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Investigation of the Interaction between ssDNA and 2-Aminophenoxazine-3-one and Development of an Electrochemical DNA Biosensor

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ABSTRACT

An electrochemical method was used to probe the interaction between 2-aminophenoxazine-3-one (AP) and the short DNA sequence related to the hepatitis B virus (HBV), and an electrochemical DNA biosensor was developed. The voltammetric signals of AP have been investigated at bare glassy carbon electrode (bare GCE), hybrid double-stranded DNA-modified GCE (dsDNA/GCE), and single-stranded DNA-modified GCE (ssDNA/GCE) by means of differential pulse voltammetry (DPV), and the peak currents increased with respect to the order of electrodes. The extent of hybridization was evaluated on the basis of the difference between signals of AP with a probe before and after hybridization with the complementary sequence. Control experiments with noncomplementary were performed to test the selectivity of the biosensor. With this approach, a sequence of the HBV could be quantified over the range from 3.53×10^{-7} to 1.08×10^{-6} M, with a linear correlation of $r = 0.9963$ and a detection limit of 1.00×10^{-7} M.

INTRODUCTION

AP (2-AMINOPHENOXAZINE-3-ONE), also known as questiomycin A, is related to the naturally occurring antibiotic actinomycin D (AMD) (Simandi et al., 1993), and has been used as a model for the behavior of AMD (Nakazawa et al.,1985). AMD is an antitumor antibiotic that contains a planar 2-aminophenoxazine-3-one chromophore and two bulky cyclic pentapeptide lactones. AMD is one of the most widely studied, biologically active, small molecules. The widespread interest in AMD is due to the drug's activity as an antibiotic and as an antitumor agent. The interaction of small organic molecules with duplex DNA is the molecular basis of many antitumor, antiviral, and antibiotic drugs. There are a number of studies on the interactions between AMD and DNA with electrospray ionization mass spectrometry (ESI-MS) (Wan et al., 2000; Mazzitelli et al., 2007), circular dichroism (Qu et al., 2003), fluorescence spectroscopy, isothermal titration calorimetry (Ren et al., 2000), angle-resolved surface plasmon resonance (SPR) imaging (Wolf et al., 2005), and electrochemical methods (Wang et al., 2003). However, there are few reports on the interaction between AP and DNA.

DNA biosensors based on nucleic acid recognition processes are rapidly being developed, with the goal of rapid and inexpensive discovery of genetic and infectious diseases. Electrochemical DNA biosensors are widely used to detect DNA hybridization events, because of their high sensitivity and compatibility with microfabrication technology (Drummond et al., 2003; Hansen et al., 2006; Castaneda et al., 2007; Hanaee et al., 2007; Shrestha et al., 2007). These sensors can be prepared by immobilizing single-stranded DNA probes on different electrodes and using electroactive indicators to measure the hybridization events between the DNA probes and their complementary DNA fragments. An indirect electrochemical DNA hybridization detection method is based on incorporation of an electroactive indicator such as anticancer agents (Erdem and Ozsoz, 2001; Jelen et

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al., 2002), organic dyes (Kobayashi et al., 2004; Ju et al., 2005; Jin et al., 2007), and metal complexes (Li et al., 2007a, 2007b). Marrazza et al. (1999, 2000) showed that daunomycin (DM) could be used as an electrochemical hybridization indicator for the detection of the hybridization of DNA. Ozsoz et al. (Erdem et al., 2000; Meric et al., 2002) reported that methylene blue was a promising indicator for electrochemical detection of mismatched bases in oligonucleotides. Ozsoz et al. (Kara et al., 2002a) also reported the electrochemical detection of hybridization between the covalently immobilized probe and its target and damage to DNA by use of hemin. To the best of our knowledge, no electrochemical DNA biosensor with AP as external indicator that interacts in a different way with ssDNA and dsDNA has been reported. The changes in the electrochemical response of AP were monitored to detect hybridization.

In our previous report, the interaction between double-stranded DNA and AP was investigated by cyclic voltammetry and UV-Vis spectroscopy (Zhang et al., 2004). Here we present an electrochemical DNA biosensor for the detection of DNA hybridization related to the Hepatitis B virus (HBV) by using a new redox indicator, AP. The results showed that there was a specific interaction between a single-stranded probe DNA, which is blocked upon hybridization. It is extended to test the specificity of the sensor using a complementary and noncomplementary DNA chain for the hybridization event. The interaction of AP with ssDNA was investigated using ssDNA modified glassy carbon electrodes and ssDNA modified carbon paste electrodes to explore the proposed interaction between AP and guanine bases.

