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Electrochemical biosensor for the detection of cauliflower mosaic virus 35 S gene sequences using lead sulfide nanoparticles as oligonucleotide labels

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ABSTRACT

Lead sulfide (PbS) nanoparticles were synthesized in aqueous solution and used as oligonucleotide labels for electrochemical detection of the 35 S promoter from cauliflower mosaic virus (CaMV) sequence. The PbS nanoparticles were modified with mercaptoacetic acid and could easily be linked with CaMV 35 S oligonucleotide probe. Target DNA sequences were covalently linked on a mercaptoacetic acid self-assembled gold electrode, and DNA hybridization of target DNA with probe DNA was completed on the electrode surface. PbS nanoparticles anchored on the hybrids were dissolved in the solution by oxidation of HNO_3 and detected using a sensitive differential pulse anodic stripping voltammetric method. The detection results can be used to monitor the hybridization reaction. The CaMV 35 S target sequence was satisfactorily detected with the detection limit as 4.38×10^{-12} mol/L (3σ). The established method extends nanoparticle-labeled electrochemical DNA analysis to specific sequences from genetically modified organisms with higher sensitivity and selectivity.

Introduction

Specific DNA sequence detection is one of the most important research fields because of its application in disease diagnosis, drug screening, epidemic prevention, and environmental protection. Various methods have been proposed for DNA analysis, and electrochemical DNA hybridization has attracted great interest for its advantages of low cost, rapidity, simplicity, and selectivity for specific DNA sequences [1,2]. Most electrochemical DNA biosensors have been devised on the basis of the electrical transduction of DNA hybridization by redox indicators, such as dyes, metal complexes, and drugs [3]. The hybridization reaction can also be monitored by the intrinsic signals of nucleic acids, by changes in interfacial properties, and by electroactive labels such as enzyme and metal nanoparticles [4].

In recent years, nanoparticle-labeled electrochemical DNA analysis has aroused great interest [5,6]. Combination of the unique optical, electrical, and biocompatible characteristics of nanoparticles with sensitive and selective electrochemical detection has led to great improvement in signal amplification for DNA detection. Different kinds of nanomaterials, such as gold or silver nanoparticles, carbon nanotubes, and semiconductor quantum dots, have been applied to DNA sequence detection. Fang et al. have reviewed DNA biosensors based on metal nanoparticles [7]. Gao has described the applications of nanoparticle-tagged DNA probes in electrochemical DNA biosensors [8]. Liu et al. have reported an effective nanocrystal-based bioelectronic strategy for coding individual single-nucleotide polymorphisms (SNPs)[1] [9]. Authier et al. have investigated a colloidal gold nanoparticle label for the quantitative electrochemical detection of amplified human cytomegalovirus DNA with a disposable microband electrode [10]. Wang et al. have also used the colloidal gold tag for the electrochemical stripping detection of DNA hybridization [11]. Storhoff et al. investigated gold nanoparticle probes for one-pot colorimetric differentiation of polynucleotides with single-base imperfections [12]. Different nanoparticles, such as gold colloid, silver colloid, Cu@Au (which is a kind of core–shell particle consisting of a core of Cu and a thin layer of Au shell), CdS, PbS, and luminol-doped nanoparticles, have been used as oligonucleotide labels for the electrochemical stripping detection of DNA hybridization [13–18].

In the work described in this article, mercaptoacetic acid (MAA)-modified lead sulfide (PbS) nanoparticles were synthesized in aqueous solution and used as specific ssDNA sequence labels. The target ssDNA was covalently fixed on the MAA self-assembled gold electrode, and the hybridization process was monitored by

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[1] *Abbreviations used:* SNPs, single-nucleotide polymorphisms; Cu@Au, core–shell particle consisting of a core of Cu and a thin layer of Au shell; CaMV, cauliflower mosaic virus; GMOs, genetically modified organisms; MAA, mercaptoacetic acid; EDC, *N*-(3-dimethylamino propyl)-*N*′-ethylcarbodiimide hydrochloride; NHS, N-hydroxysuccinimide; EIS, electrochemical impedance spectroscopy; DPASV, differential pulse anodic stripping voltammetry; GCE, glassy carbon electrode.

