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Hybridization biosensor using 2-nitroacridone as electrochemical indicator for detection of short DNA species of Chronic Myelogenous Leukemia

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ABSTRACT

A new acridone derivative 2-nitroacridone (NAD) was synthesized in this paper, and it was found that NAD had excellent electrochemical activity on the glassy carbon electrode (GCE) with a couple reversible redox peaks at 0.051 V and 0.103 V, respectively. Voltammetry was used to investigate the electrochemical behavior of NAD and the interaction between NAD and salmon sperm DNA. In pH 4.0 phosphate buffer solution, the binding ratio between NAD and salmon sperm DNA was calculated to be 2:1 and the binding constant was 3.19×10^5 L/mol. A Chronic Myelogenous Leukemia (CML, Type b_3a_2) DNA biosensor was developed by immobilizing covalently single-stranded CML DNA fragments to a modified GCE. The surface hybridization of the immobilized single-stranded CML DNA fragment with its complementary DNA fragment was evidenced by electrochemical methods using NAD as a novel electrochemical indicator, with a detection limit of 6.7×10^{-9} M and a linear response range of 1.8×10^{-8} M to 9.1×10^{-8} M for CML DNA. Selective determination of complementary ssDNA was achieved using differential pulse voltammetry (DPV).

1. Introduction

Chronic Myelogenous Leukaemia (CML) is a clonal myeloproliferative disorder resulting from the neoplastic transformation of the primitive hemopoietic stem cell (Fialkow et al., 1967; Champlin and Golde, 1985; Kantarjian et al., 1993). Generally speaking, the CML patients does not appear any observable symptoms in their early stage, and the chronic course can last 3–5 years. These phenomena bring the difficulties to the diagnosis of CML. There has been much learned about the clinical disorder and how the chimeric oncogene BCR/ABL generated from the translocation of chromosome 9 to 22 (Philadelphia Ph. Chromosome) can lead to the pathogenesis of CML. BCR/ABL gene is the traditional gene for the disease, and it exists in almost all cases of CML patients (Butturini and Ralph, 1996; Jorge et al., 1996). There are many types of BCR/ABL genes, and Type b_3a_2 is one of the most common mutation types, which has been often studied. Thus, the detection of BCR/ABL gene will afford an early diagnosis and monitor of the disease, and further improve the facility of detecting minimal residual leukaemia cells in the CML patients, especially after the bone marrow transplantation (BMT).

In recent years, the monitoring methods for clinical diagnosis and prognosis about fusion gene of CML include chromosome analysis, Southern blot, polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH) and so on, but all of these methods have some limitations. Chromosome analysis is time-consuming and insensitive; the traditional Southern blot detection is hadrospecificity, while it is very insensitive to the detection of the low replication of the gene order, its detection method is complicated, and the cycle is too long, moreover it relies on hazardous radioactive labels, which limit its extensive application in clinic. PCR, an enzyme-based DNA amplification technology, is often employed toward these applications. Although PCR is extremely sensitive, it remains to be improved from the practical point of view. Its disadvantages include relatively long assay time, high assay cost, and error-prone nature that occasionally lead to "false-positive" signals. FISH technique requires complicated fluorescence coloration system and its sensitivity is still not high, so it cannot be widely utilized in clinic. Thus, it is very significant to develop and investigate CML gene detecting technique, which is convenient, fast, accurate, sensitive and economic for solving the actual problem of the early diagnosis and prognosis monitor of CML.

