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ScienceDirect
Talanta 72 (2007) 468–471
Talanta
www.elsevier.com/locate/talanta

Electrochemical DNA biosensor for the detection of short DNA species of Chronic Myelogenous Leukemia by using methylene blue

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Received 5 July 2006; received in revised form 28 October 2006; accepted 6 November 2006
Available online 8 December 2006

Abstract

A novel assay for the voltammetric detection of 18-bases DNA sequences relating to Chronic Myelogenous Leukemia (CML, Type b3a2) using methylene blue (MB) as the hybridization indicator was reported. DNA was covalently attached onto a glassy carbon electrode (GCE) through amines of the DNA bases using *N*-hydroxysulfosuccinimide (NHS) and *N*-(3-dimethylamion)propyl-*N*′-ethyl carbodiimidehydrochloride (EDC). The covalently immobilized single-stranded DNA (ssDNA) could selectively hybridize with its complementary DNA (cDNA) in solution to form double-stranded DNA (dsDNA) on the surface. A significant increase of the peak current for methylene blue upon the hybridization of immobilized ssDNA with cDNA in the solution was observed. This peak current change was used to monitor the recognition of CML DNA sequence. This electrochemical approach is sequence specific as indicated by the control experiments in which no peak current change was observed if a non-complementary DNA sequence was used. Factors, such as DNA target concentration and hybridization conditions determining the sensitivity of the electrochemical assay were investigated. Under optimal conditions, this sensor has a good calibration range between 1.25×10^{-7} and 6.75×10^{-7} M, with CML DNA sequence detection limit of 5.9×10^{-8} M.

Keywords: Biosensors; DNA; Hybridization; CML; Methylene blue; Sequence-specific electrochemical detection

1. Introduction

Chronic Myelogenous Leukemia (CML) is a clonal myeloproliferative disorder resulting from the neoplastic transformation of the primitive hemopoietic stem cell [1–3]. Generally speaking, the CML patients do not show any observable symptoms in their early stage, and the chronic course can last for 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 [4,5]. There are many types of BCR/ABL genes, and Type b3a2 is one of the most common mutation types, which are often studied in scientific researches. Thus, the detection of BCR/ABL gene will afford an early diagnosis and monitor of the disease, which in turn improves the facility of detecting minimal residual leukemia cells in the CML patients, especially after the bone marrow transplantation (BMT).

In the recent years, electrochemical DNA biosensors based on nucleic acid hybridization have been rapidly developed due to their increasing importance in the diagnosis of disease. DNA biosensors for recognition of DNA hybridization offer a new approach for rapid, sensitive, simple and low-cost detection of specific nucleic acid sequences [6–11]. Due to the advantages of electrochemical DNA biosensors, we attempted to detect BCR/ABL genes (Type b3a2) using electrochemical methods, which will provide a new way to the diagnosis and monitor of the CML patients.

Some of anticancer drug [12–14], metal complexes [15,16] and organic dyes [17–19] exist as chiral molecules capable of selective recognition of DNA. The changes in electrochemical response of these labels are usually used to monitor the hybridization process. Methylene blue (MB) is an organic dye that belongs to the phenothiazine family. DNA biosensors using MB as chemical indicator have been widely studied [20–23].

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doi:10.1016/j.talanta.2006.11.015

According to the spectrophotometric and electrochemical results [24–28], the detection of hybridization was found to be accomplished by the specific interaction of MB with guanine bases in the DNA sequence [29]. Rohs et al. [30] reported a modeling study for MB binding to DNA with alternating guanine-cytosine base sequence. In short, the MB based DNA sensor is sequence dependent.

In this paper, a glassy carbon electrode (GCE) in combination with differential pulse voltammetry (DPV) was used to obtain information about the interaction of MB with CML DNA (Type b3a2). The DNA sensor fabrication method, such as the hybridization method and the conditions control, was modified. Changes in the voltammetric signals resulting from the interaction of MB with dsDNA and ssDNA were used to sequence-specific detection of BCR/ABL gene related to CML. Under optimal performance conditions, the present biosensor can discriminate single-base mismatch well.

2. Experimental

2.1. Apparatus and reagents

DPV measurements were performed by using CHI 660B 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). All potentials mentioned in this paper refer to this reference electrode.

