Talanta 78 (2009) 1227–1234

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

An electrochemical biosensor for detection of PML/RARA fusion gene using capture probe covalently immobilized onto poly-calcon carboxylic acid modified glassy carbon electrode

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ARTICLE INFO

Article history:
Received 2 September 2008
Received in revised form
23 December 2008
Accepted 23 December 2008
Available online 15 January 2009

Keywords:
Calcon carboxylic acid
PML/RARA fusion gene
Electrochemical biosensor

ABSTRACT

In this article, the poly-calcon carboxylic acid (poly-CCA) film modified electrode was prepared by cyclic voltammetry (CV). Then, an electrochemical DNA biosensor was developed for detection of PML/RARA fusion gene in acute promyelocytic leukemia (APL) by using 18-mer single-stranded deoxyribonucleic acid as the capture probe. The capture probe was covalently attached through free amines on the DNA bases using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydrosulfosuccinimide (NHS) cross-linking reaction on a carboxylate-terminated poly-CCA monolayer modified glassy carbon electrode (GCE). The covalent immobilized capture probe could selectively hybridize with its target DNA to form double-stranded DNA (dsDNA) on GCE surface. The aim of this work is to provide a well-defined recognition interface for the detection of DNA. Differential pulse voltammetry (DPV) was used to monitor the hybridization reaction on the capture probe electrode. The decrease of the peak current of methylene blue (MB), an electroactive indicator, was observed upon hybridization of the probe with the target DNA. The results indicated that in pH 7.0 phosphate buffer solution (PBS), the oxidation peak current was linear with the concentration of complementary strand in the range of 1.0×10^{-12} to 1.0×10^{-11} M with a detection limit of 6.7×10^{-13} M. This new method demonstrates its excellent specificity for single-base mismatch and complementary sequence (dsDNA) after hybridization, and it would be proposed to use in real sample.

1. Introduction

Acute promyelocytic leukemia (APL) is a kind of acute leukemia which often goes with bleeding severely. The bleeding mechanism of the APL patient is very complex. Almost all of the APL patients are characterized by chromosome reciprocal translocation, t(15;17) (q22;q12), resulting in the generation of fusion gene between promyelocytic leukemia (PML) and retinoic acid receptor alpha (RARA), which forms PML/RARA fusion gene. It would have crucial meanings of APL [1]. The reported methods for the detection of PML/RARA fusion gene were chromosome analysis [2], fluorescence in situ hybridization (FISH) [3], flow cytometry (FCM) [4], real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) [5], etc. Despite the chromosome analysis would analyze the abnormality of the single cellular chromosome in structure and number, it spends long time and much energies. At the same time, it has low sensitivity. FISH has high sensitivity, but it has poor precision in immobilization. Although FCM would detect 5000–10,000 cells, it identifies the cellular shape, size and fluorescence feature only in single cellular level. The result of RT-PCR detection is sensitive and accurate, but often appears the false positive and false negative. Besides, it is difficult to quantitate. Thus, it is important to develop a new effective method to detect PML/RARA fusion gene.

In recent years, it has been considerable interested in developing DNA electrochemical biosensors for the rapid and inexpensive diagnosis of genetic diseases and other applications [6–8]. Such biosensors show both high selectivity and sensitivity in detecting a specific sequence, since a specific oligonucleotide can be immobilized on the surface of electrode. The immobilization step of the capture probe could lead to a well-defined probe orientation,

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doi:10.1016/j.talanta.2008.12.053

which allows for ready accessibility of the target. Thus, specific DNA probe–target interactions and charge transfer reactions give an electrical signal which can be directly monitored. In addition, electrochemical devices offer certain advantages over the optical devices. They are easy to miniaturize, simple, rapid and inexpensive because electrochemical transduction is independent in solution turbidity or optical pathway. The immobilization of DNA onto the transducer surface plays an important role in the overall performance of the DNA biosensors. The effective immobilization of the probe not only improves the sensitivity and selectivity of biosensor, but also eliminates the non-specifically adsorption. It is of importance to effectively control the coverage of the probe on the electrode, the direction of the immobilization and the stability as to the successful detection of hybridization. However, traditional methods in immobilization of DNA onto electrode surfaces have poor hybridization efficiency [9,10]. Such as self-assembly monolayer (SAM)/Au-orientated system has been widely reported for the fabrication of biosensors [11], but gold–thiol bond can only withstand reasonably mild potentials and the alkanethiols. Moreover, the potential window of a gold electrode is limited to a relatively positive range due to its low overpotential for hydrogen evolution. Thus, it is important to develop a new effective process of immobilization.

Recently, application of chemical modified electrode in biology and chemistry has been paid more and more attention. Especially, the conducting polymers are very effective substrates for biomaterial immobilization. Some conducting polymers are doped and/or covalently or physically modified by bionanomaterials, especially proteins and nucleic acids, which exhibit unique catalysis [12] or affinity properties that can be easily employed in the design of biosensors [13]. Otherwise, polymer film modified electrodes [14–17] can form function groups of high concentration on the surface of the electrode, at the same time, they can increase the stability of fix function groups and selectivity of reactions. Therefore, the polymer film modified electrodes have found broadly studied and applied. Thus, the immobilized DNA by conducting polymeric materials have the properties of mechanical flexibilities, high surface areas, chemical specificities, tunable conductivities and easy processing, which make the conducting polymer the promising sensing materials for ultrasensitive, trace-level biological and chemical sensors [18–20].

