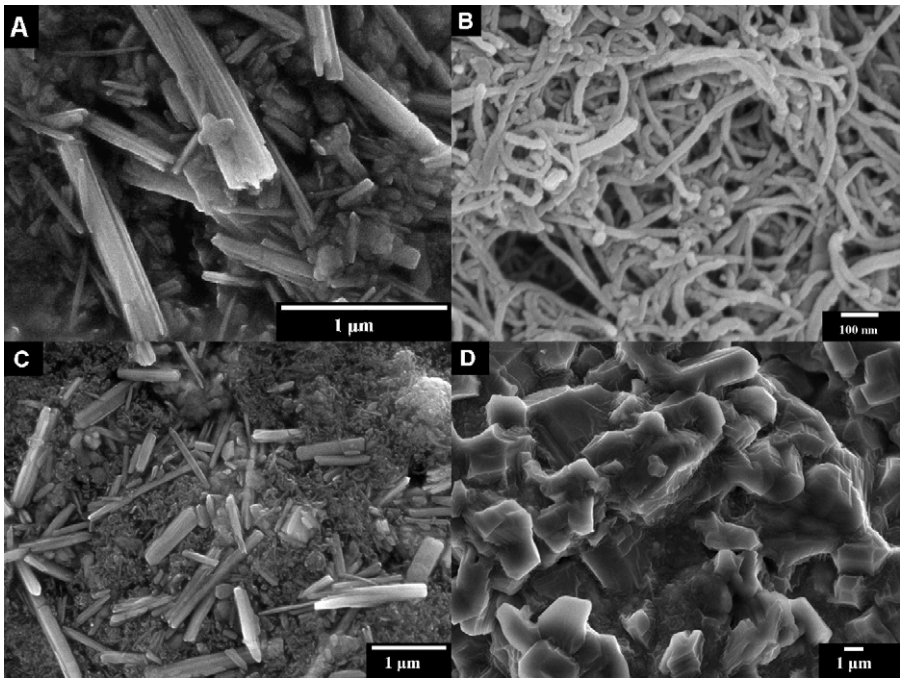

Fig. 1. SEM images of the V_2O_5 nanobelt (A), MWCNTs (B), V_2O_5–MWCNTs (C) and the CTS–V_2O_5–MWCNTs nanocomposite material (D) on the surface of CILE.

area, which provided a good interface for enhancing the loading amount of ssDNA probe on the electrode.

3.2. Electrochemical characteristics of the CTS–V_2O_5–MWCNTs/CILE

Cyclic voltammograms of CILE, CTS/CILE, CTS–V_2O_5/CILE, CTS–MWCNTs/CILE and CTS–V_2O_5–MWCNTs/CILE in a 50.0 mmol L^{-1} $K_3[Fe(CN)_6]$ solution were recorded (see Fig. S1A in supplementary data). On CILE a couple of well-defined redox peaks was observed (curve 1), which was due to the high ionic conductivity of IL present in the carbon paste. While on CTS/CILE (curve 2) the redox peak current decreased with the increase of the peak-to-peak separation (ΔEp), indicating that the presence of CTS on the electrode surface had blocking effect to the electrochemical reaction of potassium ferricyanide. While on the CTS–V_2O_5/CILE an increased electrochemical response of potassium ferricyanide was observed (curve 3), which could be attributed to the presence of V_2O_5 nanobelt on the electrode surface enhanced the effective surface area. On the CTS–MWCNTs/CILE the electrochemical response was further increased (curve 4), indicating the presence of MWCNTs greatly improved the electrode performance. The result was in agreement with a former reported DNA biosensor with chitosan film doped CNTs (Li et al., 2005). So the presence of MWCNTs in the CTS film can increase the effective surface and accelerate the electron transfer between the redox couple in bulk solution and the electrode. On the CTS–V_2O_5–MWCNTs/CILE (curve 5) the redox peak currents were further increased, indicating the synergistic amplification effects of nano-V_2O_5 and MWCNTs in the composite film effectively improved the electrochemical reaction rate. According to the Randles-Sevcik equation (Bard and Faulkner, 2001):