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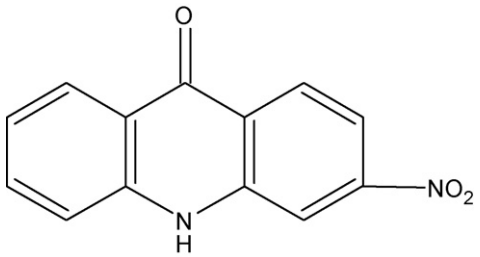
DNA biosensors based on nucleic acid hybridization, one of the most important biomolecular recognition processes, are currently undertaken wide investigation owing to their increasing importance in the diagnosis of disease with low-cost and low power requirements (Wang, 1999; Palecek and Fojta, 2001; Millan et al., 1992, 1994; Millan and Mikkelsen, 1993; Hashimoto et al., 1994a,b). Electrochemical techniques are very suitable for rapid and direct detection of specific DNA sequences with high sensitivity, small dimensions and good compatibility with transducer microfabrication technology (Wang, 2000; Mikkelsen, 1996; Yang et al., 2002; Hason et al., 2002; Wong and Gooding, 2003; Pang et al., 1996; Ozsoz et al., 2003). 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. DNA hybridization detection deals with the use of electroactive indicators interacting directly and specifically with the DNA duplex, such as intercalators, DNA groove binders, metal complexes and threading agents, has been recently reviewed by Takenaka (2003). However, concerning DNA biosensor development, there are still some limitations with these indicators, for example, metal complexes such as $Ru(bpy)_3^{2+}$ (Armistead and Thorp, 2001), Ferrocene (Eunkyung et al., 2003), $[Co(bpy)_3]Cl_2$ (bpy = 2,2-bipyridine) (Millan et al., 1994), $[Co(phen)_3]Cl_2$ (phen) 1,10-phenanthro-line) (Wang et al., 1997), $[Os(bpy)_3]Cl_2$ (Mishima et al., 2000; Gore et al., 2003), $[Os(5,6\text{-dmphen})_3]Cl_2$ (5,6-dmphen) 5,6-dimethyl-1,10-phenanthroline) (Maruyama et al., 2001) and poly(4-vinylpyridine) derivative bearing $[Os(5,6\text{-dimethyl-1,10-phenanthroline})_2Cl]^{2+}$ (Liu and Anzai, 2004), have often been used for this purpose because of their high stability and reversibility in the redox reactions. In practice, DNA hybridization is detected by measuring the redox current for redox indicators adsorbed on the dsDNA chains on the electrode. In this protocol, it is a prerequisite for the redox indicators to be adsorbed more efficiently on dsDNA chains than on single-stranded DNA (ssDNA) for affecting sensitive determination of DNA hybridization. From this point of view, the binding efficiency of the metal complexes mentioned above should be improved because their binding affinity is still inadequate, which is due to the fact that the metal complexes bind to dsDNA chains through the electrostatic force between metal complexes and DNA backbone or through hydrophobic interactions. Therefore, metal complexes have been modified with intercalative ligands to improve the binding affinity to dsDNA (Wilhelmsson et al., 2002; Metcalfe et al., 2003; Li et al., 2007; Niu et al., 2006). However, there are still some limitations with these methods, including experimental complications, such as purification of the ligands and complexes. Until now, novel electroactive indicators still need to be developed for the sensitive and selective assay of the hybridization events between the DNA probes and their complementary DNA fragments.

Acridine and acridone derivatives are well-known anti-tumor drugs and their planar structure confers on these molecules the ability to bind DNA by intercalation. The acridone is highly fluorescent and stable (Legg and Hercules, 1969; Siegmund and Bendig, 1980; Siegmund et al., 1985) against photo-degradation, oxidation, and heat. Further, acridone is a rather small molecule can be easily synthesized with high yield using inexpensive materials. Several types of acridone derivatives have been prepared (Faller et al., 1997; Reymond et al., 1996; Bahr et al., 1997), used as a fluorescent label for peptides and amino acids. One recent focus in our laboratory has been the development of some new probes of acridone for the direct monitoring of DNA hybridization events. Acridone is an azine dye, which is similar to the other planar dyes in the chemical structure belonging to the acridine, thiazine and xanthene groups. We have demonstrated the interaction of acridones with DNA spectrophotometrically (Chen et al., 2005a,b, 2006). However, based on our knowledge, acridone is not electroactive, so it cannot be used as electrochemical probe, and no electrochemical report on the interaction mechanism of acridone–DNA has been appeared in the literature. Therefore, an acridone derivative 2-nitroacridone (NAD) (see Scheme 1) has been designed and synthesized as the electrochemical label for DNA hybridization.



Scheme 1. Molecular structure of NAD.

NAD had excellent electrochemical activity on the glassy carbon electrode (GCE) with a couple reversible redox peaks. In this paper, the interaction between NAD and salmon sperm DNA was investigated in detail using CV and spectroscopic techniques. Moreover, the hybridization of the immobilized 18-base singlestranded CML DNA fragment with its complementary DNA fragment was confirmed by electrochemical methods using NAD as a novel electrochemical indicator. Based on which an electrochemical DNA biosensor was developed for detection of CML DNA with a detection limit of 6.7×10^{-9} M, which could be used to perform the clinic diagnosis and might has potential application in designing of novel anti-CML drugs.

2. Experimental

2.1. Apparatus

PerkinElmer Spectrum 2000 FT-IR spectrophotometer (USA). Elementarvario EL III elemental analyzer, Varian UNITY-500 nuclear magnetic resonance analyser. UV/vis absorbance spectra were measured with a UV-2501PC spectrophotometer (SHIMADZU, Japan) using a quartz cell with 1.0 cm optical pathway. All electrochemical measurements were carried out using CHI 660B Electrochemical Workstation (Shanghai CH Instruments, China). A three-electrode system was employed with Pt wire as auxiliary electrode, Ag/AgCl/KCl (salt) as reference electrode, and glass carbon electrode (GCE) as working electrode.

