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Electrochemical Biosensor for Detection of BCR/ABL Fusion Gene Using Locked Nucleic Acids on 4-Aminobenzenesulfonic Acid-Modified Glassy Carbon Electrode

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In this study, an electrochemical DNA biosensor was developed for detection of the breakpoint cluster region gene and the cellular abl (BCR/ABL) fusion gene in chronic myelogenous leukemia by using 18-mer locked, nucleic acid-modified, single-stranded DNA as the capture probe. The capture probe was covalently attached on the sulfonic-terminated aminobenzenesulfonic acid monolayer-modified glassy carbon electrode through the free amines of DNA bases based on the acyl chloride cross-linking reaction. The covalently immobilized capture probe could selectively hybridize with its target DNA to form double-stranded DNA (dsDNA) on the LNA/4-ABSA/GCE surface. Differential pulse voltammetry was used to monitor the hybridization reaction on the capture probe electrode. The decrease of the peak current of methylene blue, an electroactive indicator, was observed upon hybridization of the probe with the target DNA. The results indicated that, in pH 7.0 Tris-HCl buffer solution, the peak current was linear with the concentration of complementary strand in the range of 1.0×10^{-12}–1.1×10^{-11} M with a detection limit of 9.4×10^{-13} M. This new method demonstrates its excellent specificity for single-base mismatch and complementary dsDNA after hybridization, and this probe has been used for assay of PCR real sample with satisfactory results.

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder, resulting from the neoplastic transformation of the primitive hemopoietic stem cell.[1–4] The chimeric oncogene breakpoint cluster region gene and the cellular abl gene (BCR/ABL) is the traditional gene existing in almost all CML patients.[5–7] The BCR/ABL chimerical fusion gene encodes a cytoplasmatic hybrid protein with an important role in the pathogenesis of CML.[8–11] In recent years, the monitoring methods of clinical diagnosis and prognosis about the fusion gene of CML included chromosome analysis, fluorescence in situ hybridization,[12,13] flow cytometry,[14] and real-time quantitative reverse transcription PCR.[15] But there are some limitations in these methods, such as time-consuming, poor precision, and expensiveness. So it is very significant to develop a new effective method to detect the BCR/ABL fusion gene.

Electrochemical methods have prominent advantages compared with other methods, such as easy to miniaturize, simple, rapid, and inexpensive. Thus, in recent years, there has been considerable interest in developing DNA electrochemical biosensors for the rapid and inexpensive diagnosis of genetic diseases and other biological analysis.[16,17] These typical DNA electrochemical biosensors can be prepared by immobilizing short single-stranded DNA probes on different electrodes and using electroactive indicators or other methods to measure the hybridization events between the DNA probes and their cDNA fragments. So DNA-modified electrodes are very important for developing electrochemical DNA biosensors and studying the interactions