In this paper, we describe the use of an electrochemical deposition procedure, to fabricate the poly-calcon carboxylic acid (poly-CCA) film (Fig. 1, Part A (A)) modified electrode using the GCE as the working electrode. In the presence of *N*-hydrosulfosuccinimide (NHS) and 1-ethyl-3-(3-

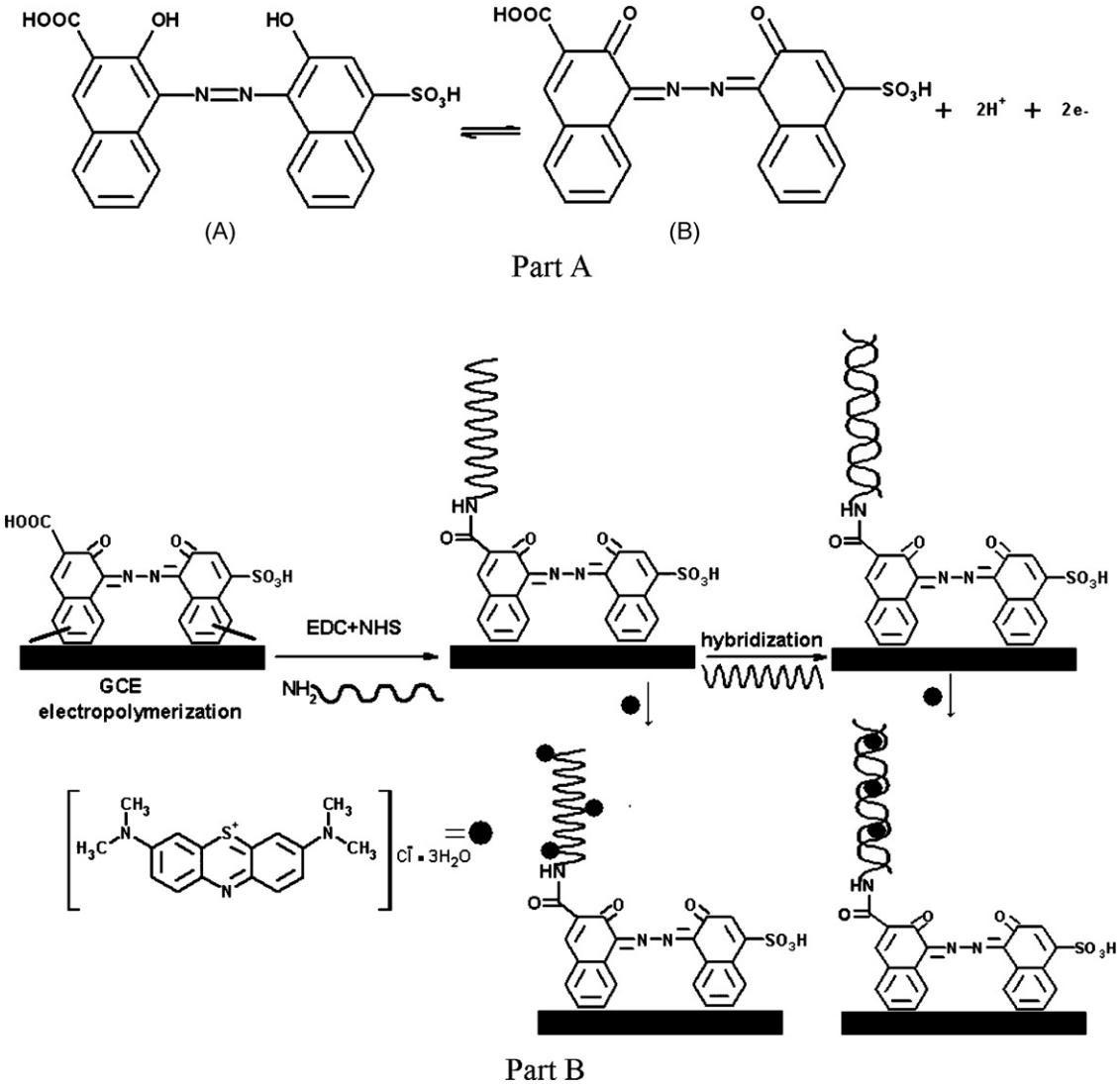

Fig. 1. (Part A) Mechanism of the poly-CCA electrode reaction: (A) the structure of CCA; (B) the benzoquinone diimine structure of CCA. (Part B) Diagram of the procedure for the fabrication of DNA biosensor for APL.

dimethylaminopropyl) carbodiimide (EDC), amines groups of oligonucleotides probe at the 5′end were covalently immobilized onto the carboxyl groups of poly-CCA film on the electrode. Hybridization was conducted by immersing the electrode immobilized DNA probe into the buffer solution containing its complementary sequence. Then DPV measurement was performed using electroactive methylene blue (MB) as an indicator. The method can effectively discriminate complementary DNA sequence from non-complementary and single mismatched sequence. The performance of the DNA biosensors with respect to the sensitivity and linear range was discussed. To our knowledge, this is the first time poly-CCA has been used for DNA detection. This method foreshows that the new immobilization of film modified electrode to detect DNA would be the basis of detecting diseases such as leukemia in early diagnosis and prognostic monitoring.