MATERIALS AND METHODS

Apparatus and reagents

All electrochemical measurements were carried out at room temperature, using a conventional three-electrode cell. Modified glassy carbon electrodes (GCEs) and carbon paste electrodes (CPEs) were used as a working electrode, and a Pt wire served as an auxiliary electrode. All potentials are reported against an Ag/AgCl/KCl (sat) reference electrode. Cyclic voltammetric (CV) and DPV studies were carried out with a Potentiostat/Galvanostat Model 263A Electrochemical Workstation (Princeton Applied Research, Oakridge, TN).

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide solution (EDC) and N-hydroxysuccinimide solution (NHS) from Sigma (St. Louis, MO) were used without further purification. The synthetic oligonucleotides were purchased from Shanghai Huashun Biological Engineering Company (Shanghai, China) for the HBV derived from HBV-DNA subtype adr nucleotide position 1734–1754 (Ono et al., 1983; Erdem et al., 2000) and the 21-mer synthetic oligonucleotides with increasing numbers of guanine bases. The base sequences for HBV were as below:

immobilized probe (S1): 5′-GAG-GAG-TTG-GGG-GAG-CAC-ATT-3′;
target (S2): 5′-AAT-GTG-CTC-CCC-CAA-CTC-CTC-3′;
noncomplementary sequence (S3): 5′-ATG-TGG-AAA-ATC-TCT-AGC-AGT-3′.

The 21-mer synthetic oligonucleotides with incresing numbers of guanine bases were shown as follow:

1: 5′- AAA-AAA-AAA-AAA-AAA-AAA-AAA-3′;
2: 5′- AAA-AAA-AAA-AGA-AAA-AAA-AAA-3′;
3: 5′- AAA-AAA-AAG-AGA-GAA-AAA-AAA-3′;
4: 5′- AAA-AAA-GAG-AGA-GAG-AAA-AAA-3′;
5: 5′- AAA-AGA-GAG-AGA-GAG-AGA-AAA-3′;
6: 5′- AAG-AGA-GAG-AGA-GAG-AGA-GAA-3′.

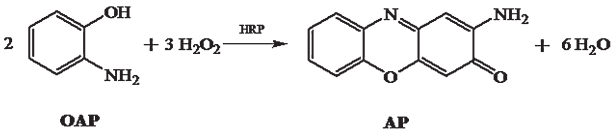
All oligonucleotides were dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH8.00 (TE buffer) and kept frozen. AP was prepared from *o*-aminophenol (OAP), H_2O_2 and HRP by enzyme-catalyzed reaction as described in the literature (Zhang et al., 1999a). HRP can catalyze enzyme substrates OAP and H_2O_2 to AP and H_2O. The reaction scheme was shown in Scheme 1. Other chemicals employed were all of analytical grade and double distilled water (DDW) was used throughout.

Preparation of the CPE working electrode

The CPE was constructed by a through hand mixing of the desired amounts of graphite powder and mineral oil [70/30 (w/w) graphite/oil]. A portion of the resulting paste was packed into the end of a 3-mm (i.d.) glass tube. The electrical contact was established via a copper wire. The surface was smoothed on a weight paper.

Probe immobilization on GCE and CPE

Direct covalent modification of GCEs with DNA was conducted according to the following procedure (Pang et al., 1996). The pretreated GCE was first oxidized at +0.50 V for 1 minute in 50 mM phosphate buffer solution (PBS, pH 7.40). Modification of the electrode was



Scheme 1. The enzyme-catalyzed reaction scheme.