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the oxidative dissolution of the PbS nanoparticles in the solution and indirect determination of the lead ion by anodic stripping voltammetry. The proposed method was successfully applied to determination of the CaMV 35 S sequence, which was derived from the cauliflower mosaic virus from *Agrobacterium tumefaciens,* and is often used as an insert and indicator of genetically modified organisms (GMOs). As for GMO products, labeling is mandatory for foodstuffs in many countries, so it is necessary to establish a sensitive analytical method for rapid qualitative and quantitative GMO assay. The commonly used method of GMO detection is the polymer chain reaction (PCR) [19,20]. But it is costly, requiring very expensive equipment and reagents and highly experienced personnel. Electrochemical DNA biosensors provide an effective alternative for GMO detection with their advantages of simplicity, low cost, and ease of use. The proposed method successfully distinguishes three-base mismatched ssDNA sequence and noncomplementary sequences with good selectivity.

Experimental

Reagents

Shenggong Bioengineering Ltd. Company (Shanghai, China) synthesized 23-mer oligunucleotides with the following sequences:

target sequence (S_1): 5′-CAT CGT TGA AGA TGC CTC TGC CG-3′;
complementary (probe) sequence (S_2): 5′-NH_2-CGG CAG AGG CAT CTT CAA CGA TG-3′;
three-base mismatched sequence (S_3): 5′-CAT TGT TGA AGA GGC CTC TGC AG-3′;
noncomplementary sequence (S_4): 5′-CGC AAT TAT ACA TTT AAT ACG CG-3′.

The target sequence was a section of the CaMV 35 S gene of GMOs.

MAA (Tianjin Yuanhang Reagent Co.), *N*-(3-dimethylamino propyl)-N′-ethylcarbodiimide hydrochloride (EDC, Sigma), and *N*-hydroxysuccinimide (NHS, Shanghai Chemical Reagent Co.) were used as received without further purification. Phosphate-buffered saline 0.24 mol/L (0.24 mol/L NaCl + 0.01 mol/L sodium phosphate buffer) was used. All other chemicals were of analytical reagent grade. Double-distilled water was used throughout.

Apparatus

All electrochemical experiments, such as electrochemical impedance spectroscopy (EIS) and differential pulse anodic stripping voltammetry (DPASV), were performed with the CHI 750B electrochemical workstation (Shanghai CH Instrument, China). The three-electrode system was composed of a glassy carbon working electrode (GCE, Φ = 4 mm, used for DPASV measurements), an Ag/AgCl reference electrode, and a platinum wire counterelectrode. A gold electrode (Φ = 2 mm) was used for target ssDNA immobilization, probe hybridization, and the EIS measurements. TEM was performed with a JEM-2000EX transmission electron microscope (JEOL Ltd., Japan). UV–Vis absorption spectra were obtained with a Cary 50 probe UV–Vis spectrophotometer (Varian Co., Australia). A TDL-16B centrifuge (Shanghai Anting Science Instrument Co., China) was used for centrifugation.

Preparation of ssDNA-labeled PbS nanoparticle

MAA-modified PbS nanoparticles were prepared according to the previously described procedure [21]. Briefly, 11.0 μL MAA was added into 50 mL 0.4 mmol/L $Pb(NO_3)_2$ solution, and the pH was adjusted to 7.0 with 0.5 mol/L NaOH. The solution was bubbled with high-purity nitrogen for 30 min, and then 1.34 mmol/L freshly prepared Na_2S solution was added dropwise to the mixture. The reaction was carried out for 24 h under bubbled nitrogen; a brown colloid solution formed gradually, which was stable for a month in the dark. One droplet of the solution was absorbed on a copper grid allowing the solvent to evaporate and be used for TEM measurement.

Oligonucleotide probe (S_2) sequences were covalently linked with MAA-modified PbS nanoparticles using the following procedure [22]. PbS colloid solution 5.0 mL was centrifuged for 30 min at 10000 rpm, and the precipitate was redispersed in 2 mL water. Then, 100 μL of 50.0 mmol/L EDC, 100 μL of 50.0 mmol/L NHS, and S_2 sequence were mixed with the redispersed PbS nanoparticles. The solution was stirred for 18 h at room temperature, and then centrifuged at 10,000 rpm for 30 min to remove unbound oligonucleotides. The PbS–ssDNA precipitate was washed and separated twice with PBS. Finally, the nano-PbS–ssDNA conjugate was resuspended in PBS and stored at −2 °C before use.