2.2. Reagents

Acridone (AR) was purchased from The Third Agents Factory of Shanghai (China). Salmon sperm DNA (1.0 mg/mL) was purchased from Aldrich. The concentrations of DNA per nucleotide phosphate ([DNA]) were calculated according to the absorbance at 260 nm by using $\varepsilon_{DNA} = 6600\,M^{-1}\,cm^{-1}$. Denatured ssDNA was produced by heating a dsDNA solution in a water bath at 100 °C for 5 min, immediately followed by rapid cooling in an ice bath. Phosphate buffer solution (PBS) with different pH were prepared by mixing the stock solutions of 0.02 M NaCl and 0.05 M NaH_2PO_4–Na_2HPO_4, and then adjusting the pH with 0.05 M H_3PO_4 or 0.05 M NaOH. The pH value of each solution was checked with pH meter prior to measurements. The 18-base synthetic oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology

Services Reagents Co., Ltd. (China), their base sequences were: immobilized probe (S1) 5′-NH_3 AGA GTT CAA AAG CCC TTC-3′; target (S2) 5′-GAA GGG CTT TTG AAC TCT-3′; single-base mismatch DNA (S3) 5′-GAA GGG CAT TTG AAC TCT-3′; non-complementary (S4) 5′-ACG TGG TCC CCA GCT CTC-3′. All oligonucleotides, dsDNA , and ssDNA stock solutions (100 mg/L) were prepared with TE solution (10 mM Tris–HCl, 1.0 mM EDTA, pH 8.00) and kept frozen. More dilute solutions were prepared with 20 mM acetate buffer (pH 4.80). Stock solutions of NAD (1 mM) were prepared with water. The stock solution of 5 mM 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC) and 8 mM *N*-hydroxysulfosuccinimide sodium salt (NHS) was prepared in 50 mM phosphate solution (pH 7.40). All the buffer solutions contained 20 mM NaCl. Other chemicals employed were all of analytical grade and double distilled water was used throughout.

2.3. Preparation of NAD

2-Nitroacridone (see Scheme 1) was synthesized starting with 9 (10H)-acridone. An amount of 0.75 g acridone was put into a beaker, 50 mL of 36% acetic acid, 3.6 mL nitric acid and 8 mL glacial acetic acid were added, the temperature was then risen to 55–60 °C and the reaction was performed for 2 h under stirring. The solution was poured into ice water and solid precipitate appeared. The resulting