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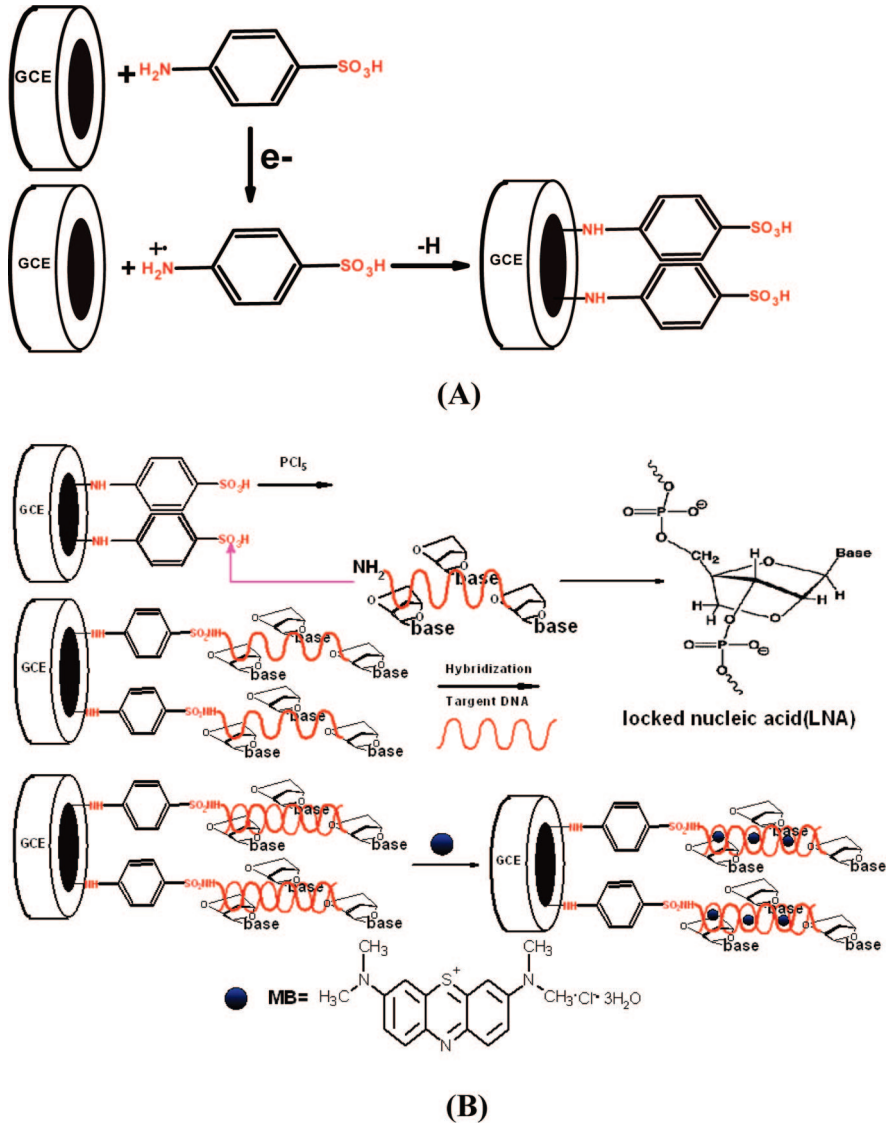

Figure 1. Diagram of the procedure for the fabrication of DNA biosensor. (A) Grafting of 4-ABSA on a GCE. (B) Covalent bond of LNA on the 4-ABSA/GCE.

of DNA with other molecules.[18−20] However, traditional methods of immobilization of DNA onto electrode surfaces have poor hybridization efficiency.[21,22]

The interactions between a specific DNA probe and target play an important role in the overall performance of the DNA biosensors. Most typical short single-stranded DNA probes designed for the sensors present poor stability or specificity. Therefore, sensitive and selective DNA probes still need to be developed for the assay of the hybridization events between the DNA probes and their cDNA fragments. Recently, some reports described the synthesis and hybridization of a novel nucleotide termed locked nucleic acid (LNA, also known as bridged nucleic acid).[23−25] LNA monomer contains a methylene bridge that connects the 2′-oxygen with the 4′-carbon of the ribose ring of RNA (see Figure 1). This bridge results in a locked 3′-endo conformation, reducing the conformational flexibility of the ribose and increasing the degree of local organization of the phosphate backbone.[26,27] This entropic constraint leads to improving the binding to complementary RNA and DNA sequences, with each LNA substitution increasing the melting temperatures (T_m) by as much as 10 °C.[28,29] Furthermore, LNAs have many advantages, including enhanced triplex forma-

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tion,[30,31] remarkable antisense activity,[32] nondetectable toxicity in vivo,[32] and nuclease resistance.[33] LNA bases can be interspersed with DNA bases, allowing binding affinity to be tailored for particular applications. The ability of LNA bases to confer dramatic increases in hybridization affinity suggests that they have great potential for optimizing nucleic acid recognition. Due to the high affinity of the LNA molecules, very short probes can be used and show an extraordinary specificity to discriminate the targets that differ by a single base.

