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Characterization of single-stranded DNA on chitosan-modified electrode and its application to the sequence-specific DNA detection

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Abstract Single-strand DNA could bind with chitosan on a platinum electrode via forming a tight DNA-chitosan complex. The salt concentration of the ssDNA solution had an obvious effect on the surface coverage, the immobilization was remarkably reduced at high salt concentration. The sample ssDNA immobilized on the chitosan-modified electrode can hybridize efficiently with the complementary sequences and be successfully used for the sequence-specific DNA detection. The same results could be obtained using a gold or graphite electrode modified with chitosan. The stability of this electrode has been also discussed.

1 Introduction

There has been considerable interest in developing reliable methods for detecting and quantifying specific DNA sequences. The traditional method always uses an enzymatic label with radioisotopes, *i.e.* ^{32}P or ^{125}I. Various techniques, such as those based on biotin [1], digoxigenin [2], fluorescent dye [3], chemiluminophore [4] and electrochemical DNA biosensor [5–21], have been proposed to overcome the main disadvantages of the traditional method, *e.g.* long assay time and the use of hazardous radioisotopes. Electrochemical techniques have been applied widely to sequence-specific DNA diagnosis and detection [5–15] with the advantages of being cheap, sensitive and rapid. Our group has also reported on several electrochemical DNA biosensors for specific DNA sequence detection [16–19] and the damage and protection of DNA [20, 21].

With the development of an electrochemical DNA biosensor, surface-immobilization of DNA becomes more important. There are many reported methods for surface immobilizing DNA on electrodes, such as chemical adsorption [5–6], covalent-binding [7, 8, 16, 17], antigen-antibody method [9] and electrostatic attraction [10–15, 18–20]. Here, we report on a novel method of single-stranded DNA (ssDNA) immobilization at a chitosan modified platinum electrode for the detection of the sequence-specific DNA with high sensitivity and selectivity. An electrochemical characterization of the electrode surface and the mechanism of immobilization were investigated.

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2 Experimental

2.1 Chemicals and solutions. The pNC_3 plasmid DNA (about 4000 bp, containing a sequence complementary to the PCR products, $A_{260}/A_{280} = 1.73$, no further purification) and the PCR products (about 200 bp, $A_{260}/A_{280} = 1.96$) were obtained from the molecular Biology Laboratory, Department of Biology, East China Normal University (Shanghai, China). λDNA and pBR322 DNA were purchased from Huamei Biotechnology Company (Shanghai, China). The DNA concentration was determined spectrophotometrically using the known molar absorption coefficient 6600 $(mol/L)^{-1}\ cm^{-1}$ at 260 nm (per P or nucleotide unit) [22]. Denatured single-stranded DNA (ssDNA) was produced by heating native double-stranded DNA (dsDNA) in a boiling water bath about 10 min followed by rapid cooling in an ice bath. Chitosan oligomer (1.0% solution in 1.0% acetic acid, *v/v*) was purchased from Aldrich company (US). Ferrocenecarboxaldehyde (FCA) was obtained from Tohoku University (Japan). 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) and hydroxymethyl-aminomethane (tris) were purchased from Sigma (US). N-Methylimidazole and sodium dodecylsulfate (SDS) were purchased from Guangyao Chemicals Company (Jiangsu, China). The following buffers were used: 2 × SSC buffer, 0.3mol/L NaCl + 0.03 mol/L sodium citrate (pH 7.0); TE buffer, 10 mmol/L Tris-HCl +1.0 mmol/L EDTA (pH 8.0). Other reagents were commercially available and were all of analytical reagent grade. Solutions were prepared with double-distilled water.

2.2 Instrumentation. A CHI 630 Electrochemical Analyzer (CHI Instruments Inc., US), a JB-1 stirring machine (Branson, Shanghai, China), a Varian Cary 50 UV spectrophotometer (Varian Com. US) and a TDL-16B centrifuge (Anting Science Instrument Inc., Shanghai, China) were used. The three-electrode system consisted of a working electrode made of gold with diameter of 2.5 mm, an Ag/AgCl reference electrode and a counter electrode made of platinum. All electrochemical measurements were carried out in a 10 mL cell.

2.3 Immobilization of DNA on platinum electrode. The chitosan-modified platinum electrode (chitosan/PE) was prepared by applying a 2.0 μL 1.0% chitosan solution uniformly on a freshly smoothed platinum electrode, then it was dried and subsequently the elec-

trode was immersed in 0.1 mol/L NaOH solution for 30 min to make the film more stable. The electrode was dried again in air. Thereby, a chitosan oligomer film was formed on the platinum electrode.