$$i_{P,c} = (2.69 \times 10^5) n^{3/2} A D^{1/2} C^* \upsilon^{1/2}$$

where $i_{P,c}$ is the reduction peak current (A), n is the electron transfer number, A is the electroactive surface area (cm^2), D is the confusion coefficient of $K_3[Fe(CN)_6]$ in the solution ($cm^2\ s^{-1}$), C^* is the concentration of $K_3[Fe(CN)_6]$ (mol cm^{-3}) and υ is the scan rate (V s^{-1}), the electroactive surface area of different modified electrodes can be estimated. Based on this method, the average electroactive area of CILE, CTS/CILE, CTS–V_2O_5/CILE, CTS–MWCNTs/CILE and CTS–V_2O_5–MWCNTs/CILE were calculated as 0.144 cm^2, 0.100 cm^2, 0.179 cm^2, 0.193 cm^2 and 0.210 cm^2, respectively. The results indicated that the presence of V_2O_5 nanobelt and MWCNTs in the CTS film greatly improved the surface area and accelerate the electron transfer between the redox couple in bulk solution and the electrodes.

EIS experiment can be used to probe the interfacial electron transfer resistance (Ret) at the modified electrodes and the semicircle portion observed at high frequencies in the Nyquist diagrams corresponds to the electron transfer limiting process. The Nyquist diagrams of different modified electrodes in 1.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 mol L^{-1} KCl with the frequencies swept from 10^5 to 0.1 Hz were recorded (see Fig. S1B in supplementary data). On the CILE (curve 1) a straight line appeared, indicating the high conductivity of the CILE. While on the CTS/CILE (curve 2), the Ret value was estimated to be 1340 Ω, which was increased dramatically and the results indicated that CTS film on the surface of CILE blocked the diffusion of the $[Fe(CN)_6]^{3-/4-}$ probe. On the CTS–V_2O_5/CILE and CTS–MWCNTs/CILE the Ret value decreased to 682 Ω (curve 3) and 502 Ω (curve 4), respectively, indicating the presence of nano-V_2O_5 and MWCNTs on the electrode surface could obviously improve the diffusion of ferricyanide towards the electrode surface and decrease the resistance. On the CTS–V_2O_5–MWCNTs/CILE the Ret value was further decreased to 379 Ω (curve 5), revealing the existence of synergistic effect of V_2O_5–MWCNTs in the membrane. The results were in agreement with the cyclic voltammetric responses of $[Fe(CN)_6]^{3-/4-}$ on the modified electrode and the results indicated that the CTS–V_2O_5–MWCNTs composite film was beneficial to the electrochemical investigation.

3.3. Differential pulse voltammogram of MB at different modified electrodes

MB is an organic dye that belongs to the phenothiazine family, which is a commonly used indicator in

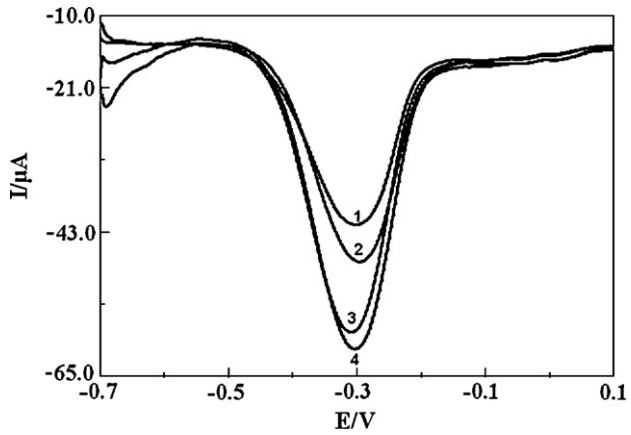

Fig. 2. Differential pulse voltammograms of (1) dsDNA/CTS/CILE, (2) dsDNA/CTS–V_2O_5/CILE, (3) dsDNA/CTS–MWCNTs/CILE and (4) dsDNA/CTS–V_2O_5–MWCNTs/CILE using MB as the indicator in 50 mmol L^{-1} Tris–HCl buffer solution (pH 7.4).

electrochemical DNA biosensor. Fig. 2 showed the differential pulse voltammograms of MB on dsDNA/CTS/CILE (curve 1), dsDNA/CTS–V_2O_5/CILE (curve 2), dsDNA/CTS–MWCNTs/CILE (curve 3) and dsDNA/CTS–V_2O_5–MWCNTs/CILE (curve 4) in 50 mM Tris–HCl buffer solution (pH 7.4). It can be seen that the reduction peak current of MB increased gradually, indicating more dsDNA was adsorbed on the modified electrode surface. dsDNA could interact with MB and accumulate more MB on the electrode, so the increase of the reduction peak current appeared. On the dsDNA/CTS–V_2O_5–MWCNTs/CILE the largest signal response appeared, which indicated that the biggest amounts of the MB molecules accumulated due to the interaction with dsDNA present on the electrode surface. CTS is a polycationic polymer and has been used for effectively immobilization of DNA and other polyanions on the surface of CTS film through electrostatic attraction. With the addition of nanomaterials in the CTS film, an increase of surface area with more roughness appeared, which helped to increase the loading of DNA amount and improved the sensitivity of the biosensor. So the DPV signal of MB on the modified electrodes confirmed that the synergistic effect of nano-V_2O_5 and MWCNTs in the nanocomposite film was more favorable to improve the immobilization amount of dsDNA on the electrode surface.