2. Experimental

2.1. Reagents and apparatus

The 18-base synthetic oligonucleotides were purchased from Shanghai Sheng gong Bioengineering Ltd. Company (Shanghai, China). Their base sequences were: immobilized probe (18-base sequence, S1)-5′-NH_2-TCT CAA TGG CTG CCT CCC-3′; target (S2)-5′-GGG AGG CAG CCA TTG AGA-3′; non-complementary (S3)-5′-ACT TCA TCC TTC GCT CTC-3′; single-base mismatch (S4)-5′-GGG AGG CAG CCA TTG AAA-3′. Stock solutions (100 μM) of all oligonucleotides 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). MB was purchased from Aldrich. Stock solutions of MB (1.0 mM) were prepared with deionized water. CCA was purchased from Sigma. EDC and NHS from Sigma were used without further purification. Other all chemicals were of analytical reagent grade. Phosphate buffer solution (PBS) was prepared by mixing the stock solutions of NaCl and NaH_2PO_4–Na_2HPO_4, and then adjusting the pH with H_3PO_4 or NaOH. All the buffer solutions contained 50 mM NaCl. Sterilized and deionized water was used in all solutions.

Electrochemical impedence spectroscopy (EIS), cyclic voltammetry (CV) and DPV measurements were performed by using CHI 660C Electrochemical Workstation (CH Instrument, USA). The electrochemical system consisted of GCE (3 mm diameter) as the working electrode, a platinum wire as the auxiliary electrode, and the reference electrode (Ag/AgCl, 3 mol L^{-1} KCl). All potentials mentioned in this paper referred to this reference electrode.

2.2. The preparation of surface of biosensor and its modification with DNA

The diagram for preparation of the electrochemical DNA biosensor is illustrated in Fig. 1.

2.2.1. GCE modification to form poly-CCA/GCE

We had ever reported the method that a novel poly-CCA modified GCE was fabricated by electropolymerization. Then the characterization of electrochemically synthesized poly-CCA film was investigated by atomic force microscopy (AFM), EIS and voltammetric methods [21]. So, GCE modification to form poly-CCA/GCE was done according to the procedure previously described by the method [21]. Before surface modification, the GCE was polished in sequential order with 1.0, 0.3 and 0.05 μm alumina (AlfaAesar, USA). The electrode was thoroughly washed with water, sonicated in ethanol, and finally dried thoroughly under N_2 flow. The surface modification of the GCE was performed by procedure reported in Ref. [21]. Briefly, GCE was polarized in 0.1 M H_2SO_4 by cyclic scanning between −0.40 and +1.60 V for 5 min at scan rate of 100 mV s^{-1}. Then the polarized electrode was carried out in 0.05 M NaOH containing 0.3 mM CCA solution by cyclic scanning between −0.40 and +1.80 V for 40 cyclic times at scan rate of 100 mV s^{-1}.

2.2.2. Immobilization of single-stranded DNA (ssDNA, S1) on poly-CCA/GCE

A versatile method for covalently attaching S1 to the poly-CCA/GCE was by using EDC and NHS linking reaction. The terminal carboxylic acid groups of the poly-CCA/GCE were activated by immersion in the 50 mM PBS (pH 7.40) containing 2 mM EDC and 5 mM NHS for 1 h. The linker/poly-CCA/GCE was rinsed with 50 mM PBS (pH 7.40) to wash off the redundant EDC and NHS. Then, S1 immobilization onto the surface was performed by the following procedure: 10 μl of 1.0×10^{-12} M S1 was pipetted onto the chemically modified GCE. The S1 droplet was left to dry. Thus, a S1-modified GCE was obtained. This electrode S1/poly-CCA/GCE was designated as probe electrode. Then the electrode was washed with 0.1% SDS phosphate buffer (pH 7.30) for 5 min to remove the unbound DNA probes.

2.2.3. Hybridization with synthetic oligonucleotides

The hybridization was performed by immersing the S1/poly-CCA/GCE into 0.1 M PBS (pH 7.00) containing 5 μl of different concentrations of S2. This hybridization process was left for 30 min at 45 °C [22]. Thus, a hybrid-modified GCE was obtained. The electrode surface was then washed with ultra-pure water and 0.1% SDS phosphate buffer (pH 7.30) for 5 min to remove the unbound oligonucleotides. The same protocol as above was applied at S1-modified GCEs for hybridization reactions of S1 with S3 and also with S4.