2.2. Chemicals

The 18-base synthetic oligonucleotides were purchased from Shanghai Chemical Reagents Company (China); their base sequences were:

- immobilized probe(18-base sequence A)-5′-NH_3 AGA GTT CAA AAG CCC TTC-3′;
- target (18-base sequence B)-5′-GAA GGG CTT TTG AAC TCT-3′;
- non-complementary(18-base sequence B′)-5′-ACG TGG TCC CCA GCT CTC-3′.

All oligonucleotides, dsDNA and ssDNA stock solutions (100 mg L^{-1}) 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 (AR) was purchased from The Third Agents Factory of Shanghai (China). Stock solutions of MB (1 mM) were prepared with deionized water. *N*-hydroxysulfosuccinimide and *N*-(3-dimethylamion)propyl-*N*′-ethyl carbodiimidehydrochloride were obtained from Sigma. The stock solution of 5 mM DEC and 8 mM NHS was prepared in 50 mM phosphate solution (pH 7.40). Other chemicals were of analytical reagent grade. All the buffer solutions contained 20 mM NaCl. Sterilized and deionized water was used in all solution.

2.3. Experimental procedures

2.3.1. GCE pretreatment

The GCE was polished with 0.3 and 0.05 μm alumina, sonicated in water, and oxidized at 0.5 V for 1 min in 50 mM phosphate buffer solution (pH 7.40). After the oxidation step, the electrode was rinsed with distilled water.

2.3.2. Covalent bond modification

GCE was inverted and 20 μL 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-drying of this solution, the electrode was rinsed with distilled water.

2.3.3. Immobilization of DNA on covalently modified GCE

SsDNA (100 μg mL^{-1}, 10 μL) was pipetted onto the GCE surface and air-dried to dryness. The dsDNA modified GCE was prepared by immersing the above electrode into 20 mM Tris–HCl buffer solution (pH 7.00) containing cDNA at 45 °C for 30 min. The electrode was then rinsed with pH 4.80 acetate buffer solution.

2.3.4. MB accumulation and voltammetric transduction

The DNA modified electrode was immersed in 20 mM Tris–HCl buffer (pH 7.00) containing 20 μM MB for 5 min. After this modified electrode was transferred into the blank 20 mM Tris–HCl buffer (pH 7.00), differential pulse voltammograms were collected from 0.1 to −0.6 V with amplitude of 10 mV at 50 mV/s scan rate.

Replicate measurements were performed by renewing the surface and repeating the above assay preparation procedure.

3. Results and discussion

Fig. 1 displays the differential pulse voltammograms of the bare, ssDNA-modified and dsDNA-modified GCE previously accumulated with MB in blank buffer solution. For all these

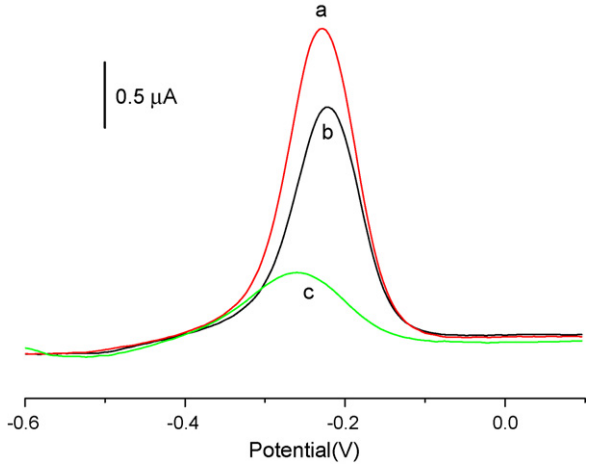

Fig. 1. Differential pulse voltammograms of MB accumulated on the ssDNA (curve a) and dsDNA (curve b) modified electrode in a 0.50 M acetate buffer (pH 4.80) with 20 mM NaCl. For comparison, the DPV of MB accumulated on a bare GCE under the same experimental conditions is also displayed (curve c).

electrodes, a current peak for the reduction of MB appears at −220 mV. The bare GCE shows the smallest current peak, which could be due to the specific adsorption of MB at the bare GC electrode surface (curve c). As the GCEs were modified with ssDNA (curve a) and dsDNA (curve b), the current peak for the reduction of MB increased, indicating that MB was successfully immobilized on the modified GCE due to the interaction between the MB and the DNA. In addition, the current for the ssDNA-modified electrode is higher than that for the dsDNA-modified electrode. This difference should be due to the lesser interaction between dsDNA and MB than that for ssDNA and MB, resulting in decreased intercalation of MB on the dsDNA-modified electrode. This also indicates that MB has strong affinity to the free guanine bases, and thus the greatest amount of MB accumulated at this ssDNA-modified electrode [31]. After MB was intercalated between the base pairs of hybrid, the electrochemical signal decreased (Fig. 1b). This decrease was attributed to the steric inhibition of MB packing between the double helix of the hybrid. Besides the change in peak current, the peak potentials for the reduction of MB on the ssDNA- and dsDNA-modified GCEs shift to positive value as compared to that for bare GCE, which demonstrates the MB interacts with the DNA molecules.