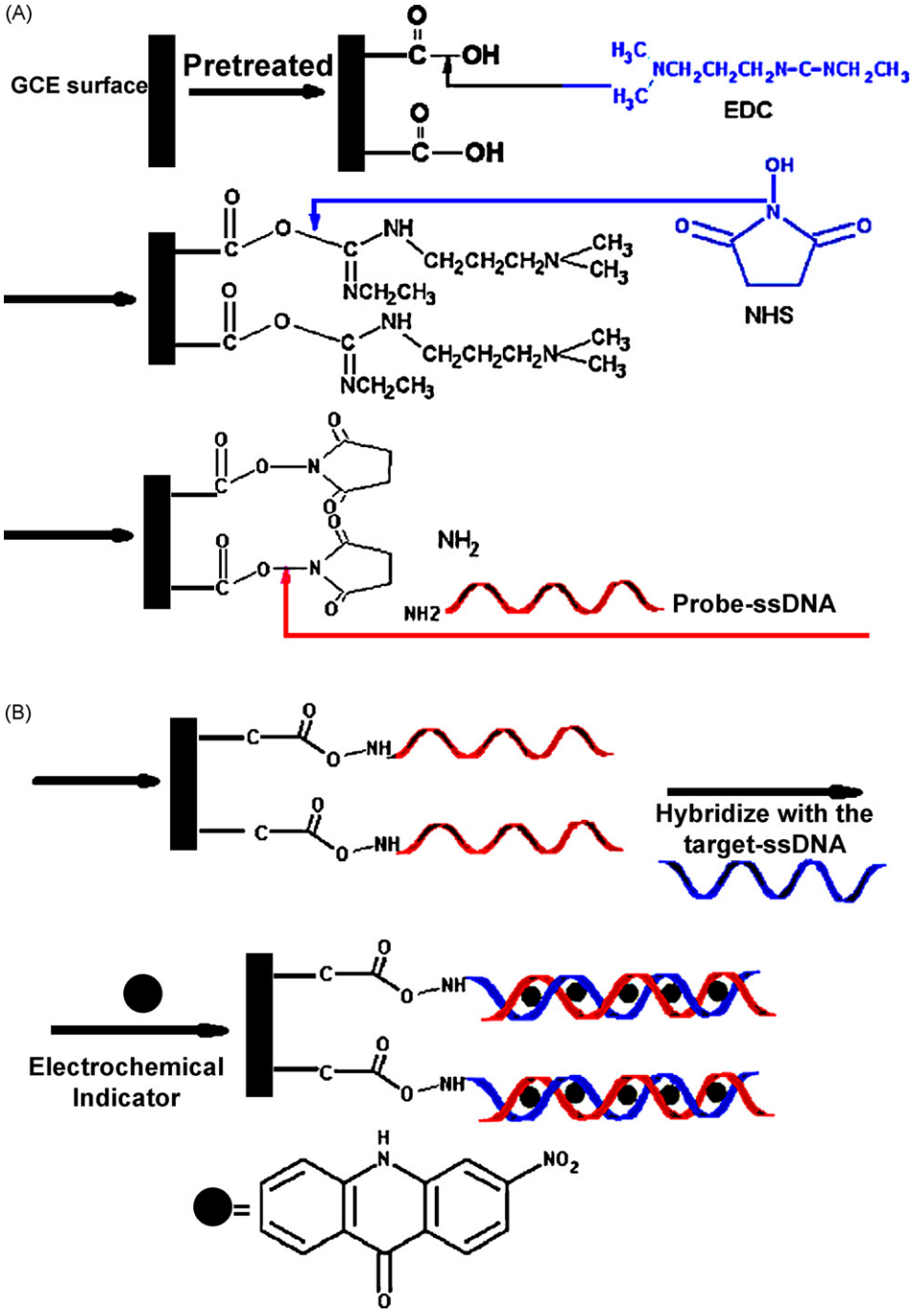


Fig. 1. Scheme for preparation of the electrochemical DNA biosensor and principle of electrochemical detection of the target-specific DNA hybridization.

solid was filtered off, washed with a few water and further purified by recrystallization in absolute alcohol. The solid was then oven-dried at 80 °C and 1.12 g of yellow crystal of 2-nitroacridone could be obtained, the yield was 59.8%.

Analytical: for $C_{13}H_8N_2O_3$; calculated: C, 65.0%; H, 3.36%; N, 11.66%; found: C, 65.22%; H, 4.12%; N, 11.02%. The infrared spectrum of NAD was examined, and IR (KBr) ν: 1584 (υ_{C-C}), 1650 ($\upsilon_{C=C}$), 1380 (υ_{C-NO_2}). FAB-MS: m/z 241 ([M+1]$^+$). ^{1}H NMR ($CDCl_3$, δ) 8.30 (d, ArH), 8.42 (s, ArH), 7.62 (t, ArH), 7.48 (d, ArH), 6.92 (d, ArH), 6.76 (t, ArH), 6.50 (d, ArH), 4.22 (s, NH).

2.4. Electrochemical study on the interaction between NAD and DNA

A 1.0 mL of 1.0 mM NAD solution was transferred into 10 mL colorimetric tubes containing pH 4.0 PBS buffer, and then DNA was added. Each mixture was diluted to the mark using the same PBS buffer. The changes on characteristics of CVs and the DPVs were investigated. CV scanning was performed in the potential range of −0.40 V to +0.40 V with the scanning rate of 0.10 V/s, the sample interval of 0.001 V and the standing time of 2 s. DPV scanning was performed in the range of −0.4 V to 0.4 V with pulse width of 0.05 V, pulse frequency of 0.05 s, pulse cycle of 0.2 s, pulse interval of 0.004 V and standing time of 2 s.

2.5. Preparation of the electrochemical DNA biosensor

The electrochemical DNA biosensor was developed based on the covalent immobilization of ssDNA on the modified electrode. The scheme for preparation of the electrochemical DNA biosensor is shown in Fig. 1A. Prior to the experiment, the GCE was firstly polished using 1.0 μM, 0.3 μM and 0.05 μM Al_2O_3 suspension, respectively, and then extensively rinsed in water with ultrasonic. Afterwards, the electrode was oxidized at +0.50 V for 1 min in 5.0×10^{-2} M pH 7.4 PBS, followed by thorough rinse with water. Twenty microlitres of solution containing 5 mM DEC and 8 mM NHS in 50 mM phosphate buffer solution (pH 7.40) was pipetted onto the GCE surface. After air-dry of this solution, the electrode was rinsed with water. ssDNA (S1) was pipetted onto the GCE surface and air-dried to dryness. Then the electrode was rinsed with water to eliminate the DNA adsorbed. The DNA-immobilized electrode was stored in pH 7.0 Tris–HCl buffer solution.