Immobilization of S_1 sequence on MAA self-assembled gold electrode

Target (S_1) sequence was covalently linked on the MAA self-assembled gold electrode. The surface of the gold electrode was subjected step by step to the following procedure. First, the gold electrode was heated in a Pirrana solution (30% H_2O_2:concentrated H_2SO_4 3:7) for about 5 min to remove any previous organic layer and then polished with a 0.05-μm albumin/water slurry on polishing cloth to obtain a mirror-like surface. The electrode was sonicated in ethanol and water for 2 min each, respectively. The freshly pretreated gold electrode was dipped into a 10.0 mmol/L MAA solution for about 4 h to produce a MAA self-assembled gold electrode. The electrode was rinsed with water to remove the physically adsorbed MAA and then stored in distilled water. The MAA/Au electrode was immersed in a mixture of 5.0 mmol/L EDC and 8.0 mmol/L NHS in pH 7.4 PBS for 30 min to obtain an active surface, and then dipped in S_1 solution (pH 7.4, PBS) for 12 h at 25 °C. With the help of EDC and NHS, ssDNA was immobilized on the surface of the MAA/Au electrode through deoxyguanosine (dG) residues of DNA with carboxyl group on the surface of the modified electrode to form a S_1/SAM/Au interface.

Hybridization of S_2-labeled PbS nanoparticles with S_1 on the gold electrode

The S_1/SAM/Au electrode was immersed in a hybridization solution (PBS buffer) containing the nano-PbS–ssDNA conjugate for 40 min at 40 °C with stirring. After hybridization, the electrode was washed with PBS, pH 7.4 (containing 0.1% SDS), three times to remove the unhybridized S_2 probes.

Dissolution of PbS nanoparticles and electrochemical measurements

After hybridization, the Au electrode containing nano-PbS–dsDNA was immersed in a 150-μL 1.0 mol/L HNO_3 solution for 5 min to completely dissolve the PbS nanoparticles hybridized on the gold electrode. The resulting solution was transferred into 3 mL HOAc–NaOAc buffer (pH 4.7, containing 1.8×10^{-4} mol/L Hg^{2+}). To detect the dissolved Pb^{2+} sensitively, DPASV was used with the in situ preparation of mercury film on the surface of a GCE with a deposition time of 300 s and deposition potential of −1.1 V. After a 15-s rest time, the anodic stripping peak current ($i_{p,a}$) located at −0.84 V was measured and taken as the analytical response. The conditions selected for DPASV were: increment in potential 0.004 V, amplitude 0.05 V, pulse width 0.06 s, sample width 0.02 s, pulse period 0.2 s.

Results and discussion

Preparation and characterization of S_2-modified PbS nanoparticles

Fig. 1 is the transmission electron micrograph of synthesized PbS nanoparticles. It can be seen that the average diameter of MAA-modified PbS nanoparticles is 25 nm with good dispersion, which was ideal for the following ssDNA labeling procedure without aggregation and precipitation. Because the PbS nanoparticles are modified by MAA molecules, the free carboxyl group outside of PbS can be covalently coupled to the 5′-NH_2 of the S_2 sequence with the help of EDC and NHS [23]. The UV–Vis spectrum of the S_2 sequence-functionalized PbS nanoparticle is shown in Fig. 2. Compared with that of the PbS nanoparticle (curve 1), a new band at 255 nm appeared (curve 2), which was attributed to the absorbance of ssDNA labeled on the PbS nanoparticle. The results indicate that the ssDNA molecules had been successfully labeled on the PbS nanoparticle.

EIS of different modified electrode

Electrochemical impedance spectroscopy has proven to be one of the most powerful tools for investigation of interfacial reaction mechanisms [24–26]. EIS measures the response (current and its phase) of an electrochemical system to an applied oscillating potential as a function of frequency. Based on the charge-transfer kinetics of the $Fe(CN)_6^{3-/4-}$ redox couple, Faradaic impedance spectra with an $Fe(CN)_6^{3-/4-}$ redox probe are modeled using the equivalent circuit approach of Randles. The monolayer of electrode surface is treated as a resistor and capacitor in parallel, and the solution resistance is represented by a resistor. The interfacial electron-transfer resistance (R_{et}) between the monolayer and the $Fe(CN)_6^{3-/4-}$ redox couple varies greatly between monolayers of the different classes of compounds. The Nyquist plot, a widely used format of EIS, can be described as a semicircle near the origin at high frequencies followed by a linear tail. The magnitude of R_{et} can is a reflection of the semicircle diameter of the Nyquist plot.