Control experiments were also performed to estimate whether the biosensor responds selectively via hybridization. The histogram of the reduction current of MB intercalated on the ssDNA modified GCE (1), after hybridization with non-complementary sequence (2), complementary sequence (3) and one-base mismatch sequence (4) in solution is shown in Fig. 2. As expected, there was no significant current difference observed for the ssDNA modified GCE and its hybridization with non-complementary sequence, 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 was not changed after its interaction with non-complementary sequence. However, when the ssDNA modified GCE interacted with the complementary sequence in solution, the peak current for the MB reduction decreased significantly. This decrease in current clearly showed that the ssDNA modified on the GCE successfully hybridized with its complementary sequence, decreasing the intercalation amount of MB on the modified GCE due to the steric inhibition of MB packing. The effective discrimination against single-base mismatch was also studied. In the presence of oligonucleotide containing a single-base mismatch, significant increased voltammetric signal was obtained as compared to the complementary sequence. This difference indicates that the complete hybridization was not accomplished due to the base mismatch. A series of three repetitive measurements resulted in reproducible results. The relative standard deviation is 3.90%, 4.75%, 6.18%, 7.98% and 9.85% for bare GCE, ssDNA-GCE, dsDNA-GCE, one-base mismatch detection and non-complementary sequence detection, respectively.

The influence of experimental parameters including MB accumulation time and concentration, hybridization time and hybridization temperature on the sensitivity of the present assay were explored for optimum analytical performance. Fig. 3 shows that the DPV signals for MB increased as a function of accumulation time. The peak current increased exponentially with the accumulation time in the first 5 min. Beyond this accumulation time, the MB signal reached constant, which indicated that intercalation of MB to DNA reached a saturation value. Thus, accumulation time of 5 min was chosen as the optimal accumulation time.

The influence of hybridization time and hybridization temperature is shown in Fig. 4. The co-influence of hybridization time and hybridization temperature was investigated in these experiments. We can see that hybridization time decreases with the increase of hybridization temperature due to the fact that higher temperature speed the movement of DNA molecules. On the other hand, higher hybridization temperature accelerates the denaturation of dsDNA, resulting in the decrease of the absolute hybridization number. Taking consideration of the above two factors, the ssDNA modified electrode reacting with cDNA in solution is chosen at 45 °C for 30 min, which is the optimal hybridization condition.

The electrochemical response for the intercalated MB on the ssDNA modified GCE interacting with different level of the target DNA in solution was studied and the results are displayed

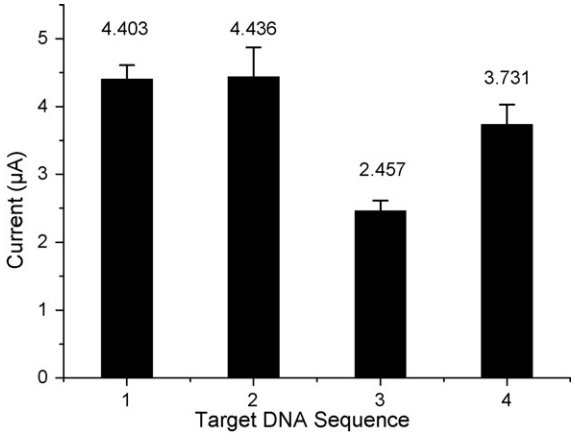

Fig. 2. Histogram of DPV signals for the electrochemical reduction of MB intercalated on the ssDNA/GCE (1); after its hybridization with non-complementary sequence (2); after its hybridization with complementary sequence (3); after its hybridization with one-base mismatch sequence (4).