The aim of this work is to develop a simple and specific DNA immobilization strategy, which provides a well-defined recognition surface for hybridization. In this study, an electrochemical DNA biosensor was developed for recognition of target DNA by using 18-mer single-stranded LNA as the capture probe for hybridization with the BCR/ABL fusion gene in CML. This new probe demonstrates excellent specificity for single-base mismatch and complementary double-stranded DNA (dsDNA) after hybridization, and this probe has been used for assay of a PCR real sample with satisfactory results.

EXPERIMENTAL SECTION

Preparation of the Biosensor Surface and Its Modification with DNA. The diagram for preparation of the electrochemical DNA biosensor is illustrated in Figure 1. First, the electrochemical modification of the clean glassy carbon electrode (GCE) was carried out in 0.025 M KH_2PO_4 and Na_2HPO_4 solution containing 20 mM sulfonic-terminated aminobenzenesulfonic acid (4-ABSA) by cyclic voltammetry (CV) scanning between ±0.5 and +1.40 V (versus SCE) for four cycles with scan rate of 10 mV s^{-1}. Second, the capture probe was covalently attached on the 4-ABSA monolayer-modified GCE through the free amines of DNA bases based on the acyl chloride cross-linking reaction. The terminal sulfonic acid groups of the 4-ABSA/GCE were activated by immersing this modified electrode in acetone solution containing 40 mM PCl_5 for 0.5 h.[34] The linker/4-ABSA/GCE was rinsed with 20 mM Tris-HCl buffer (pH 7.00) to wash off the excess PCl_5. A 5-μL solution containing 0.1 nM LNA probe was then pipetted onto the modified GCE and air-dried to dryness. Thus, a probe-modified GCE was obtained. The electrode surface was then washed with water to remove the unbound oligonucleotides. Finally, the hybridization was performed by pipetting 5 μL of different concentrations of target DNA (18-mer sequence S2) onto the probe-modified GCE for 30 min at 45 °C.[4] Thus, a hybrid-modified GCE was obtained. The electrode surface was then washed with water to remove the unbound oligonucleotides. The same protocol as mentioned above was applied to probe-modified GCEs for hybridization of probe (18-mer sequence S1) with one-base mismatch (18-mer sequence S3) and also with noncomplementary sequences (18-mer sequence S4). Details of the LNA electrochemical probe and target DNA sequences were shown in Table 1.

Table 1. Details of the LNA Electrochemical Probe and Target DNA Sequence

LNA and target DNA	sequence
LNA-modified probe (S1)	5′-NH_2-A^LGA GTT CA^LA AAG CCC^L TTC-3′ (L: 2′-O,4′-C-methylene-(D-ribofuranosyl) nucleotides LNA)
target (S2)	5′-GAA GGG CTT TTG AAC TCT-3′
single-base mismatch (S3)	5′-GAA GGG CAT TTG AAC TCT-3′
noncomplementary (S4)	5′-ACG TGG TCC CCA GCT CTC-3′

Intercalation of Methylene Blue (MB) and Voltammetric Transduction. MB was accumulated onto the surface of hybrid-modified GCE by immersing the electrode into the stirring 20 mM Tris-HCl buffer solution (pH 7.0) containing 20 μM MB and NaCl for 5 min without applying any potential.[4] After accumulation of MB, the electrode was rinsed with 20 mM Tris-HCl buffer (pH 7.0) in an ultrasonic bath for 10 s to remove the nonspecifically bound MB. MB was intercalated into the DNA to form a DNA/MB system on the probe electrode after hybridization. Differential pulse voltammetry (DPV) was then scanned from +0.40 to −0.50 V with an amplitude of 5 mV.