3.4. Optimization of the experimental conditions

The experimental conditions, such as the component of film materials, the concentration and accumulation time of hybridization indicator, the temperature and time of hybridization reaction, can influence the performance of prepared DNA biosensor. In order to maximize the efficiency and sensitivity of the DNA biosensor, the optimization of the experimental conditions was investigated.

The ratio of V_2O_5 nanobelt and MWCNTs influenced the electron transfer rate and the maximum loading amount of ssDNA probe on the electrode surface. Different mass ratios of V_2O_5 nanobelt and MWCNTs in 5:1, 2:1. 1:1, 1:2 and 1:5 were studied and the largest current value of MB was obtained with the component of 0.2 mg V_2O_5 nanobelt and 0.4 mg MWCNTs in 1.0 mL 1.0 mg mL^{-1} CTS (1.0% HAc solution). So this ratio was selected as the optimum component.

The concentration of MB also intensely influenced the sensitivity of biosensor. The current response increased with the MB concentration increasing from 1.0×10^{-6} to 2.0×10^{-5} mol L^{-1} and intended to be stable at the concentration of 2.0×10^{-5} mol L^{-1} or above. Therefore, 2.0×10^{-5} mol L^{-1} of MB was employed for the prepared DNA biosensor.

The accumulation time of MB was further investigated. The highest current signal of MB was sampled after 10 min accumulation and a signal plateau appeared when the accumulation time was more than 10 min. So, 10 min was chosen as the optimum accumulation time of MB in this paper.

The hybridization efficiency was a critical parameter concerning to discriminate specific sequence and enhance the sensitivity of DNA biosensor, while it was depended on the hybridization conditions like temperature and time. The influence of temperature upon MB signal was investigated by adjusting the temperature of water bath from 30 to 60 °C. The maximum current of MB was obtained at 40 °C. Consequently, a hybridization temperature of 40 °C was used. The hybridization time was investigated by hybridizing with target sequence from 30 to 60 min. The maximum DPV signal of MB was obtained at 40 min. As a result, the hybridization time of 40 min was selected through the experiment.

3.5. Analytical performance of the DNA biosensor

The selectivity of this electrochemical DNA biosensor was investigated by using the ssDNA/CTS–V_2O_5–MWCNTs/CILE to hybridize with different ssDNA sequences related to target ssDNA sequence. Fig. 3 showed the DPV reductive signals of MB at the ssDNA probe-modified electrode (curve 1) and after hybridization with the complementary DNA sequence (curve 2), one-base mismatched sequence (curve 3), three-base mismatched sequence (curve 4) and the noncomplementary sequence (curve 5). The highest MB reduction signal was obtained at the electrode hybridization with complementary ssDNA sequence (curve 2), indicating that the largest attachment of MB occurred at this electrode because of the strong affinity of MB with the formed dsDNA. The peak current value of MB was very small after the ssDNA probe was reacted with the noncomplementary sequence (curve 5), indicating that the hybridization reaction was not achieved in the solution. The little increase of the peak current was due to the non-specific adsorption of ssDNA on the electrode surface. After the ssDNA probe was hybridized with the one-base mismatched sequence (curve 3) and three-base mismatched sequence (curve 4), the peak current increased was much smaller than that obtained from the hybridization with the complementary DNA sequence. Also the one-base mismatched sequence and the three-base mismatched sequence could be recognized via the different changes of the peak current of MB. The results demonstrated that this DNA biosensor displayed the high selectivity for the hybridization detection.