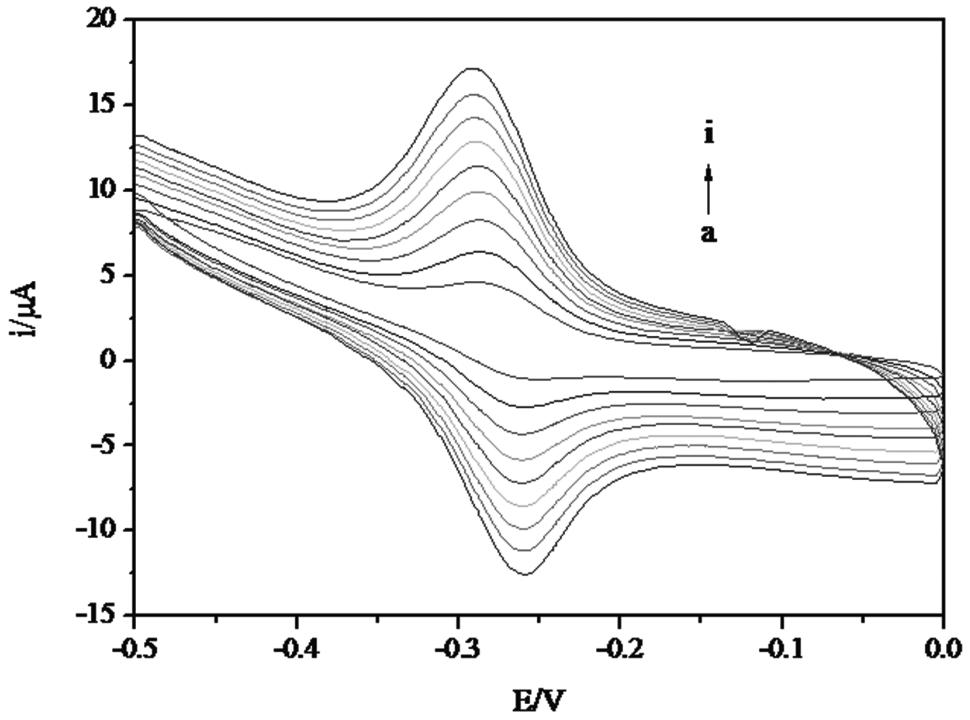


FIG. 1. Cyclic voltammograms bare GCE in 0.2 M PBS (pH 6.80) containing 4.39×10^{-5} M AP at (**a**) 20, (**b**) 40, (**c**) 60, (**d**) 80, (**e**) 100, (**f**) 120, (**g**) 140, (**h**) 160, (**i**) 180 mV/s.

achieved by dropping 20 μL PBS containing 5 mM EDC and 8 mM NHS on the electrode surface. Finally, ssDNA solution was dropped on the modified GCE surface. Then the electrode was rinsed with DDW to eliminate the DNA adsorbed. The DNA-immobilized electrode was stored in 20 mM Tris–HCl buffer (pH 7.00).

The CPE surface was pretreated by applying +1.70 V for 1 minute in 50 mM phosphate buffer solution (pH 7.40) without stirring. ssDNA was adsorbed on the surface of the pretreated CPE by applying a potential of +0.50 V for 5 minutes in ssDNA solution with 200 rpm stirring. The electrode was rinsed with DDW for 10 seconds.

Hybridization

The ssDNA-modified electrode was immersed in 20 mM Tris–HCl buffer (pH 7.00) containing target ssDNA for 45 minutes at 38°C with shaking to form dsDNA at the electrode surface. After hybridization, the dsDNA electrodes were washed with Tris–HCl buffer and then water to remove oligonucleotide bound nonspecifically.

Indicator binding to the hybrid

The DNA-modified electrode was immersed in a 0.2 M PBS aqueous AP solution for 10 minutes, then the electrodes were rinsed with the Tris–HCl buffer and water, placed in a PBS containing no dissolved complex, and subsequently subjected to electrochemical study.

Voltammetric studies

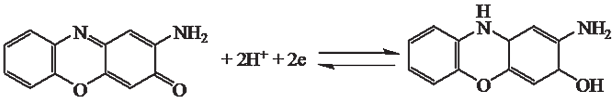
CV and DPV experiments were carried out by immersing the desired electrode in 0.2 M PBS (pH 6.80) solution. For CV, the potential scanning range was from −0.50 V to 0 V. For DPV, the initial potential was −0.50 V; the high potential was 0 V; pulse height was 50 mV for 60 milliseconds, step height was 2 mV, and step time was 0.1 seconds.

RESULTS AND DISCUSSION

The new biosensor relies on the different interaction between the ssDNA and dsDNA with AP, where reversible electroactivity and strong association with the immobilized ssDNA segment lead to significantly enhanced voltammetric signals. The decrease in the magnitude of the voltammetric indicator peak reflects the extent of the hybrid formation.