RESULTS AND DISCUSSION

Thermostability of the LNA and DNA Capture Probe. Melting temperature (T_m) is the temperature at which, under a given set of conditions, double-stranded DNA is changed (50%) to single-stranded DNA. The T_m is a physical property of nucleic acids that gives information about the stability of duplexes in a specified environment. T_m values are useful in a variety of fields ranging from practical assay design in molecular biology to theoretical biophysics. [35] Therefore, the T_m for the hybridization of LNA with their complemental DNA was examined to confirm their potential for selective recognition of complementary sequences (see Table 2). As seen in Table 2, the T_m value of the LNA capture probe (62.8 °C) was greatly higher than that of its corresponding DNA probes (48.3 °C). Compared with the analogous DNA−DNA hybrids, the T_m value for LNA binding to DNA was increased 14.5 °C, which was equivalent to 4.8 °C/LNA base. It could be also seen from Table 2 that the T_m value for LNA binding to single-base-mismatched DNA was decreased to 38.3 °C. The results indicated that the T_m of oligonucleotides could be changed by the incorporation of LNA residues into the oligo sequence, which made it possible to discriminate fully complementary and single mismatch sequences better.

Theoretically, the optimum hybridization temperature was ~20 °C lower than T_m. As seen in Table 2, the T_m value of the LNA capture probe binding to cDNA was 62.8 °C, which for single-base-mismatched DNA was decreased to 38.3 °C. Therefore, the theoretical hybridization temperature was 42.8 and 18.3 °C for the cDNA and single-base-mismatched DNA, respectively. The earlier experiments indicated that when the selected hybridization temperature was farther away from the theoretical hybridization temperature, the speed of hybridization would be slower. On the other hand, a higher hybridization temperature would accelerate

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Table 2. Thermostability of the LNA and DNA Capture Probe Sequences against Complementary and Single-Base-Mismatched DNA Targets

		T_m, °C		
capture probe	sequence	DNA target (5′-GAAGGGCTTTTGAACTCT-3′)	m-DNA target, (5′-GAAGGGCATTTGAACTCT-3′)	ΔT_m, °C
DNA	5′-AGAGTTCAAAAGCCCTTC-3′	48.3	41.1	7.2
LNA	5′-$\underline{A}^L$GAGTTC$\underline{A}^L$AAAGCC$\underline{C}^L$TTC-3′	62.8	38.3	24.5

the denaturation of dsDNA, resulting in the decrease of the absolute hybridization number.[4] Accordingly, 45 °C was selected as hybridization temperature. At this temperature, hybridization could occur for the cDNA strand but not for the single-base-mismatched DNA strand. Therefore, the hybridization specificity was dramatically increased.

Covalent Modification of GCE with 4-ABSA. The covalent modification of the GCE was performed by repeated CV scanning between the potential of +0.50 and +1.40 V (versus SCE) in 0.025 M KH_2PO_4 and Na_2HPO_4 solution containing 20 mM 4-ABSA with a scan rate of 10 mV s^{-1} for four cycles. There is an irreversible oxidation peak attributed to the formation of amino cation radical and subsequently to chemical bonding of the radical to GCE surface.[36,37] Typically, it was found that, when the potential was repeatedly scanned, the peak gradually diminished, which indicated that the GCE surface had been modified with a sulfonic-terminated monolayer to form the 4-ABSA/GCE.

In order to further confirm that 4-ABSA had been modified on GCE, the CV behavior of this modified electrode was investigated in 5 mM $K_3Fe(CN)_6$ solution. Figure 2 shows the CV of 5 mM $K_3Fe(CN)_6$ in 0.1 M phosphate buffer solution on (a) a bare GCE and (b) a 4-ABSA/GCE. Figure 2 clearly shows that the electron transfer of $Fe(CN)_6^{3-}$ is completely blocked on the 4-ABSA/GCE (see Figure 2b). It can be explained by the electrostatic interactions between the modified surface and the electroactive probes.[37−41] In pH 7.0 solution, the sulfonic acid groups of the modified GCE surface are expected to fully dissociate if we assume that its pK_a is near to that of 4-ABSA (pK_a 3.24).[42] Thus, on the negatively charged 4-ABSA film, the electrostatic repulsion resists access of $Fe(CN)_6^{3-}$ to the electrode surface and blocks its electron transfer on the electrode surface.