Fig. 3 is the Nyquist plot of the gold electrode subjected to the step-by-step modification process. After the MAA monolayer self-assembled on the Au electrode, the interfacial R_{et} increased greatly (curve 2) compared with that of the bare Au electrode (curve 1); this was attributed to the large quantity of negative charges from $-COO^-$ groups that perturbed the rates of interfacial electron transfer between the electrode and the electrolyte solution. After the MAA/Au electrode surface was activated with EDC and NHS, the negative charges of $-COO^-$ groups were sealed, so R_{et} decreased evidently (curve 3). After the target ssDNA was immobilized on the electrode surface, R_{et} increased because of the strong electrostatic repulsion between the negatively charged $Fe(CN)_6^{3-/4-}$ molecules and the negative charges on the DNA phosphate backbone. This phenomenon was further enhanced after hybridization was completed on the electrode surface, and an obvious increase in R_{et} was observed (as shown in curve 5). The EIS results indicate that modification and hybridization were successfully accomplished on the electrode surface.

DPASV for the determination of Pb^{2+}

Differential pulse anodic stripping analysis is known to be one of the most sensitive techniques and is widely used for trace heavy metal analysis in various samples because of its ability to preconcentrate analytes on the surface of the electrode [27]. First, the metals are preconcentrated by electrodeposition into a small-volume mercury electrode. The preconcentration is achieved by

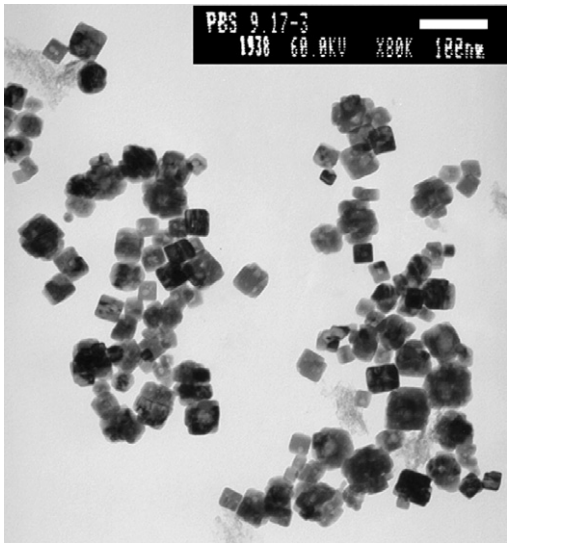

Fig. 1. Transmission electron micrograph of MAA modified PbS nanoparticles.

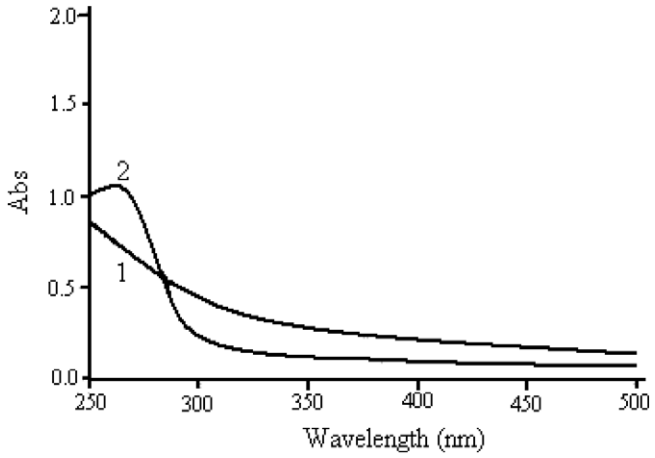

Fig. 2. UV–Vis absorption spectra of PbS colloid (1) and PbS–ssDNA conjugate (2).

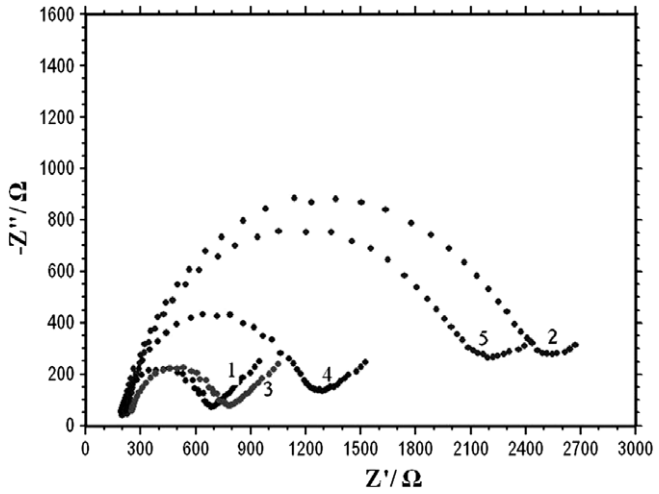

Fig. 3. Nyquist plot of the Faradaic impedance measured in 0.1 mol/L KCl solution containing 10 mmol/L $Fe(CN)_6^{3-/4-}$ at a potential of 0.15 V (vs Ag/AgCl) in the frequency range from 10^4 to 1 Hz. (1) Bare gold electrode; (2) SAM/Au electrode; (3) activated SAM/Au electrode; (4) S_1/SAM/Au electrode; (5) PbS/dsDNA/SAM/Au electrode.