The chitosan-modified electrode was immersed in a 1.0 mL TE solution containing 6.06 μmol/L denatured plasmid DNA solution (ssDNA). The solution was stirred at room temperature (25 ± 0.5 °C) for 120 min. Thus, the ssDNA was immobilized on the chitosan-modified platinum electrode (ssDNA/chitosan/PE). Subsequently, the electrode was washed with 0.1% SDS phosphate buffer (pH 7.0) three times and immersed in a 0.01 mol/L TE buffer (pH 8.0).

2.4 Measurement of apparent capacitance. The capacitance was measured by cyclic voltammetry (CV) in 1.0 mol/L KNO_3 between +0.40 and +0.60 V (*vs.* Ag/AgCl). The charging current at +0.50 V was recorded in the third cycle of cyclic voltammetry and was plotted versus scan rates (< 100 mV/s). The slope of the plot represents the apparent capacitance.

2.5 Hybridization. Hybridization reaction was done by immersing the immobilized ssDNA electrode in a 5.0 mL 2 × SSC buffer containing the 5.0 μmol/L FCA labeled PCR product, marked as FCA-ssDNA (its synthesis was carried out according to [18]). After incubating at 42 °C for 1 h with shaking, the electrode was washed three times with a 0.1% SDS phosphate buffer (pH 7.0) to remove the unhybridized DNA.

2.6 Ultraviolet-visible spectrophotometric detection. UV-vis spectrophotometric detection was carried out using a Varian Cary 50 UV spectrophotometer. The ssDNA immobilized electrode was immersed in a 2.0 mL 0.1 mol/L HCl solution and incubated at 37 °C by shaking, the solution was cooled gradually to room temperature. Then the absorbance at 260 nm was recorded.

2.7 Electrochemical detection. CV and differential pulse voltammetry (DPV) were carried out at 25 °C in a 10 mL electrochemical cell with a normal three-electrode configuration consisting of a Ag/AgCl reference electrode, a gold wire counter electrode and a working electrode. Both modes of voltammetry were performed at 100 mV/s in a TE buffer (pH 8.0).

3 Results and discussion

3.1 Evidence for the immobilization of ssDNA at chitosan modified platinum

Chitosan oligomer is a kind of natural cationic polymer [23]. The formation of a chitosan oligomer film at a platinum electrode could greatly increase the electrochemical response of the ferrocyanide/ferricyanide due to an accumulation of the negatively charged ferricyanide ions. Curve *a* in Fig. 1 shows the electrochemical response of bare platinum electrode in a 0.1 mol/L phosphate buffer (pH 7.0) containing 1.0 mmol/L $K_3Fe(CN)_6$, curve *b* shows that of the chitosan/PE and curve *c* shows that of the ssDNA/chitosan/PE. The contact time of ssDNA/chitosan/PE with $K_3Fe(CN)_6$ solution was 1.0 min. As can be seen, the peak current of curve *b* increased obviously indicating the effect mentioned above, hence the presence of a chitosan film at the platinum electrode. However, compared with the peak current of the electrode modified only with the chitosan oligomer (curve *b*), the peak current of ssDNA/chitosan/PE (curve *c*) decreased obviously. All the results were the means of seven measurements, the RSD (relative standard deviation) were 2.0%, 2.3% and 2.5% for curve *a*, curve *b* and curve *c*, respectively. The reason was that the immobilized ssDNA had partially neutralized the positive charge

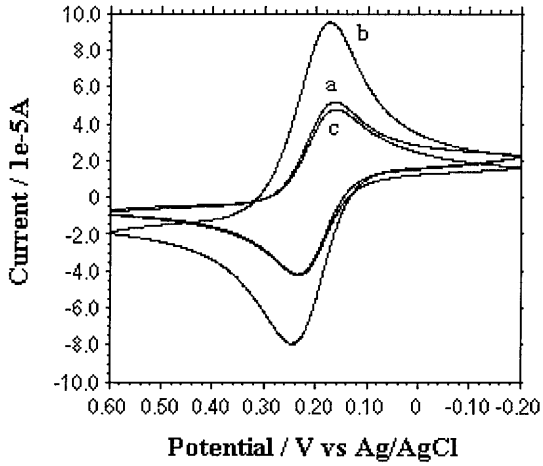

Fig. 1 Cyclic voltammograms for $K_3Fe(CN)_6$ on (*a*) bare platinum electrode, (*b*) chitosan modified platinum electrode and (*c*) ssDNA-chitosan modified platinum electrode. $K_3Fe(CN)_6$ concentration, 1.0 mmol/L; contact time of ssDNA/chitosan/PE with $K_3Fe(CN)_6$ solution, 1.0 min; scan from –0.20 to +0.60 V (vs. Ag/AgCl); scan rate, 100 mV/s

on the chitosan oligomer film. The decrease of the peak current of ferricyanide at the ssDNA/chitosan/PE indicated the formation of an ssDNA-chitosan film modified at the platinum electrode surface.