2.6. Principle of electrochemical detection of the target-specific DNA hybridization

The principle of electrochemical detection of the target-specific DNA hybridization is shown in Fig. 1B. The S1-immobilized electrode was immersed in Tris–HCl buffer containing complementary S2, single-base mismatch S3 or uncomplementary S4, respectively. The hybridization was allowed for 30 min with stirring at 45 °C. Afterwards, the electrode was dried at room temperature and then thoroughly rinsed with the same Tris–HCl buffer and water successively to eliminate the adsorbed S2, S3 or S4. For DPV detection of the DNA hybridization, NAD was used as hybridization indicator. To accumulate NAD on the electrode surface, S1-immobilized GCE, dsDNA-immobilized GCE and the electrodes obtained after DNA hybridization were immersed in 1.0×10^{-4} M NAD solution dissolving in Tris–HCl buffer for 5 min at room temperature. Then, the electrodes were rinsed with the Tris–HCl buffer and water successively. Subsequently, the electrochemical investigation of hybridization was carried out in a 10 mL electrochemical cell using the above functioned GCE as working electrode, an Ag/AgCl as the reference electrode and a platinum wire as the auxiliary electrode. The DPV measurements were performed in Tris–HCl buffer solution and the scan range was from −0.60 V to 0.40 V. The peak related to the oxidation of NAD at approximately −0.116 V was taken as the electrochemical detection signal.

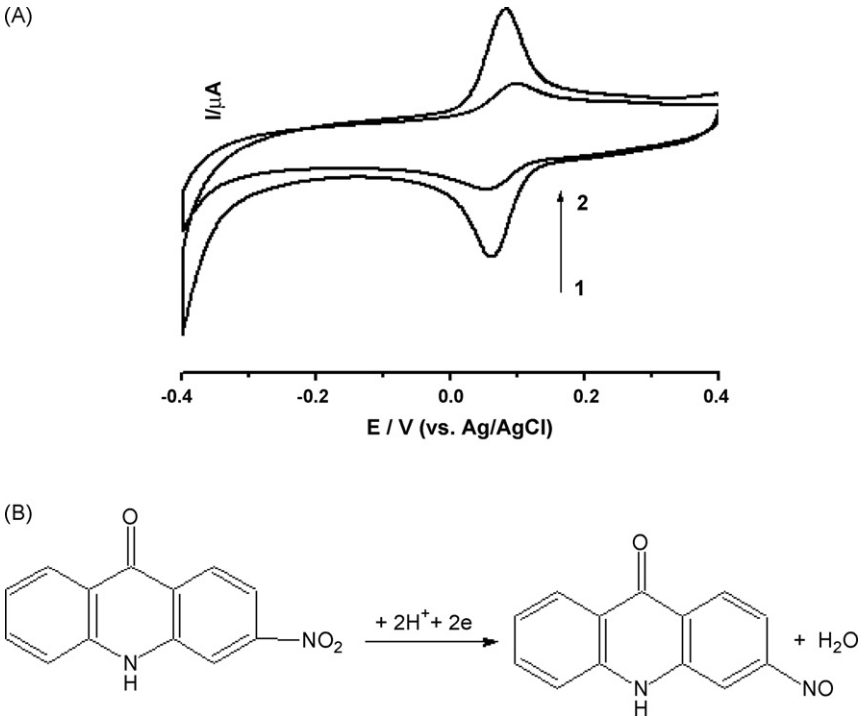


Fig. 2. (A) CVs of NAD in the absence and presence of DNA: (1) 1.0×10^{-4} M NAD; (2) 1 + 6.2×10^{-5} M DNA. (B) Electrode reaction mechanism of NAD.

3. Results and discussion

3.1. Electrochemical study on the interaction between NAD and DNA

CVs of NAD in the absence and presence of DNA were shown in Fig. 2A. As shown in Fig. 2A-1 that there is a pair of peaks with good shape in the range of −0.4 V to +0.4 V, the cathodic peak potential (E_{pc}) and the anodic peak potential (E_{pa}) are 0.056 V and 0.083 V, respectively, $\Delta E = 27$ mV. $I_{pa}:I_{pc} = 2.144:1.848 \approx 1$, which indicates that the reaction is reversible, According to $\Delta E_p = 2.3RT/nF$ (59/n mV at 25 °C) (Bard and Faulkner, 1980), the number of electrons involved in the reaction was 2 ($n = 2.18 \approx 2$), and $\Delta E_{1/2} = 69$ mV. According to the equation

$$\Delta E_{1/2} = \frac{62.5}{(1-\alpha)n}$$

the electron transfer coefficient is $\alpha = 0.54$.

The effect of scan rate on the peak current of NAD was studied. The peak current (I_{pa} and I_{pc}) increased with the scan rate, and was proportional to the root of scan rate over the range of 20–200 mV s^{-1} ($R = 0.9992$), which suggests that the electrochemical reaction is a diffusion-controlled process.