cathodic deposition at a controlled time and potential; the metal ions reach the mercury electrode by diffusion and convection, where they are reduced and concentrated as amalgams. Then the forced convection is stopped, and the potential is scanned anodically. During this anodic scan, the amalgamated metals are reoxidized and stripped out of the electrode.

By in situ preparation of mercury film on the GCE and electrodeposition of lead ions in the mercury film at the potential of −1.1 V for 300 s, the stripping procedure was carried out in the range of potentials from −1.1 to −0.5 V after 15 s of rest. At the optimal conditions for DPASV of Pb^{2+} selected and in pH 4.7 HOAc–NaOAc buffer solution, a good anodic stripping peak appeared at −0.84 V (vs Ag/AgCl). Under optimal conditions, the anodic peak currents were in good linear relationship with the concentration of lead ion in the range 2.0×10^{-8} to 2.0×10^{-6} mol/L according to the linear regression equation $i_{p,a}$ (μA) = 7.57*C* (μmol/L) − 0.064 (n = 8, γ = 0.997). The detection limit calculated was 8.5×10^{-9} mol/L (3σ).

Electrochemical DNA detection with PbS-nanoparticle labels

Fig. 4 illustrates the principle of electrochemical DNA detection using PbS nanoparticles as labels, which comprises four main steps: (a) preparation of self-assembled gold electrode; (b) immobilization of target oligonucleotides on the SAM/Au electrode; (c) hybridization with PbS-labeled oligonucleotide probes; (d) dissolution of PbS and DPASV detection of the Pb^{2+} released.

On the basis of this principle, the proposed method was applied to detection of the CaMV 35 S sequence from GMOs. The sensitivity of this protocol was carefully investigated by varying the target oligonudeotide concentration. As shown in Fig. 5A, the more fixed the S_1 sequence, the higher the anodic peak current. The results indicate that the anodic stripping peak current of lead ion has a good linear relationship with the logarithmic value of the target DNA concentration ranging from 1.2×10^{-11} to 4.8×10^{-8} mol/L; the results are shown in Fig. 5B. The linear regression equation was calculated as $i_{p,a}$ (μA) = 0.123 log *C* (pmol/L) + 0.616 with a correlation coefficient as 0.994 (n = 7). The detection limit obtained for S_1 was 4.38×10^{-12} mol/L (3σ). With respect to sensitivity, this method was better than electrochemical stripping detection of DNA hybridization based on gold nanoparticle labels [14] and was competitive with the DNA hybridization assay by impedance measurement based on CdS nanoparticles [20].

In Fig. 6 are the DPASV signals for the detection of different kinds of oligonucleotides including complementary sequence, noncomplementary sequence, and three-base mismatched sequence. Blank measurement (without target DNA immobilized on the electrode) also gave a DPV signal, which may be attributed to the physical adsorption of PbS nanoparticles on the MAA/Au electrode (curve 1). The noncomplementary sequence had a response almost equivalent to that of the blank measurement (curve 2). Compared

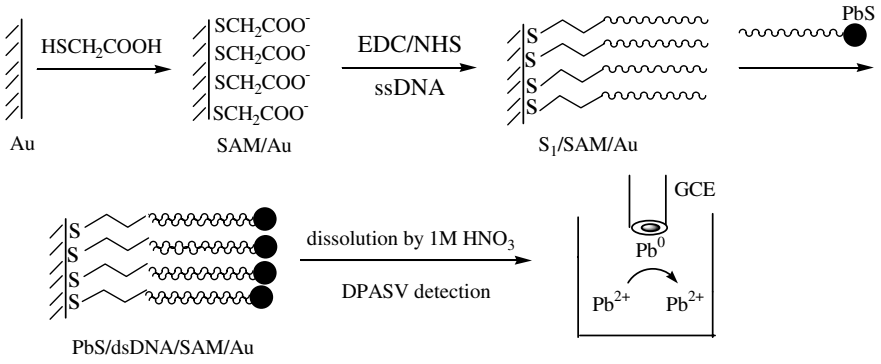

Fig. 4. Schematic representation of electrochemical DNA detection with PbS nanoparticle labels.