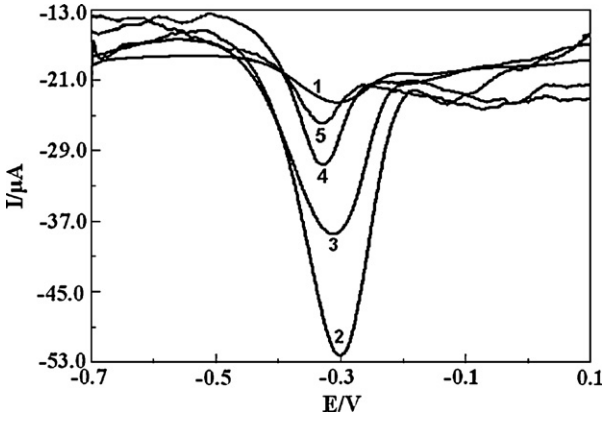

Fig. 3. Differential pulse voltammograms of MB at the ssDNA probe-modified electrode (1) and after hybridization with the complementary DNA sequence (2), one-base mismatched sequence (3), three-base mismatched sequence (4) and the noncomplementary sequence (5).

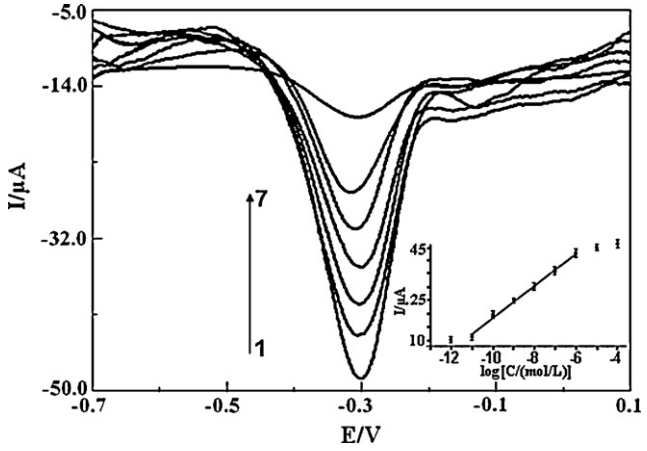

Fig. 4. Differential pulse voltammograms of MB on ssDNA probe-modified electrode after hybridization with different concentrations of target ssDNA sequence. Concentrations of target ssDNA sequence from 1 to 7 are 1.0×10^{-6}, 1.0×10^{-7}, 1.0×10^{-8}, 1.0×10^{-9}, 1.0×10^{-10}, 1.0×10^{-11} and 0 mol L^{-1}, respectively. Insert is plot of I versus logarithm of target ssDNA sequence concentration.

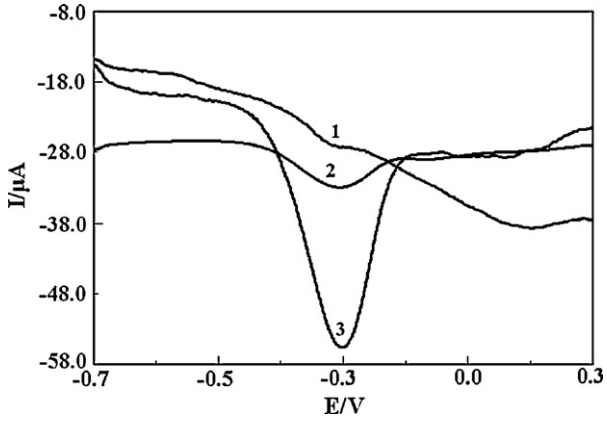

Fig. 5. Differential pulse voltammograms of MB on (1) CTS–V_2O_5–MWCNTs/CILE, probe ssDNA/CTS–V_2O_5–MWCNTs/CILE (2) and hybridization with 20.0 mg mL^{-1} LAMP product of *Yersinia enterocolitica* gene sample (3).

3.6. Detection of Y. enterocolitica gene specific sequence

The sensitivity of this electrochemical DNA biosensor was explored by using the immobilized ssDNA probe to hybridize with the *Y. enterocolitica gene* target sequence of different concentrations. With the increase of the target ssDNA concentration in the solution the reduction peak current of MB also increased gradually and the typical differential pulse voltammograms were shown in Fig. 4. The difference of MB cathodic peak current before and after hybridization was linear with the logarithmic value of the *Y. enterocolitica gene* target sequence concentration in the range from 1.0×10^{-11} to 1.0×10^{-6} mol L^{-1}. The linear regression equation was calculated as $I/\mu A = 77.513 \pm 2.325 \log[C/(\text{mol L}^{-1})] + 5.946 \pm 0.178$ ($n = 6$, $\gamma = 0.994$) (where C is the *Y. enterocolitica gene* target sequence concentration; I is the difference of MB peak current before and after hybridization) and the relation standard deviation (R.S.D.) of peak current was 3.0%. A detection limit of the *Y. enterocolitica* gene target sequence was estimated to be 1.76×10^{-12} mol L^{-1} (3σ) (where σ is the relative standard deviation of the blank solution, $n = 11$).