Another experiment for the confirmation of the formation of an ssDNA-chitosan film immobilized at the platinum electrode surface was done according to the procedures described in experimental section 2.3 except the replacement of the unlabeled sample plasmid ssDNA with the FCA-ssDNA. The modified electrode in this experiment was marked as FCA-ssDNA/chitosan/PE. DPV was used in 0.1 mol/L acetate buffer solution (pH 4.2) with the FCA-ssDNA/chitosan/PE as working electrode. An anodic peak of FCA was observed at +0.25 V (*vs.* Ag/AgCl, curve *b*). For comparison, the DPV curve of a bare PE was also shown in Fig. 2 (curve *a*). The anodic peak of FCA in

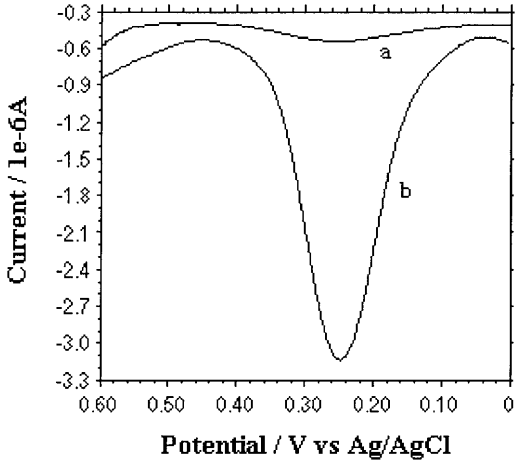

Fig. 2 DPV response of FCA labeled ssDNA probe at chitosan/PE. Pulse amplitude, 2.5×10^{-2} V; scan from 0.00 to +0.60 V (vs. Ag/AgCl); scan rate, 100 mV/s

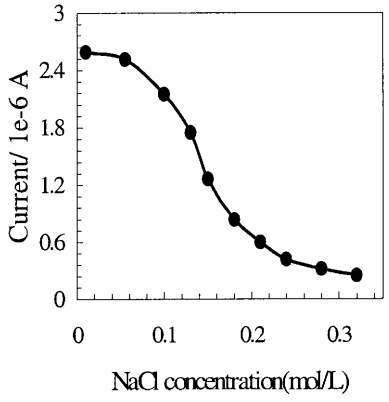

Fig. 3 Effect of salt concentration on the immobilization of FCA labeled ssDNA at the chitosan modified electrode measured as the DPV response of FCA. Conditions as in Fig. 2

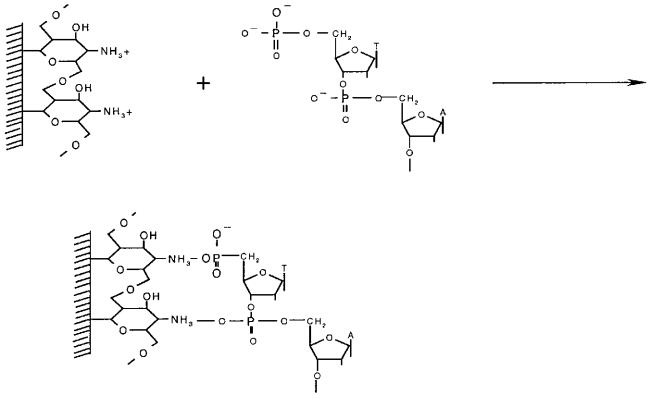

Fig. 4 Schematic immobilization diagram of ssDNA on chitosan modified platinum electrode

curve *a* was much lower than that on the FCA-ssDNA/chitosan/PE in curve *b*. That also confirmed the immobilization of ssDNA at chitosan modified electrode.