Electrochemical study of AP

Figure 1 shows the cyclic voltammograms of bare GCE in 0.2 M PBS (pH 6.80) containing 4.39×10^{-5} M AP at different scan rates. It can be seen that AP has well-defined voltammetric peaks at −270 mV and −280 mV, and ΔE_p is 10 mV at the scan rate 60 mV/s. It indicated that this process might be a reversible, two-electron re-



Scheme 2. The two-electron redox reaction scheme for AP.

dox reaction. The reaction scheme was given in Scheme 2 (Zhang et al., 1999b). The cathodic peak current of AP increased and the peak potential did not change with the increase of scan rate over the entire range of sweep rates studied. The peak current was proportional neither to the square root of the scan rate ($v^{1/2}$) nor to the scan rate (v), indicating that the process is not controlled by pure adsorption or diffusion.

Interaction of ssDNA/GCE and dsDNA/GCE with AP

Figure 2 showed the CV curves of AP at bare GCE, ssDNA-modified GCE (ssDNA/GCE) and after hybridization with the same amount of complementary DNA sequence (dsDNA-modified GCE, dsDNA/GCE). The signal of AP on the bare GCE is attributed to the nonspecific adsorption of phenoxazine compounds on GCE. It is evident that the current corresponding to AP reduction increased owing to preconcentration of AP by ssDNA or dsDNA immobilized on GCEs, respectively. The ssDNA/GCE displayed significantly larger current than that of dsDNA/GCE, indicating that AP was concentrated at the surface of the ssDNA-modified GCE. When AP reacts with DNA, the phenoxazone ring of AP intercalates between the stacked base pairs of DNA and then loses its electroactivity (Wang et al., 2003). The obvious electrochemical response proved that AP bound tightly, and was coupled to the modified electrode surface. This result was consistent with the interaction between AMD and ssDNA. Actinomycin D was shown to bind with high affinity to single-stranded DNA, which has been confirmed by the discovery of a few single-stranded oligonucleotides that bind the drug with affinities matching or exceeding those for binding to preferred sites in double-stranded DNA (Wadkins et al., 1991). Due to the lesser interaction between the dsDNA/GCE and AP, a relatively smaller peak current was observed when compared with the one obtained with the ssDNA/GCE. Thus, AP can be used as an electroactive indicator for recognition of the surface hybridization process.

The cyclic voltammetric peak currents of AP at ssDNA/GCE and dsDNA/GCE increased as a function of scan rate. The linear plot of cathodic current versus scan rate was obtained for adsorption electroactive species up to 180 mV/s and tend to level off at high scan rates, indicating that AP was strongly bound to the DNA modified surface. All subsequent experiments employed a 60 mV/s for optimum scan rate.