To further investigate the mechanism of electrochemical reactions of NAD on the electrode, effect of pH on the formal potential and the peak current was investigated using CV. The CV curves showed that the peak potential of NAD at GCE shifted toward negative direction with increasing of pH, which indicated that proton transfer was involved in the electrode reaction process. E^0 is the formal potential, the linear regression equation between E^0 and pH was $E^0 = -49.61$ pH + 245.37, with a slope of 49.6 = 59m/n (n is the number of electrons transfer, m is the number of protons transfer), as $n = 2$ (see above), therefore the number of protons involved was also 2. According to the references (Rance and Coulson, 1969; Marquez and Pletcher, 1980; Christopher and Handady, 1952), a mechanism for the NAD oxidation can be proposed in Fig. 2B.

Fig. 2A-2 shows the voltammogram obtained when NAD interacted with DNA for 15 min. The E_{pc} and the E_{pa} are 0.051 V and 0.103 V, respectively. The peak potential separation (ΔE_p) was 77 mV, and its formal potential (E^0) was 0.159 V. As can be seen, both the cathodic and anodic peak currents (I_{pc} and I_{pa}) decrease and their formal potential (E^0) shift toward the positive direction. This phenomenon indicated the formation of a new association complex. The literature (Carter and Bard, 1989) indicated that intercalative binding of small molecules to DNA might make E^0 shift to more positive value, while electrostatic binding might make E^0 shift to more negative value. In fact, the intercalative character of the complex maybe mainly due to the acridone, since its planar aromatic ring helps it to be inserted into the double helix of dsDNA (Cusumano et al., 2006).

The phenomena mentioned above were further studied by using DPV. As shown in Fig. 3, curve 1 is the voltammogram of the NAD solution, while the curves 2–6 are those of NAD interacted with DNA for 15 min, the decrease of peak current can be observed with increasing of DNA. As far as ΔI_{pa} was concerned, we found that ΔI_{pa} was linear proportion to the concentration of DNA in the range of 1.24×10^{-7} M to 4.65×10^{-7} M with a regression equation, the detection limit of dsDNA was 5.8×10^{-8} M. The relative standard deviation was 3.4% for 3.1×10^{-7} M dsDNA ($n = 5$).

$$\Delta I_{pa} = 0.7565 C_{DNA} + 0.1652, \quad R = 0.9919$$

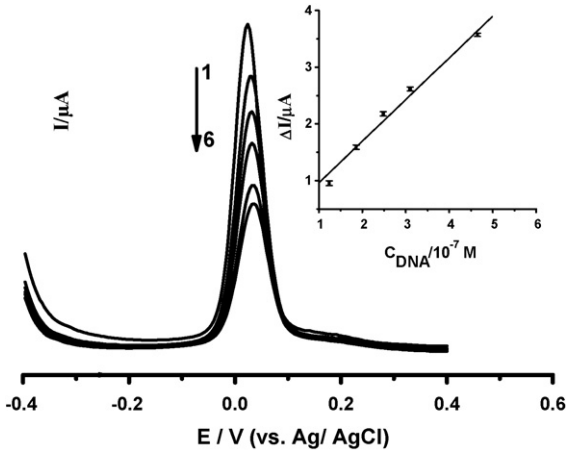


Fig. 3. DPVs of NAD in the absence and presence of DNA: (1) 1.0×10^{-4} M NAD; (2) 1 + 1.24×10^{-7} M DNA; (3) 1 + 1.86×10^{-7} M DNA; (4) 1 + 2.48×10^{-7} M DNA; (5) 1 + 3.1×10^{-7} M DNA; (6) 1 + 4.65×10^{-7} M DNA. *Other conditions*: in pH 4.0 PBS; the initial potential was −0.4 V; the high potential was 0.4 V; the pulse scope was 0.05 V; the pulse extent was 0.05 s; the pulse cycle was 0.2 s; the sample interval was 0.004 V and the standing time was 2 s.

3.2. Binding ratio and the binding constant between NAD and DNA

In order to study the binding ratio and binding constant between NAD and DNA, it was assumed that interaction of DNA and NAD only produced one single complex: DNA–nNAD, as shown in the following equation (Liu et al., 2000):

$$\text{DNA} + n\text{NAD} = \text{DNA} - n\text{NAD} \quad \left(n = 1, 2, 3, \ldots \text{ or } \frac{1}{2}, \frac{1}{3}, \ldots\right) \Delta I_{pa}^{-1}$$