3.2 Mechanism of ssDNA immobilizing on platinum electrode

The adsorption of ssDNA onto the electrode mostly takes place at the bases of DNA, which influences the hybridization and the bio-affinity of complementary DNA [16]. The polycationic polymer of glucosamine, chitosan, could bind to DNA and other polyanions [24]. DNA could form an insoluble complex with chitosan in solution. The content of DNA in the complex was about 50% and the DNA remained insoluble in aqueous media of pH 2–7 [25]. In this approach, we changed the anion concentration (in this case, NaCl was used as a typical substance) in the ssDNA solution used for immobilization and found that the anion concentration had an obvious effect on the DNA binding with the chitosan film. Using FCA-ssDNA as an indicator, the peak current of FCA decreased from 2.60 to 0.25 μA with increasing anion concentration from 0.01 to 0.30 mol/L. When the NaCl concentration was over 0.30 mol/L, the observed FCA peak current was less than one tenth of the peak current at the NaCl concentration of 0.01 mol/L (shown in Fig. 3). This result indicated that the immobilization of ssDNA was inhibited by the high salt concentration. All the results suggested that DNA and chitosan formed a tight complex because of ionic interaction, which were consistent with that described in literature [25] and could be confirmed by the apparent capacitance test. The schematic mechanism diagram is shown in Fig. 4.

On the other hand, the effect of high ionic strength can be successfully utilized to avoid a non-specific DNA adsorption. As the hybridization solution was 2 × SSC buffer and the salt concentration of this buffer was about 0.3 mol/L, the nonspecifically adsorbed DNA was inhibited by the high anion concentration in the hybridization buffer. Thus, the surface-bound ssDNA were accessible for specific hybridization with the already labeled complementary oligonucleotides and were able to discriminate between complementary and noncomplementary DNA molecules. That is the primary advantage of using this chitosan oligomer film to immobilize ssDNA.

SsDNA was immobilized also at chitosan modified gold electrode and graphite electrodes with the procedure described. The immobilization mechanism was similar to that at chitosan modified platinum electrode and the immobilization relies on the salt concentration, too.

3.3 Apparent capacitance measurements

The cyclic voltammograms were recorded in a 1.0 mol/L KNO_3 solution between +0.40 and +0.60 V (*vs.* Ag/AgCl) at PE, chitosan/PE and ssDNA/chitosan/PE, respectively. The capacitive current densities (j_c) at +0.50 V (*vs.* Ag/AgCl) were measured with different scan rates (ν < 100 mV/s). The slope of j_c versus ν is called the apparent capacitance [26–28], The capacitive currents are lower than predicated values when ν > 100 mV/s because of the limited ion transfer rates in the solution [26, 28]. When the apparent capacitance is measured in concentrated electrolytes at low scan rate (< 100 mV/s), j_c is proportional to ν. The values of the calculated apparent capacitance are shown in Table 1 (the geometric area of the electrodes was calculated by measuring the peak current in $K_3Fe(CN)_6$ solution).

It could be seen that the apparent capacitance of the chitosan/PE was lower than that of the bare PE. Since the capacitance per unit area is:

$$C = \varepsilon_r \varepsilon_0 / d \quad (1)$$

where ε_r is the relative permittivity of the layer coated on the electrode, d is its thickness and ε_0 is the permittivity of free space. The capacitance is inversely proportional to the layer thickness (*d)* [26]. However, it increases if the electronic double layer is formed on the electrode surface. The immobilization of chitosan increased the layer thickness on the chitosan/PE surface, so the C values decreased. The immobilization of chitosan/ssDNA also increased the layer

Table 1 Apparent capacitance data for current densities evaluated at +0.50 V (vs. Ag/AgCl) from cyclic voltammograms in solution of 1.0 mol/L KNO_3[a]

Electrodes	Apparent capacitance ($\mu F\ cm^{-2}$)	RSD(%)
Bare PE	43.12	2.0
Chitosan/PE	34.20	2.3
SsDNA/chitosan/PE	44.68	2.3

[a] The number of independent measurements on different electrodes was 7

thickness, but at the same time it formed an electric double layer on the ssDNA/chitosan/PE surface, so the C values increased slightly (compared with the bare PE). The capacity order of these electrodes was chitosan/PE < bare PE < ssDNA/chitosan/PE due to the two influence factors. This also proved that ssDNA could be immobilized on chitosan/PE.