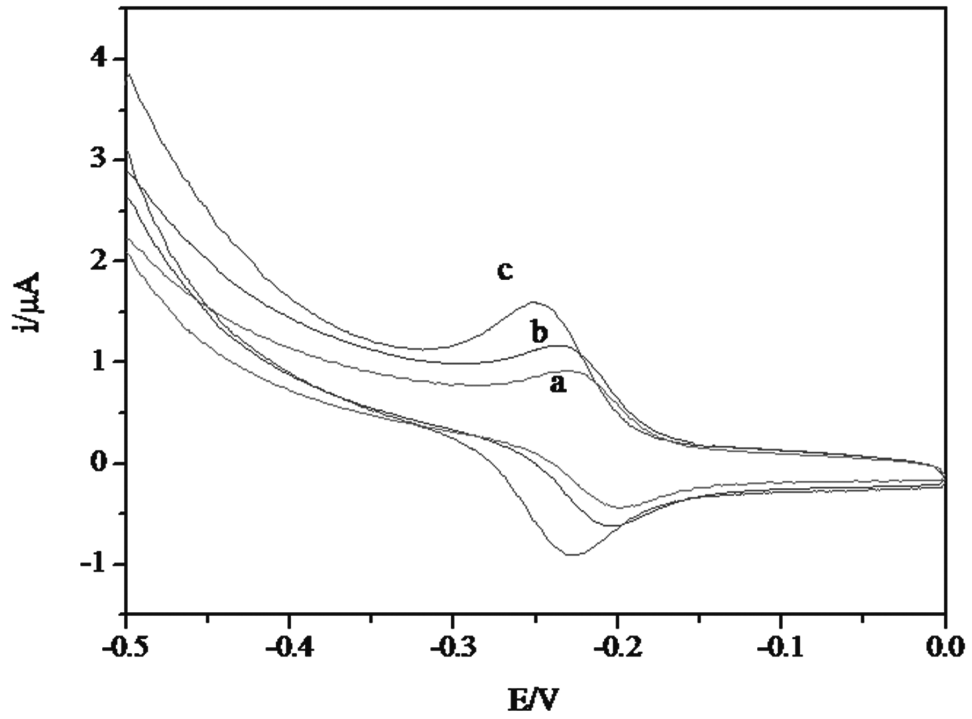


FIG. 2. Cyclic voltammograms of AP on bare/GCE (**a**), dsDNA/GCE (**b**), ssDNA/GCE (**c**) in 0.2 M PBS (pH 6.80) at 60 mV/s.

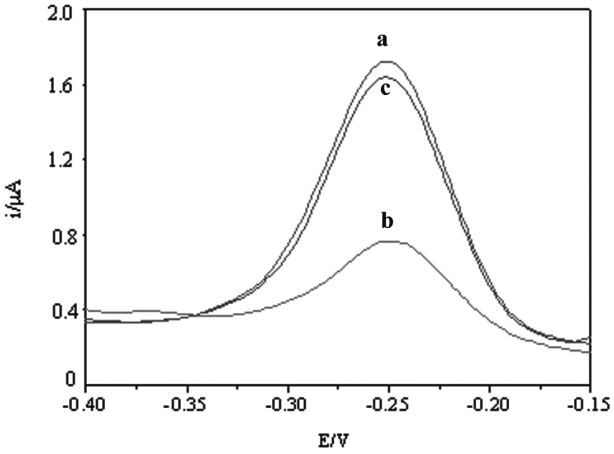


FIG. 3. Differential pulse voltammograms of AP on (**a**) S_1/GCE; (**b**) S_1–S_2/GCE; (**c**) S_1–S_3/GCE. The scan rate, pulse amplitude, and pulse period were 20 mV/s, 50 mV, and 60 ms, respectively.

Hybridization detection

Because it was observed that there is a significant difference between the voltammetric signals of AP obtained with ssDNA and dsDNA on the probe electrode, subsequently the capture probe was investigated for the response of noncomplementary oligonucleotide. Figure 3 showed the DPV for the signal of AP at synthetic single 21-mer sequence of the hepatitis B virus probe immobilized electrode (Fig. 3a), after hybridization with the same amount of complementary DNA sequence (Fig. 3b), and the noncomplementary sequence (Fig. 3c). The highest AP reduction signal was observed with the probe-modified GCE, indicating that AP had a strong affinity for ssDNA and the greatest amount of AP on the GCE surface. Significant decreases in the voltammetric signals were observed after hybridization with the complementary ssDNA. This may be attributed to less AP accumulation on the dsDNA caused by the bulky double helix of the DNA hybrids. The peak current almost did not decrease when the capture probe was exposed to the noncomplementary sequence in the control experiment. This result indicated that only a complementary sequence could form a double-strand DNA on the probe-modified GCE and cause a significant decrease of the signal. No significant change in signal was observed for noncomplementary sequence, showing the high selectivity of the hybridization detection.

Electrochemical response of the complementary target with different concentration

Under constant AP, the response in the presence of hybridization between probe and increasing concentration levels of target was presented in Figure 4. The DPV peak currents of the proposed DNA sensor decreased with increasing target concentration in the hybridization solution after the hybridization reaction. The decrease of DPV peak current was proportional to target concentration in the range of 3.53×10^{-7} to 1.08×10^{-6} M and the linear regression equation is $i = 0.2206–0.139\ C$ (C is the concentration of target DNA, 10^{-7} M; i is the cathodic current of AP, 10^{-7} A) and the regression coefficient $r = 0.9963$. In this concentration, all of the available probe on the electrode surface were involved in hybridization. The relative standard deviation over three independently modified capture probe electrodes measured at 4.0×10^{-7} M of target HBV DNA was 4.63%, implying a remarkable reproducibility of the detection method, and the detection limit was 1.00×10^{-7} M.