The equilibrium constant β could be expressed as Eqs. (1)–(7)

$$\beta = \frac{[\text{DNA} - n[\text{NAD}]]}{[\text{DNA}][\text{NAD}]^n} \quad (1)$$

$$\Delta I_{pa,max} = KC_{DNA} \quad (2)$$

$$\Delta I_{pa} = K[\text{DNA} - n[\text{NAD}]] \quad (3)$$

$$[\text{DNA}] + [\text{DNA} - n[\text{NAD}]] = C_{DNA} \quad (4)$$

$$\Delta I_{pa,max} - \Delta I_{pa} = K(C_{DNA} - [\text{DNA} - n[\text{NAD}]]) \quad (5)$$

$$\Delta I_{pa,max} - \Delta I_{pa} = K[\text{DNA}] \quad (6)$$

$$\frac{1}{\Delta I_{pa}} = \frac{1}{\Delta I_{pa,\,max}} + \frac{1}{\beta \Delta I_{pa,\,max}[\text{NAD}]^n} \quad (7)$$

where C_{DNA} denoted the analytical concentration of DNA while [DNA] and [NAD] represented the equilibrium concentration of DNA and the NAD. According to Eq. (7), different n might result in different curves of ΔI_{pa}^{-1} versus $[\text{NAD}]^{-n}$. With the suitable n, the curve of ΔI_{pa}^{-1} versus $[\text{NAD}]^{-n}$ should be a straight line if there was only one complex formed when [NAD] bound to DNA. From the slope and intercept of the straight line, the binding constant β could be calculated and the n could be regarded as the binding ratio.

The dependence of the oxidation peak current (I_{pa}) on the analytical concentration of [NAD] in the absence (curve 1) and presence (curve 2) of DNA was shown in Fig. 4A. The relationship between ΔI_{pa} (the difference of I_{pa1} and I_{pa2}, $\Delta I_{pa} = I_{pa1} - I_{pa2}$) and the analytical concentration of [NAD] was also displayed (curve 3). The curves of ΔI_{pa}^{-1} versus $[\text{NAD}]^{-1/2}$, ΔI_{pa}^{-1} versus $[\text{NAD}]^{-1}$, ΔI_{pa}^{-1} versus $[\text{NAD}]^{-2}$ were displayed in Fig. 4B, where [NAD] represented the equilibrium concentration of [NAD] and calculated from data

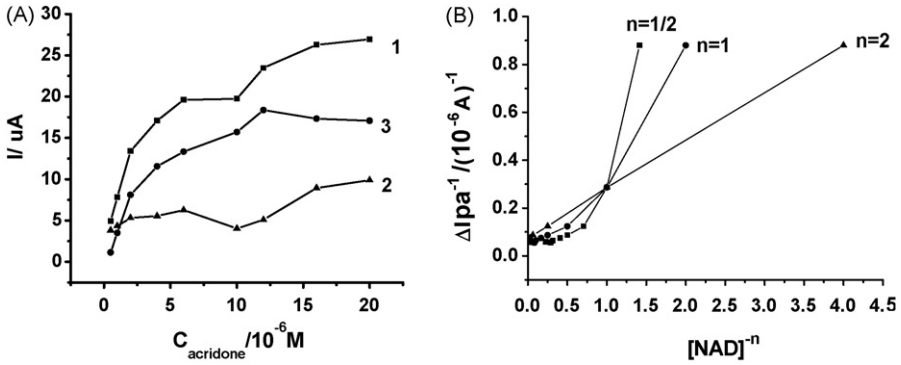


Fig. 4. (A) Relationship between I_{pa1}, I_{pa2}, ΔI_{pa} and the concentration of [NAD]: (1) without DNA; (2) 1.55×10^{-5} M DNA; (3) $\Delta I_{pa} = I_{pa1} - I_{pa2}$. (B) Curves of ΔI_{pa}^{-1} versus $[NAD]^{-n}$; *Conditions*: in pH 4.0 PBS; the initial potential was −0.4 V; the high potential was 0.4 V; the pulse scope was 0.05 V; the pulse extent was 0.05 s; the pulse cycle was 0.2 s; the sample interval was 0.004 V and the standing time was 2 s.

in Fig. 4. For $n = 0.5$ and 1, the curves bent up, respectively. While for $n = 2$, the curve was a straight line ($R = 0.9988$), indicating the formation of a 2:1 association between [NAD] and DNA. From the slope and intercept of the straight line, the binding constant β was calculated to be 3.19×10^5 L/mol, which was corresponding to the following equation:

$$DNA + 2[NAD] = DNA - 2[NAD]$$

3.3. Electrochemical detection of the target-specific DNA hybridization

Since NAD could intercalate into the bases of dsDNA, the DNA biosensor using NAD as electrochemical hybridization label was developed. Fig. 5 shows the DPV curves for different modified GCEs obtained in PBS.