3.7. Specific sequence detection of LAMP product of Y. enterocolitica gene sequence

The hybridization detection of the LAMP amplified real sample of *Y. enterocolitica* gene was further conducted on this electrochemical DNA biosensor under the selected conditions. The *Y. enterocolitica* amplified samples were diluted with 5.0 mmol L^{-1} Tris–HCl buffer solution (pH 7.4) and denatured by heating it in boiling water bath for 10 min, and then frozen in an ice bath for 2 min. The ssDNA probe sequence was immobilized on the CTS–V_2O_5–MWCNTs/CILE and the ssDNA modified electrode was immersed into the solution of the denatured LAMP amplification product from *Y. enterocolitica* gene sample for hybridization. After the hybridization the electrode was immersed into MB solution for detection with the results shown in Fig. 5. The MB signal at the *Y. enterocolitica* gene probe electrode (curve 2) was bigger than that of the CTS–V_2O_5–MWCNTs/CILE (curve 1), indicating that the presence of ssDNA on the electrode surface can adsorb MB molecules. An obvious increase of the MB reduction peak current was observed after the hybridization of the *Y. enterocolitica* gene probe with the denatured *Y. enterocolitica* real sample (curve 3), indicating the successful accomplishment of the hybridization reaction on the electrode surface, which resulted in the formation of dsDNA on the electrode surface. This significant difference of the MB signals between the *Y. enterocolitica* gene probe electrode and at the hybridized electrode confirmed that this DNA biosensor could effectively detect the LAMP product of *Y. enterocolitica* sample.

The stability of ssDNA probe on the modified electrode was further investigated and the results indicated that ssDNA/CTS–V_2O_5–MWCNTs/CILE had good stability with the ssDNA sequence tightly adsorbed on the electrode surface. After 10 days storage of the modified electrode at 4 °C, 97.4% of the initial sensitivity remained, indicating this modified electrode was a stable platform for ssDNA probe immobilization.

4. Conclusions

In this paper, an efficient DNA electrochemical sensor based on the CTS–V_2O_5–MWCNTs/CILE was developed and further applied to the detection of the LAMP product from *Y. enterocolitica* gene. The surface of the modified electrodes was characterized by different methods including SEM, cyclic voltammetry and electrochemical impedance spectroscopy. The presence of V_2O_5 nanobelts and MWCNTs on the electrode surface had synergistic effects on the immobilization of ssDNA probe with its large surface area and good charge transport characteristics, which increased the ssDNA loading quantity and improved the detection sensitivity for DNA hybridization. The DNA electrochemical sensor was applied to the

Table 1
Comparison of the analytical parameters with other modified electrodes.

	Zhang et al. (2008)	Zhu et al. (2004)	Yang et al. (2007b)	This work
Substrate electrode	GCE	GCE	GCE	CILE
Modifying films	ZrO_2/nanogold	ZrO_2/Au	MWCNTs/nano-ZrO_2	CTS–V_2O_5–MWCTs
Detection method	DPV with MB as indicator	DPV with MB as indicator	DPV with daunomycin as indicator	DPV with MB as indicator
Linear range (mol L^{-1})	1.0×10^{-10} to 1.0×10^{-6}	2.25×10^{-10} to 2.15×10^{-8}	1.49×10^{-10} to 9.32×10^{-8}	1.0×10^{-11} to 1.0×10^{-6}
Detection limit (mol L^{-1})	3.1×10^{-11}	1.0×10^{-10}	7.5×10^{-11}	1.76×10^{-12}

GCE, glassy carbon electrode.

detection of the *Y. enterocolitica* gene sequence with a dynamic concentration range from 1.0×10^{-11} to 1.0×10^{-6} mol L^{-1} and the detection limit of 1.76×10^{-12} mol L^{-1} (3σ). The results indicated that the biosensor showed the advantages such as simple preparation procedure, high selectivity, low cost, fast response and wider linear range. The LAMP product of the *Y. enterocolitica* gene from the pathogenic pork meat sample was also detected satisfactorily. The performance of the proposed biosensor was compared with that of other DNA electrochemical biosensors based on zirconia nanoparticles (Zhang et al., 2008; Zhu et al., 2004; Yang et al., 2007b) with the results shown in Table 1. It can be seen that the proposed DNA biosensor had a lower detection limit and wider detection range for the target ssDNA sequence assay.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.10.011.

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