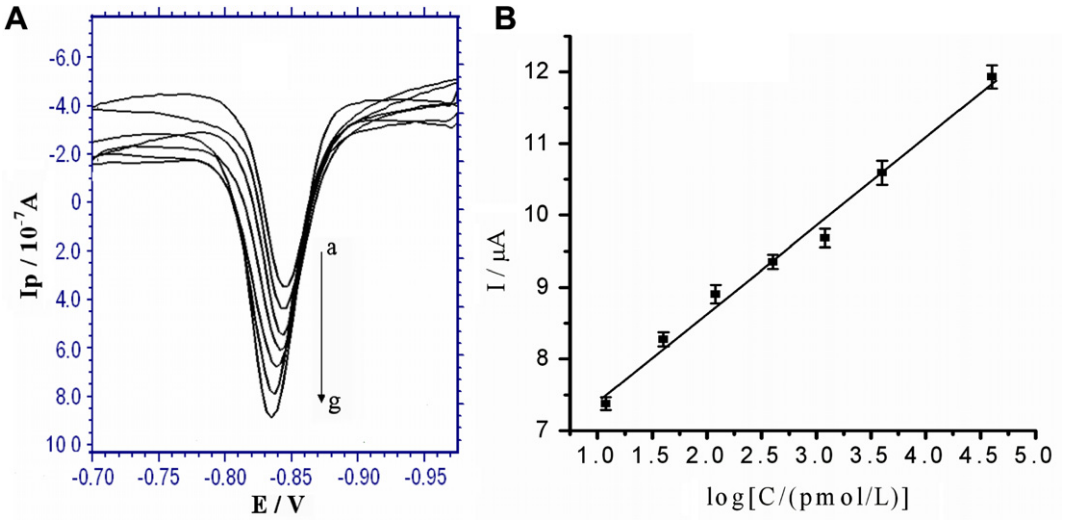

Fig. 5. (A) Differential pulse anodic stripping voltammograms for different target concentrations of S_1 sequence (pmol/L): (a–g) 12, 48, 120, 480, 1200, 4800, and 48,000. (B) Plot of *I* versus logarithm of target DNA concentration. *I* is the anodic stripping peak current of lead ion.

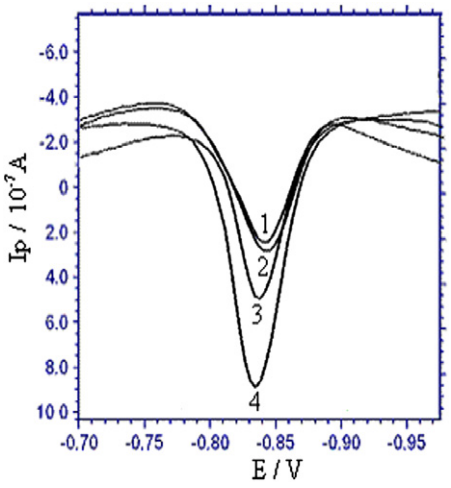

Fig. 6. Differential pulse voltammograms of hybridization with different kinds of 4.8×10^{-8} mol/L oligunucleotides. (1) No sequence; (2) noncomplementary sequence; (3) three-base mismatched sequence; (4) complementary sequence. The concentration of probe sequences was 5.0×10^{-6} mol/L.

with the complementary sequence (curve 4), the three-base mismatched sequence gave a smaller DPV signal (curve 3). Thus, the results indicate that this analytical protocol has good selectivity for the different ssDNA sequence.

Conclusions

CaMV 35 S sequences from GMOs were successfully detected with the PbS nanoparticle oligonucleotide labeled electrochemical DNA biosensor. By immobilizing target ssDNA on the MAA self-assembled gold electrode, the PbS nanoparticle-labeled probe ssDNA was hybridized with target ssDNA on the electrode surface. PbS nanoparticles in the hybrids were dissolved in the solution and detected sensitively with the DPASV method. The current responses had good linear relationship with the concentration of target ssDNA sequence in the range 1.2×10^{-11} to 4.8×10^{-8} mol/L. The DNA biosensor fabricated had good ability to distinguish complementary, noncomplementary, and three-base mismatched ssDNA sequences.

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