2.2.4. MB accumulation and voltammetric transduction

MB for DNA hybridization detection is illustrated in Fig. 1. MB was accumulated onto the surface of hybrid-modified GCE by immersing the electrode into stirred 0.1 M PBS (pH 7.00) containing 20 μM MB with 50 mM NaCl for 5 min without applying any potential. In the optimal condition, the concentration of MB was chosen as 20 μM and the accumulation time of MB was chosen as 5 min for optimum analytical performance. After accumulation of MB, the electrode was rinsed with 0.1 M PBS (pH 7.00) in ultrasonic bath for 10 s to remove the non-specific MB. MB was intercalated into the DNA to form DNA/MB system on the probe electrode after hybridization. Then, CVs were collected between −0.50 and +0.40 V with scan rate of 100 mV s^{-1}; differential pulse voltammetries were collected from −0.50 V to 0.40 V with amplitude of 5 mV.

2.2.5. Regeneration of modified electrode surface

The probe modified GCE surface denaturation/regeneration cycles were investigated. Both hybridization and detection involve reversible-binding processes, so that the biosensor could be reusable for five cycles of hybridization and regeneration. Surface denaturation/regeneration cycles using MB as indicator were carried out as follows. A probe modified GCE electrode was first characterized by DPV in a stirred 0.1 M PBS (pH 7.00) containing 20 μM MB with 50 mM NaCl for 5 min without applying any potential. The reduction signal of MB was measured as in Section 2.2.2. Then, hybridization of the complementary was performed as in Section 2.2.3 and intercalation of MB was performed as in Section 2.2.4. Subsequently, the reduction signal of MB was measured as in Section 2.2.4. After this measurement, the surface-immobilized dsDNA was then denatured in ultra-pure water at 95 °C for 1 min, thus, this gave rise to the probe modified GC electrode. Then a new cycle of denaturation/regeneration would start again.

3. Results and discussion

3.1. Covalent modification of GCE with CCA

The potential scan range, especially the positive potential, affects considerably the formation of polymerization film. It is found that it is difficult to initiate the polymerization reaction by the electrochemical method when the positive limited potential was less than +1.3 V [21]. It is clear that the anodic peak at about 0.4 V corresponding to the oxidation of CCA monomer decreased sharply for the first two cycles (figure not shown). Upon further potential cycling, it decreased gradually. This phenomenon implies the formation of poly-CCA membrane on GCE [21]. The electro-deposited behavior of CCA at the GCE is similar to some reports [23,24] referring to the electrochemical responses of a few azo compounds at solid electrode. The reaction mechanism could be explained as follows (Fig. 1): CCA (A) was first deposited at the surface of GCE and oxidized to form a benzoquinone diimine structure (B); and then the benzoquinone diimine structure (B) was reduced to CCA (A) at the surface of GCE. After electropolymerization, the modified electrode was carefully rinsed with doubly distilled water and then kept in pH 7.00 PBS. This modified electrode was used within 4 weeks.

3.2. Electrochemical behavior of $Fe(CN)_6^{3-/4-}$ on different modified GCE

Prior to hybridization of the target DNA on the surface of the S1-modified electrodes, we examined the surface coverage of S1 on the electrode by CV in the presence of $Fe(CN)_6^{3-}$ marker ion in solution, according to the reported procedure [25,26]. The bare GCE, poly-CCA/GCE, S1/poly-CCA/GCE, were used as the working electrodes, respectively (figure not shown). The result showed that a symmetric, reversible voltammogram was obtained for a bare GCE, whereas the poly-CCA modified electrode showed an asymmetric, irreversible wave. Both the anodic and cathodic currents are severely reduced for the poly-CCA modified electrode, due to the electrostatic repulsion between $Fe(CN)_6^{3-}$ anion and negatively charged poly-CCA film on the electrode surface. When the probe DNA immobilized on the modified electrode, compared with bare GCE and poly-CCA/GCE, the peak current decreased further and the potential deviation increased. That is, the probe DNA had negative charge, the negative charge on the surface would further increase, so hampered $Fe(CN)_6^{3-}$ to participate the electrode reaction on the surface [27]. These results suggest that the electrode surface is well covered with the probe DNA under the modification conditions.

The EIS was also used to identify the immobilization of S1 on the GCE. In the Nyquist diagram the semicircle diameter of the electrochemical impedance spectra equaled to the electron transfer resistance R Nyquist diagrams of $[Fe(CN)_6]^{3-/4-}$ at different modified electrodes were illustrated in Fig. 2. After the poly-CCA film modified on the GCE, the interfacial Ret increased greatly (curve b) compared with that of the bare GCE (curve a); this was attributed to the large quantity of negative charges from $-COO^-$ groups or $-SO_3^-$ groups that perturbed the rates of interfacial electron transfer between the electrode and the electrolyte solution. After the probe DNA S1 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 [28]. 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 c and curve d). The surface coverage (θ) of poly-CCA film on a bare GCE can be calculated from the EIS in terms of the