Impedance Characterization of the 4-ABSA/GCE. The 4-ABSA/GCE was then characterized by electrochemical impedance spectroscopy (EIS). Impedance spectra of 4-ABSA/GCE with different cyclic times for electropolymerization of ABSA in a solution of 0.1 M KCl containing 5 mM $[Fe(CN)_6]^{3-}$ and 5 mM $[Fe(CN)_6]^{4-}$ were collected at a potential of 0.18 V (versus Ag/AgCl) in the frequency range of $0.1-10^5$ Hz. The results are shown in Figure 3. At a bare GCE, the redox process of the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ system showed an electron-transfer resistance of ~65 Ω (see Figure 3, curve a). When a bare GCE was electropolymerized with 4-ABSA for different cyclic times, the electron-transfer resistance increased significantly for the first four cycles (R_{ct} = 9000 Ω) and then did not increase with the electropolymerization times (see Figure 3, curves b−f). The surface coverage (θ) of 4-ABSA film on a bare GCE can be calculated from the EIS in terms of the equation[43−45]

$$\theta = 1 - R^{bare}ct/R^{4\text{-ABSA}}ct \quad (1)$$

where R_{ct}^{bare} denotes the charge-transfer resistance of the bare GCE, $R_{ct}^{4\text{-ABSA}}$is the corresponding resistance of the 4-ABSA/GCE with different electropolymerization cyclic times. It is evident that a saturated monolayer of 4-ABSA film on the bare GCE surface was already formed after electropolymerization for four cycle times; then, the surface coverage (θ) did not change with the increase of cycle times. In 0.1 M KCl solution with 5 mM $Fe(CN)_6^{3-/4-}$ (pH 7.05), R_{ct}^{bare} is 65 Ω. Under the same conditions, $R_{ct}^{4\text{-ABSA}}$ is ~9000 Ω. Using eq 1, the coverage was calculated to be 99.3%.

Electrochemical Responses of MB-Bound LNA-Modified Probe. It had been reported that MB interacted in a different way with ssDNA and dsDNA.[46,47] Here, MB was used as an electrochemical indicator to study hybridization between the LNA and the target DNA. The CV responses of 2.0 μM MB at a LNA-modified electrode are shown in Figure 4. The plot of cathodic peak current (I) versus scan rate (v) is linear (see Figure 4A inset), indicating that MB was strongly bound to the LNA-modified surface. MB could bind specifically to the guanine bases and readily intercalate into dsDNA as well. Different binding modes of MB with LNA-modified ssDNA and dsDNA resulted in variation in electrochemical responses of the LNA probe. Figure 4B shows the CV signals at the LNA/4-ABSA/GCE before and after hybridization with 18-mer complementary target DNA in Tris-HCl buffer solution containing 20 μM MB. The voltammetric reduction signals of MB at LNA/4-ABSA/GCE decreased after hybridizing with complementary target DNA. Thus, the decrease of current signal after DNA hybridization was due to the inaccessibility of the guanine bases in dsDNA.

Hybridization Specificity of LNA Probe. Detection of target DNA was also monitored by measuring the peak current of MB on the LNA probe electrode with DPV. Typically, there is a small signal of MB on a 4-ABSA/GCE obtained as shown in Figure 5a, which is due to the small amount of MB adsorbed on the 4-ABSA-

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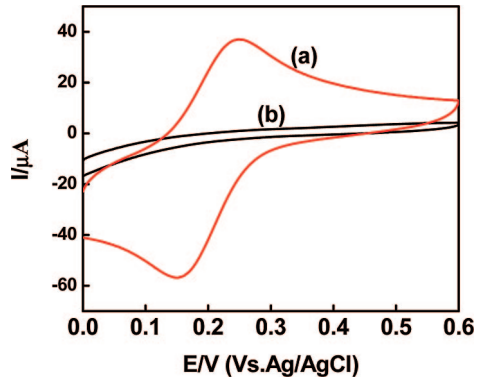

Figure 2. Cyclic voltammograms for (a) a bare GCE and (b) a 4-ABSA-modified GCE for 5 mM $K_3Fe(CN)_6$ in 0.1 M phosphate buffer solution (pH 7.0) and scan rate of 100 mV s^{-1}.