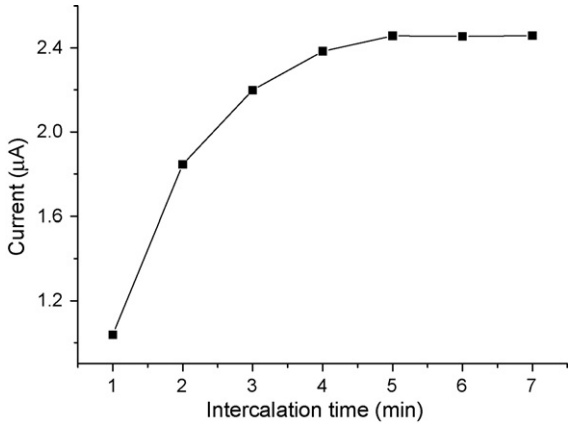

Fig. 3. Plot of the peak current for MB at the modified electrode as a function of MB accumulation time.

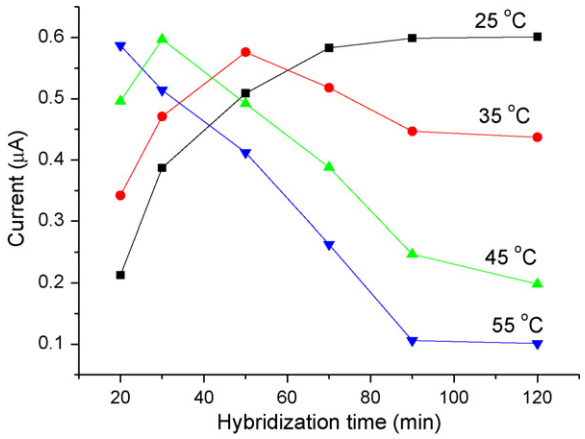

Fig. 4. DPV response as function of hybridization time and temperature using MB as indicator.

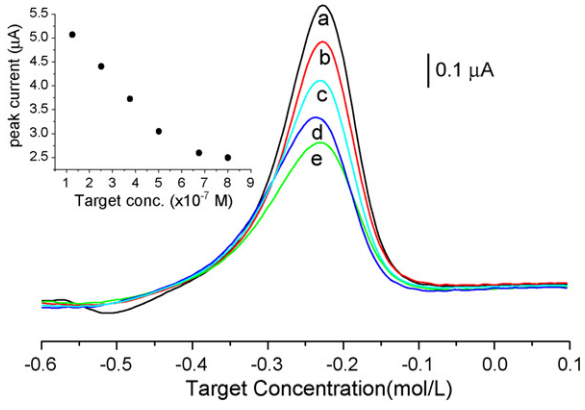

Fig. 5. Differential pulse voltammograms of MB accumulated on the ssDNA after its hybridization with different concentration of the target sequence in a 0.50 M acetate buffer (pH 4.80) with 20 mM NaCl. Target concentration ($\times 10^{-7}$ M): (a) 1.25, (b) 2.5, (c) 3.75, (d) 5.0 and (e) 6.75. Inset shows the plot of the peak current of MB as a function of the target concentration.

in Fig. 5. The signal for the reduction of MB after hybridization with target DNA decreased with the target concentration up to 6.75×10^{-7} M, and then remained constant with further increase of target concentration, which indicates that all the immobilized probes on GCE surface have been involved in hybridization at the concentration of 6.75×10^{-7} M. Before this threadhold concentration, the MB reduction signal decreased accordingly with the increase of the target concentration, which was resulted from more hybrid formation preventing the interaction of guanine bases with MB. The constant MB signal also indicated that all the hybridization sites on the ssDNA GCE have been covered. The reduction peak current of MB was linearly relating to the concentration of the target oligonucleotide sequence of the b3a2 type CML between 1.25×10^{-7} and 6.75×10^{-7} M ($R^2 = 0.9951$). The detection limit was 5.9×10^{-8} M based on the ratio of signal-to-noise of 3.

4. Conclusion

In conclusion, we have reported a new hybridization biosensor for electrochemical detection of sequences specific to CML DNA of Type b3a2. This effort addresses the urgent needs and analytical challenges of detecting BCR/ABL gene in the CML patients. The present method using MB as indicator for the detection of short DNA segments relating to CML (b3a2) is simple, sensitive and rapid and is promising for clinical diagnostic testing for CML.

Acknowledgements

This work was supported by Science & Technology Department of Fujian Province (2006I0016) and the grant from the National Natural Science Foundation of China (no. 20535010 and 20675015).

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