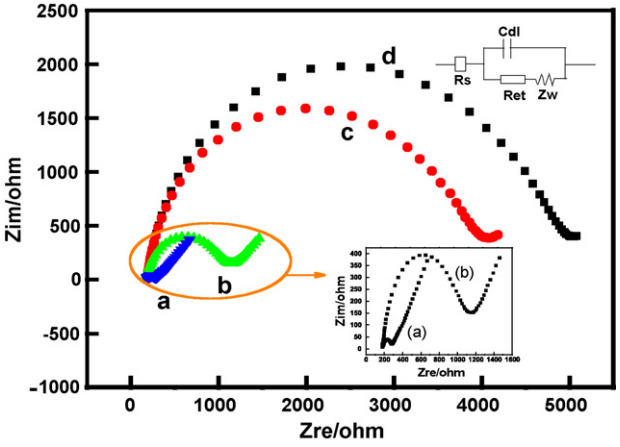

Fig. 2. Impedance plots of 10 mM $Fe(CN)_6^{3-/4-}$ in 1.0 M KCl on different modified electrode: (a) bare GCE; (b) poly-CCA/GCE; (c) S1/poly-CCA/GCE; (d) S1–S2/poly-CCA/GCE.

equation [21,29–33]:

$$\theta = 1 - \frac{R_{ct}^{Bare}}{R_{ct}^{poly\text{-}CCA}} \quad (1)$$

where R_{ct}^{Bare} denotes the charge transfer resistance of the bare GCE, and $R_{ct}^{poly\text{-}CCA}$ is the corresponding resistance of the poly-CCA/GCE with different electropolymerization cyclic times. It is evident that a saturated monolayer of poly-CCA film on the bare GCE surface was formed after electropolymerization for 40 cyclic times; in 0.1 M KCl solution with 5 mM $Fe(CN)_6^{3-/4-}$ (pH 7.0), R_{ct}^{Bare} is 65 Ω. Under the same conditions, $R_{ct}^{poly\text{-}CCA}$ is about 9000 Ω. Using Eq. (1), the coverage was calculated to be 99.3%. The EIS results indicated that modification and hybridization were successfully accomplished on the electrode surface.

3.3. The electrochemical behavior of MB on different electrodes

Fig. 3 shows that bare GCE, poly-CCA/GCE, S1/poly-CCA/GCE and S1–S2/poly-CCA/GCE interacted with pH 7.00 PBS containing 20 μM MB, respectively. There is a rather low signal of MB on bare GCE obtained as shown in Fig. 3A (curve a). The oxidoreduction peak potential for non-specifically adsorbed MB peak was at −100 mV versus Ag/AgCl. This is due to the relatively low quantity of MB adsorbed on the GCE surface [25,34]. When the poly-CCA modified on the electrode, the peak current increased (Fig. 3A, curve b). As CCA was a kind of chelometric titration indicator, and the whole name was 1-(2-hydroxy-4-sulfo-1-naphthylazo)-2-hydroxy-3-naphthoic acid, which containing a sulfonic group($-SO_3H$) in the structure. As it was known, the pK_a value of $R-SO_3H$ was usually between 3.00 and 4.00 [35,36]. When the solution pH was equal to 7.00, the $-SO_3H$ group of poly-CCA film could dissociate favorably into a negative charge group $-SO_3^-$. Under this condition, the MB in the solution would interact with proton (H^+) and form the positive ion of MBH^+ (pK_a: 5.18–6.03) [37]. Therefore, the negative charge group $-SO_3^-$ on the surface of poly-CCA modified electrode had a well affinity to the MBH^+ positive ions and could catalyze and promote the oxidation of MB in the pH 7.00 PBS (mechanism shown in Fig. 3C). The highest MB oxidoreduction signal was observed with capture probe on the electrode because the electronegative phosphate skeletons of capture probe immobilized on the electrode had a powerful attracting force to MB cation. At the same time, many more free guanine bases of DNA molecules were exposed out and had a strong affinity with MB, and hence the greatest amount of MB accumulation occurs at this sur-