Regeneration of DNA biosensor

Regeneration of modified electrodes with single-stranded oligonucleotides bound to the probe electrode surface was accomplished by rinsing the hybridized surfaces with hot (95°C) ultrapure water for 5 minutes, followed by rapid cooling in ice bath. The reusability of the model biosensor was tested by repetitive hybridization with ssDNA and reusability. Following this procedure, the peak current obtained for AP was the same as those obtained at the modified GCE surface prior to regeneration. No significant deterioration of peak currents occured over four cycles after hybridization/regeneration, respectively. Over five cycles, the peak currents began to decrease gradually. The regeneration of the modified GCEs with single-stranded oligonucleotides was useful for continuous monitoring of the target DNA in the future research.

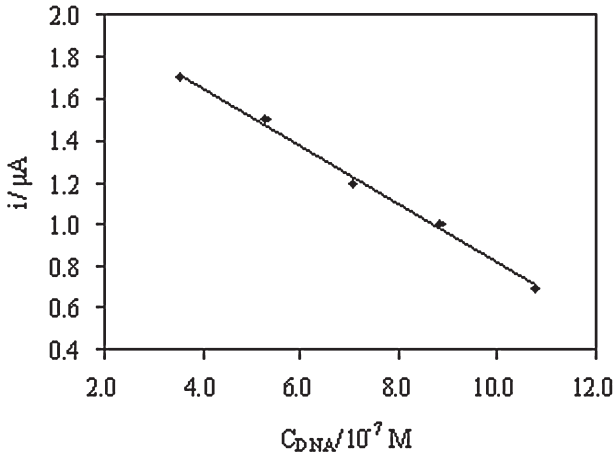


FIG. 4. The response of the capture probe with increasing concentration of complementary targets.

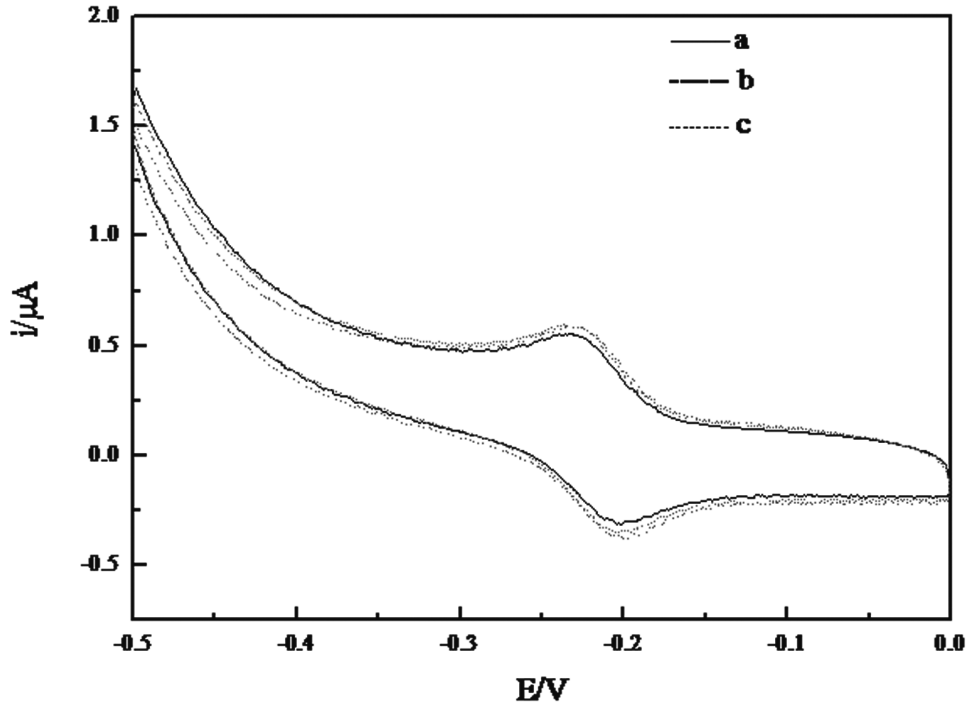


FIG. 5. Cyclic voltammograms of AP bound to dsDNA/GCE in solutions containing (**a**) 5.0 mM KNO_3 + 0.65 mM KCl; (**b**) 5.0 mM KNO_3 + 16 mM KCl; (**c**) 5.0 mM KNO_3 + 80 mM KCl. Scan rate 60 mV/s.