3.4 The stability of ssDNA/chitosan/PE

To investigate the stability of ssDNA/chitosan/PE, the electrode was incubated in a 0.1 mol/L HCl solution at 37 °C by shaking for 4 h, then the solution was recorded by UV-vis. Almost no DNA absorption at 260 nm was observed using the 0.15 mol/L HCl solution as baseline correction, even when the incubation time was over 4 h. This indicated that the immobilized ssDNA was stable in the 0.1 mol/L HCl solution. The UV-vis absorption of DNA was linearly with the amount of ssDNA in solution ranging from 0.15 to 30.0 μmol/L. A detection limit to the ssDNA was 50.0 nmol/L using 3*s* (where *s* is the standard deviation of blank solution, n = 11). The same experiment was done except using a 0.1 mol/L NaOH solution for incubation. The DNA absorption at 260 nm (A_{260} = 0.03, A_{260}/A_{280} = 1.72) was observed using the 0.1 mol/L NaOH as baseline correction only when the incubation time was 1 h, which indicated that the immobilized ssDNA was unstable in the 0.1 mol/L NaOH solution. In addition, when the ssDNA/chitosan/PE was incubated in a 0.02 mol/L phosphate-buffered saline (pH 7.0) at 37 °C for about 26 h, DNA absorption at 260 nm was hardly observed, which indicated that the immobilized ssDNA was stable in the phosphate-buffered saline at pH 7.0.

Moreover, the temperature stability of the surface-bound ssDNA was explored. The ssDNA/chitosan/PE was incubated in a 2.0 mL 2 × SSC buffer (pH 7.0) for 1 h at different temperatures. The solution was cooled gradually to room temperature before recording UV absorption. Almost no absorption at 260 nm was observed up to 100 °C, which indicated that the modified electrode was stable at high temperatures.

3.5 Sequence-specific DNA detection

The sample DNA, namely plasmid DNA, λDNA and pBR322 DNA (all at the concentration of 0.2 μmol/L), were first denatured in a boiling water bath about 10 min followed by rapid cooling in an ice bath. Then they were immobilized at different chitosan/platinum electrodes according to the procedure specified. After hybridization with the denatured FCA-PCR product (its bases sequence already known), these platinum electrodes were subjected to electrochemical measurement in a TE buffer. The differential pulse voltammograms are shown in Fig. 5. The results showed that only the plasmid DNA has a complementary sequence to the PCR product, other samples did not have a complementary sequence to the PCR product. The anodic currents of FCA were in sections linearly with the amount of immobilized plasmid DNA ranging from 0.01 to 5.0 μmol/L. The regressing equation ranging from 0.01 to 0.20 μmol/L was y = 2.073x + 0.238 (x is the concentration of denatured plasmid DNA, $\times 10^7$) and the regressing coefficient (r^2) of the linear curve was 0.9972. A detection limit to the plasmid DNA was 5.0 nmol/L using 3*s* (where *s* is the standard deviation of blank solution, n = 11). The relative standard deviation based on 11 measurements for plasmid DNA was 3.6%.

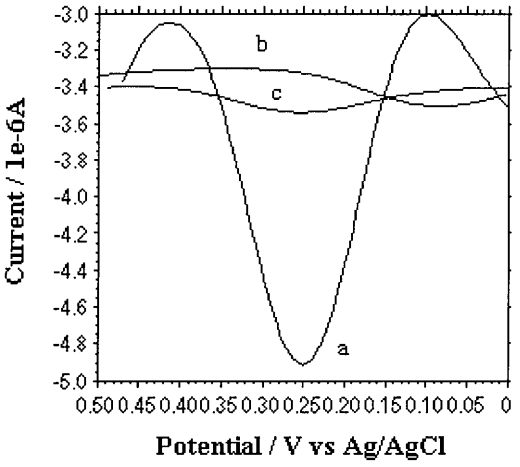

Fig. 5 Differential pulse voltammograms of FCA at (*a*) denatured Plasmid DNA chitosan/modified platinum electrode, (*b*) denatured λDNA modified chitosan/platinum electrode and (*c*) denatured pBR322 DNA modified chitosan/platinum electrode after a hybridization with FCA-PCR product; scan from 0.00 to +0.50 V (vs. Ag/AgCl). Other conditions as in Fig. 2

4 Conclusion

Single-strand DNA could bind with the chitosan at a platinum electrode via forming a tight DNA-chitosan complex. The ionic strength of the ssDNA solution had an obvious effect on surface coverage, with immobilization remarkably reduced at high ionic strength. The main advantage of using this chitosan oligomer film to immobilize ssDNA is that the effect of high ionic strength can be successfully utilized to avoid a non-specific DNA adsorption. The immobilized ssDNA at the chitosan modified platinum electrode was very stable in a phosphate-buffer saline (pH 7.0)

and a HCl solution (pH 1.0), and unstable in a NaOH solution (pH 13.0). The ssDNA/chitosan/PE can be satisfactorily used in DNA recognition and detection. Future works include the further application of electrochemical DNA probe on DNA recognition and detection, and the study on DNA damage and protection.

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