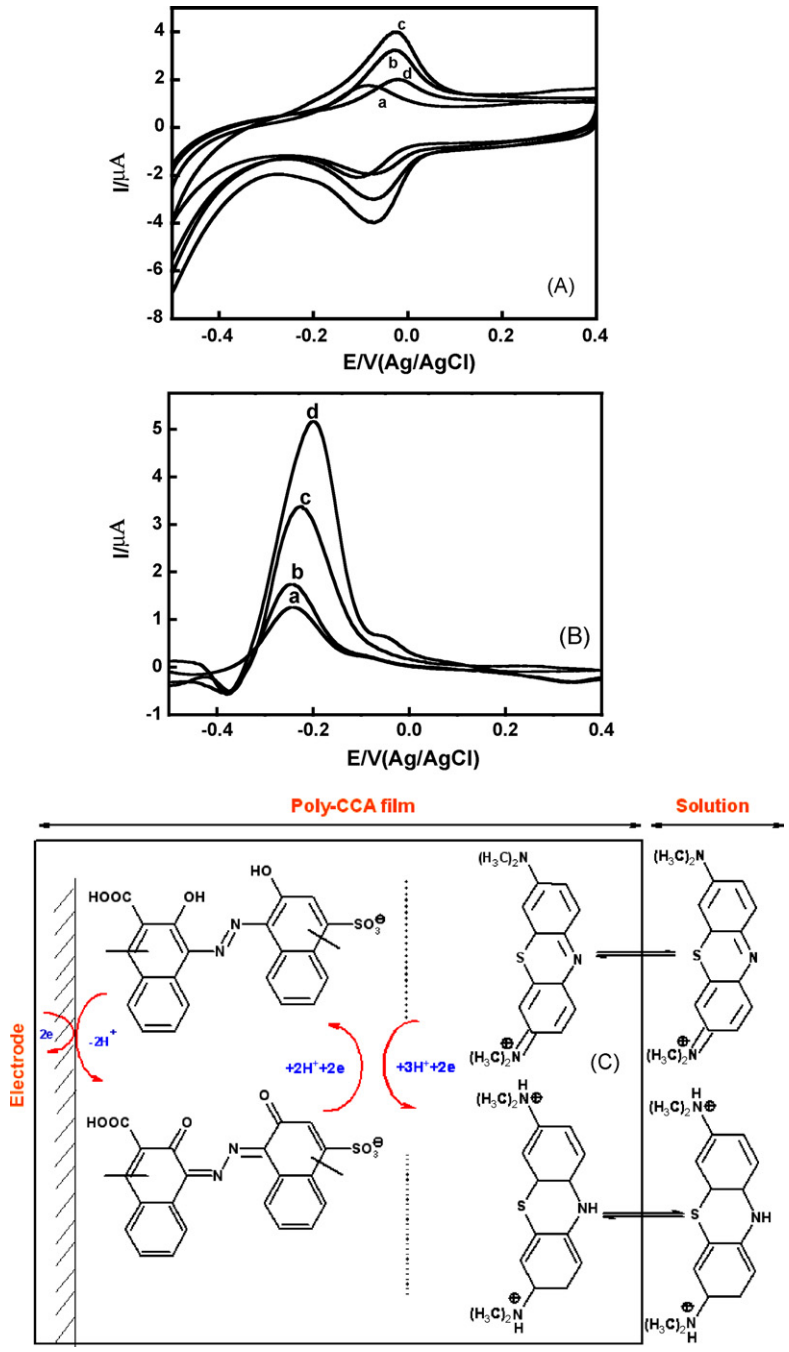

Fig. 3. (A) Cyclic voltammetries of 20 μM MB interacted on bare GCE (a), poly-CCA/GCE (b), 1.0×10^{-12} M S1/poly-CCA/GCE (c), and 1.0×10^{-12} M S1–S2/poly-CCA/GCE (d). (B) Differential pulse voltammetries of 20 μM MB on bare GCE (a), poly-CCA/GCE (b), 1.0×10^{-12} M S1/poly-CCA/GCE (c), and 1.0×10^{-12} M S1–S2/poly-CCA/GCE (d). Pulse amplitude, pulse width and pulse period were 0.05 V, 0.05 s, and 0.2 s, respectively. (C) Mechanism of MB reaction at the poly-CCA/GCE. Cyclic voltammetries were collected between −0.50 and +0.40 V with scan rate of 50 mV s^{-1}; differential pulse voltammetries were collected from −0.50 V to 0.40 V with amplitude of 5 mV.

face [22,38–45] (Fig. 3A, curve c). However, a significant decrease in the voltammetric peak current of MB was observed after complementary target sequence S2 was allowed to hybridize with the capture probe electrode (Fig. 3A, curve d). After hybridization, the current signal of MB decreased due to less MB binding to dsDNA, caused by the inaccessibility of MB to the guanine bases [38,46–49]. This decrease is attributed to the steric inhibition of the reducible groups of MB packed between the bulky double helix of the hybrid. Another interaction may be related to majority of the MB molecules starting intercalating between the double helix of dsDNA at high ionic strength conditions, because the ionic shielding of the negative charges on the DNA was established. It showed that less MB was associated with dsDNA in a high ionic strength solution. The result indicated that less charge was being passed through dsDNA and upon intercalation the reduction potential became less cathodic [47].

Fig. 3B displays DPV of the bare, poly-CCA-modified, S1-modified and S1–S2-modified GCE previously accumulated with MB in blank buffer solution. The results were in good agreement with those obtained from CV. The decrease in the magnitude of the voltammetric peak current of MB intercalator thus reflects the extent of the hybridization at the electrode surface [50].

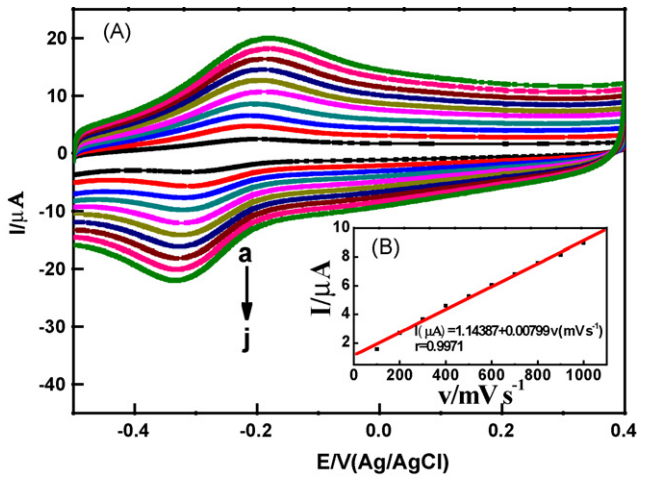

Fig. 4. (A) Cyclic voltammetries of 2.0 μM MB (PBS pH 7.00) on probe modified electrode at various scan rates (mV s^{-1}): (a) 100; (b) 200; (c) 300; (d) 400; (f) 500; (g) 600; (h) 700; (i) 800; (j) 900. (B) The relationship of peak current I (μA) and scan rate v (mV s^{-1}).