Hypothesis of interaction between AP and dsDNA and ssDNA

Detailed spectroscopic and hydrodynamic studies led to the conclusion that AMD binds to DNA via intercalating its phenoxazone chromophore between the DNA base pairs and anchoring its pentapeptide rings on the minor groove (Krugh and Neely, 1973). The binding is, however, quite sequence specific and had been shown to greatly prefer the GpC sequence. We extend our studies of AP–DNA interactions to describe the dependence of the electrochemical behavior on the nature of the compound. If the ionic strength of the solution is increased with a potassium salt, there will be a charge compensation of the nucleotide phosphates by the potassium ions, hindering the electrostatic binding to the complex. Consequently, the quantity of complex accumulated this way would decrease, whereas the binding by an intercalative mechanism should remain virtually unaffected (del Pozo et al., 2005). To investigate this issue in more details, we carried out a set of experiments in which we added increasing amounts of potassium chloride to the initial background electrolyte (KNO_3 5.0 mM + 0.65 mM KCl) and monitored the changes in peak current. The results for AP are presented in Figure 5. When the ionic strength of the solution was increased from 5.65 to 85 mM, the peak currents and the peak potential did not change. This suggests that, at both high and low ionic strength, the interaction of AP and dsDNA is mainly intercalative, so the nature of the interaction did not change with ionic strength. In fact, maybe AP's planar aromatic ring helps it to be inserted between the double helix of dsDNA, so the current keeps constant. The electrochemically active centers of AP were enveloped by the bulky DNA molecule immobilized on the GCE surface, and the signal of AP was decreased compared with that of ssDNA.

The nature of the complex between AP and ssDNA is

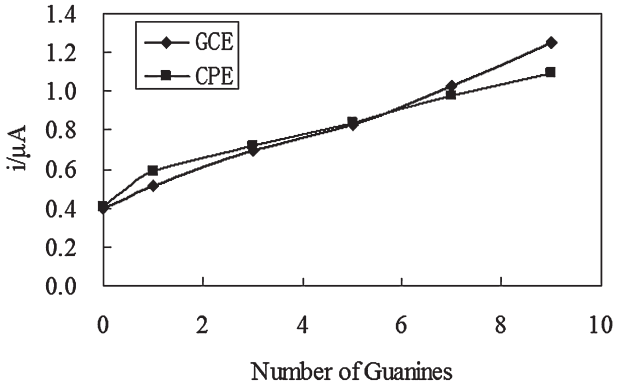


FIG. 6. Peak current as a function of a different numbers of guanines in a 21-mer synthetic oligonucleotide sequence on GCE and CPE.

important, in as much as the AP–ssDNA complex may yield clues to important DNA secondary structures occurring during transcription. Methylene blue (MB), which is a tricyclic heteroaromatic compound similar to AP, has been reported to interact with the guanine bases specifically rather than the bases of ssDNA in general with CPE (Kara et al., 2002b) and GCE (Teh et al., 2005). To investigate whether AP interacted specifically with the guanine bases, a series of DNA sequences with different numbers of guanines inserted in a 21-mer oligonucleotide were designed (Yang et al., 2002). A plot of the cathodic currents against the number of guanines per oligomer obtained from GCE and CPE is shown in Figure 6. The results from the two electrodes were consistent with each other. With the increase of the number of guanine bases, the cathodic currents were increased. AP preferred to interact with guanine bases as did MB. This base sequence specificity derives mainly from the formation of strong hydrogen bonds between the N-2 amino group and O-3 ring nitrogen of guanine residues and the amino oxygen atom and amino groups of the phenoxazine, respectively. Additional stability derives from hydrophobic interactions between groups on the phenoxazine and sugar residues as well as from other specific weaker hydrogen bonds. However, the mechanism of the interaction between AP and ssDNA is not fully understood at this time, and merits further study.

CONCLUSIONS

The results showed that the interaction of AP and dsDNA was mainly intercalative, whereas AP interacted with guanine bases in ssDNA. Based on the significant difference between the voltammetric signals of AP obtained with ssDNA and dsDNA on the probe electrode, an electrochemical DNA sensor was developed to the study the hybridization specificity of HBV using AP as a hybridization indicator, which was a simple and cost-effective method. The discovery of AP with site-specific affinities for G bases in single-stranded DNA may assist development of drugs against HBV and other retroviruses because replication of their single-stranded RNA genomes proceedes through a single-stranded DNA intermediate.

ACKNOWLEDGEMENTS

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