As the GCEs were modified with ssDNA (curve d) and after hybridization with non-complementary sequence (curve c), single-base mismatch DNA (curve b), complementary sequence (curve a). It can be seen from Fig. 5 that when the ssDNA modified GCE interacts with the complementary sequence in solution, the peak current for the NAD reduction increases significantly (see curve a), which clearly indicates that the ssDNA modified on the GCE has been successfully hybridized with its complementary sequence. The greater peak current indicates that more NAD molecules are concentrated or bound to the adsorbed dsDNA helix than to the adsorbed ssDNA helix. In the presence of oligonucleotide containing a single-base mismatch, significantly decreased voltammetric signal can be observed (see curve b), which indicates that the complete hybridization is not accomplished due to the base mismatch. In addition, as expected, there is no significant current difference observed for the ssDNA modified GCE and its hybridization with non-complementary sequence (see curves d and c), since no successful hybridization occurs due to the sequence mismatch between the modified ssDNA and the non-complementary sequence. This means that the surface properties of the ssDNA modified GCE is not changed after its interaction with non-complementary sequence.

The sensitivity of the electrochemical hybridization assay was investigated by varying the target oligonucleotide concentration according to the procedure described in Section 2. The different current value obtained in the DPV response of NAD after hybridization of probe with target is recorded with three repetitive measurements. The current response at about −116 mV increased in proportion to the amount of the target sequence used. The average current response shows excellent correlation with the amount of the complementary oligonucleotides in the range of 1.82×10^{-8} M to 9.1×10^{-8} M (see Fig. 6). The regression equation was

$$y = 0.2247x + 1.275, \quad R = 0.9985$$

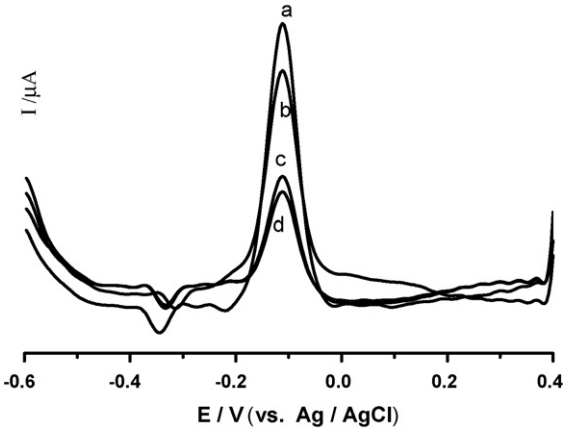


Fig. 5. DPVs of NAD accumulated on the S1–S2/GCE (a), S1–S3/GCE (b), S1–S4/GCE (c) and S1/GCE (d) in a 50 mM PBS (pH 4.40) with 20 mM NaCl.

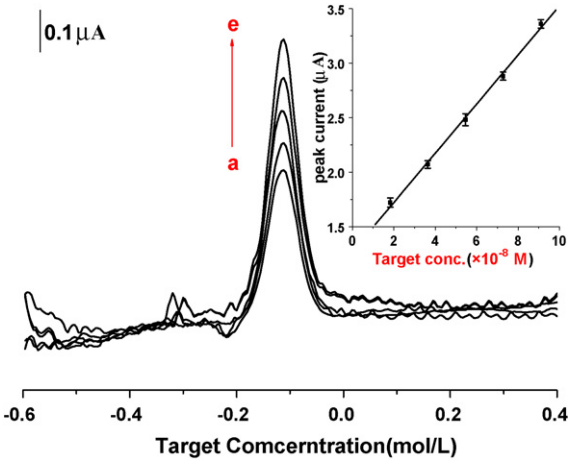


Fig. 6. Differential pulse voltammograms of NAD accumulated on the ssDNA after its hybridization with different concentration of the target sequence. Target concentration ($\times 10^{-8}$ M): (a) 1.82; (b) 3.64; (c) 5.46; (d) 7.28; (e) 9.10. Inset shows the plot of the peak current of NAD as a function of the target concentration. Error bars = ±relative standard deviation.

where x is the amount of the target DNA (mol) and y is the DPV peak current of NAD (μA). A detection limit of 6.7×10^{-9} M for the target DNA can be estimated using 3σ (where σ is the standard deviation of the blank solution, $n = 8$). The reproducibility of the biosensor was assessed by analyzing samples of 5.46×10^{-8} M target DNA and the R.S.D. of eight replicate determination was 6.42%.

4. Conclusion

Interaction between NAD and salmon sperm DNA was studied using cyclic voltammetry. All the experimental results indicated that the binding mode of NAD to dsDNA was intercalation. The association formed was a 2:1 complex with the binding constant of 3.19×10^5 L/mol. 18-Base CML ssDNA was covalently immobilized on the EDC-NHS modified GCE as the probe. The surface hybridization of the immobilized single-stranded CML DNA fragment with its complementary DNA fragment was confirmed by electrochemical methods using NAD as a novel electrochemical indicator with a detection limit of 6.7×10^{-9} M for CML DNA. Using of the new electrochemical hybridization indicator could provide a simple, rapid detection method for CML DNA fragment, and might have a promising future in transducing DNA hybridization. The developed electrochemical DNA sensor might have the potential application in diagnosis of diseases.

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