3.4. The CV study of MB interacted with the probe modified electrode along with change of scan rate

The probe modified electrode immersed in pH 7.00 PBS containing 20 μM MB and CV were collected with the scan rate of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 V s^{-1}. As shown in Fig. 4A, a pair of evident oxidoreduction peak appeared, and the peak current increased along with the increasing of the scan rate. Fig. 4B shows that the reduction peak current was linear with the scan rate. The regression equation was I (μA) = 1.14387 + 0.00799v (mV s^{-1}), and the coefficient correlation r = 0.9971. It also indicated that MB strongly bound to the probe modified electrode [27].

3.5. Hybridization specificity of probe-DNA/poly-CCA/GCE

The selectivity of this assay was investigated by using the DNA/poly-CCA/GCE as the capture probe (S1) to hybridize with various DNA sequences complementary oligonucleotide (S2), non-complementary oligonucleotide (S3), and one base mismatch oligonucleotide (S4). Fig. 5 shows the DPV signal of MB at S1 before hybridization (Fig. 5, curve a), and after hybridization with the same amount of S2 (Fig. 5, curve d), S3 (see Fig. 5, curve b) and S4 (see Fig. 5, curve c). As can be seen, the largest DPV peak current of MB was observed when exposed the S1/poly-CCA/GCE to the S3 (Fig. 5, curve b), which was similar to that of the blank measurement (Fig. 5, curve c) (a blank measurement means the response of MB at the S1/poly-CCA/GCE, indicating that no change occurred at the electrode surface, and the greatest amount of MB accumulated on the electrode because of the strong affinity of MB with the free guanines; an obviously decreased peak current of MB was obtained when incubating with the S2 (Fig. 5, curve d), suggesting that hybrids (dsDNA) formed at the electrode surface, and the interaction of MB and guanine residues of the probe was prevented by duplex formation on the electrode surface; after S1 hybridized with S4, the peak current of MB was significantly increased and approached to that of S1/poly-CCA/GCE (Fig. 5, curve c), indicating the complete hybridization was not accomplished due to the base mismatch). Thus, response of MB on the probe electrode can be considered as an efficient intercalator to distinguish between hybrids, non-complementary and single-base mismatch oligonucleotides.

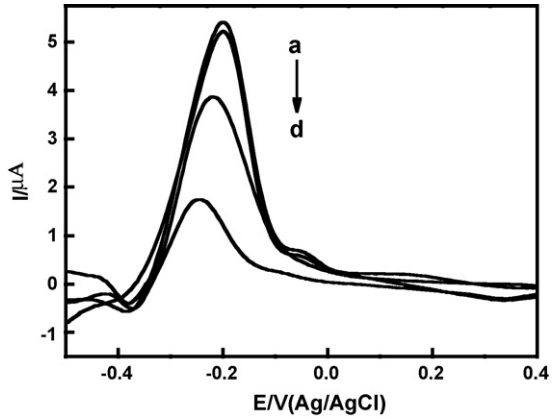

Fig. 5. Differential pulse voltammetries of 20 μM MB accumulated on the 1.0×10^{-12} M S1/GCE (a), 1.0×10^{-12} M S1–S3/GCE (b), 1.0×10^{-12} M S1–S4/GCE (c) and 1.0×10^{-12} M S1–S2/GCE (d) in 0.10 M PBS (pH 7.00) with 50 mM NaCl. Differential pulse voltammetries were collected from −0.50 V to 0.40 V with amplitude of 5 mV.

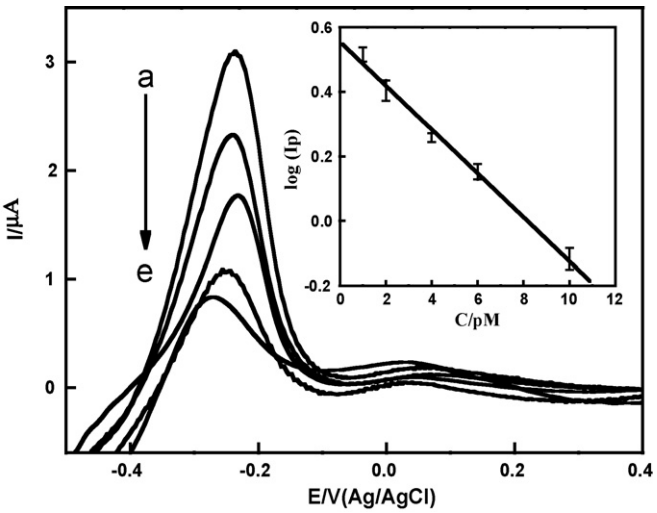

Fig. 6. Differential pulse voltammetries of 20 μM MB accumulated on the probe -1.0×10^{-12} M S1 after its hybridization with different concentration of the S2 in 0.10 M pH 7.00 PBS buffer solution. Target concentration ($\times 10^{-12}$ M): (a) 1.0, (b) 2.0, (c) 4.0, (d) 6.0 and (e) 10. Inset shows the plot of the peak current of MB as a function of the target concentration. Error bars = ±relative standard deviation.

The sensitivity of this electrochemical biosensor for the target DNA 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 MB after hybridization of probe with target was recorded with three repetitive measurements. The current response at about −0.24 V decreased in proportion to the amount of the target sequence used. The average logarithmic current response shows excellent correlation with the amount of the complementary oligonucleotides in the range of 1.0×10^{-12} to 1.0×10^{-11} M (shown in Fig. 6). The regression equation is $\log I_p$ (μA) = 0.068C (pM) + 0.555 (r = 0.9963). A detection limit of 6.7×10^{-13} 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 for detection of 1.0×10^{-12} M target DNA is 7.32% (n = 8). Compared with the other method which our laboratory had been established [51], the novel covalently modification method can get a lower detection limit and has higher sensitivity.

3.6. Optimization of conditions

3.6.1. The selectivity of hybridization time and hybridization temperature

The co-influence of hybridization time and hybridization temperature were studied in these experiments. The result indicated that hybridization time decreased with the increasing of hybridization temperature due to the fact that higher temperature sped the movement of DNA molecules. On the other hand, higher hybridization temperature accelerated the denaturation of dsDNA [52], resulting in the decreasing of the absolute hybridization number. Taking consideration of the above two factors, the probe modified electrode reacted with target DNA in solution was chosen at 45 °C for 30 min, which was the optimal hybridization condition.

3.6.2. The selectivity of the concentration about MB

The indicator of DNA electrochemical sensor was a kind of electroactive compound which would be interacted with probe and dsDNA in different ways. The conjugation way of indicator and DNA had three kinds of fundamental modes [46,53]. The good functional indicator would be the different selectivity in the binding ability of the probe and dsDNA. So the indicator in the dsDNA and probe-DNA modified electrode would be the different level of accumulation and different current response. MB was selected as indicator, that's because MB was of good specificity to recognize the different modified electrode [50]. The concentration about MB would be the great influence on the electrochemical signal. Therefore, the concentration of MB was examined.

In order to examine the effect of MB concentration on the enhanced DPV signal (ΔI_p), DNA modified electrode was prepared, and selected different concentrations of MB to detect DPV signal, respectively. The result showed that ΔI_p was increased with the increasing of concentration of MB after hybridization. When MB concentration was 2.0×10^{-5} M, the peak current reached the maximum. When the concentration of MB was increased more, the ΔI_p kept constant, which indicated that the concentration of MB reached saturation, thus 2.0×10^{-5} M was selected as experimental concentration.

3.6.3. The selectivity of MB accumulation time

The effect of accumulation time of MB on ΔI_p was examined, and the results showed that ΔI_p increased exponentially with the accumulation time of MB in the first 5 min. Beyond this accumulation time, ΔI_p tended to constant, which indicated that intercalation of MB into DNA reached a saturation value. Thus, accumulation time of 5 min was chosen as the optimal accumulation time.

3.6.4. The effect of pH

CVs for the electro-oxidation of MB were performed at the DNA modified GCE in various pHs of PBS, and the results showed that the peak potential shifted to negative value with increasing of pH. The peak current of MB showed a maximum at pH 7.00, so the electro-oxidation of MB was preformed better in pH 7.00 PBS.

3.6.5. The selectivity of the concentration about NaCl in the hybridization solution

The monovalent cation such as NaCl would increase the speed of the generation about the heterogenous heteroduplexes [54]. In the hybridization solution, since changing of the concentration of NaCl would greatly affect the hybridization signal, the effect of NaCl concentration on the hybridization of 1.0×10^{-12} M complementary strand was examined, when the concentration of NaCl was lower than 0.05 M, the oxidation peak current of MB increased along with the increasing of the NaCl concentration. On the contrary, when the concentration of NaCl was higher than 0.1 M, the oxidation peak current of MB decreased with increasing of NaCl. While the peak current reached maximum and constant when Na^+ was between 0.05 and 0.1 M. Therefore, 0.05 M of NaCl was selected for subsequent experiments.

4. Conclusion

In this article, through using poly-CCA films, the new immobilization was studied to modify covalently on the carbon surface in order to detect APL PML/RARA fusion gene. The results of new covalent bond manifested that the probe would show the specificity of the signal compared with that of hybridization. Conferred to the same kind of immobilization method, it provided a simple, stable detection and might have a promising future in the electrochemical DNA biosensors. Along with the deep study of immobilization, it would play the potential predominance in diagnosis of diseases and the new APL DNA biosensor would be proposed to use in real sample.

Acknowledgements

The authors gratefully acknowledge the financial support of the National High Technology and Development of China (863 Project: 2006AA02Z4Z1 and 2008AA02Z433); the National Natural Science Foundation of China (20675015, the Foundation of Fujian Provincial Technology International Cooperation (2006I0016) and the Foundation of Fujian Education Department (2005K051) and the Foundation of Fujian Health Department (2005123).

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