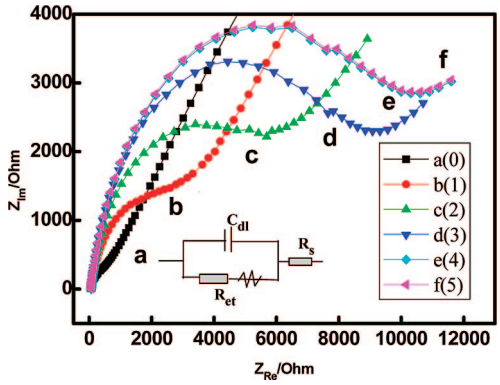

Figure 3. Impedance plots on a 4-ABSA-modified GCE in 5 mM $Fe(CN)^{3-/4-}$ for the different cycles of cyclic voltammograms on a bare GCE in 0.025 M KH_2PO_4 and Na_2HPO_4 solution containing 20 mM 4-ABSA for the (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5 cyclic times. Scan rate:,10 mV s^{-1}.

modified GCE surface. The selectivity of this assay was investigated by using the LNA/4-ABSA/GCE as the capture probe to hybridize with various DNA sequences (complementary oligonucleotide S_2, one-base-mismatch oligonucleotide S_3, and noncomplementary oligonucleotide sequence S_4). Figure 5 shows the DPV signal of MB at capture probe before hybridization (see Figure 5e) and after hybridization with S_2 (see Figure 5b), S_3 (see Figure 5c), and S_4 (see Figure 5d). It is clear that Figure 5e shows the highest peak current of MB on the LNA/4-ABSA/GCE before hybridization, and after hybridization with cDNA, the peak current of MB is dramatically decreased (see Figure 5b). It can be also known that the LNA capture probe has high hybridization specificity; it can easily discriminate the complementary from single-base-mismatch target DNA. In the presence of oligonucleotide containing a single-base mismatch, significantly increased voltammetric signal can be observed (see Figure 5b and c), which indicates that the complete hybridization is not accomplished due to the base mismatch. In addition, as expected, no significant difference of peak current can be observed for the LNA-modified ssDNA GCE and its hybridization with noncomplementary sequence (see Figure 5d and e), since no successful hybridization occurs due to the sequence mismatch between the LNA-modified ssDNA and the noncomplementary sequence.

The sensitivity of this electrochemical biosensor for the target DNA was investigated by varying the target oligonucleotide concentration according to the procedure described in the

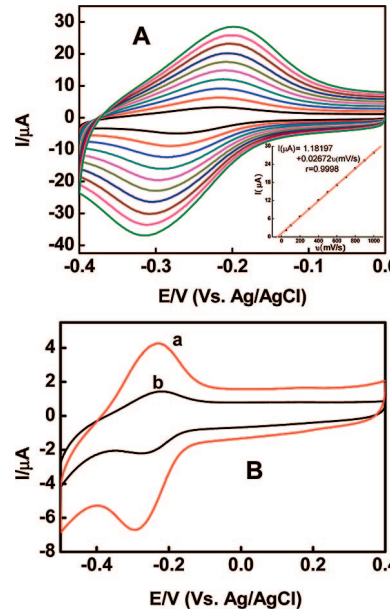

Figure 4. (A) Cyclic votammograms after incubation of 2.0 μM MB in 20 mM Tris-HCl buffer (pH 7.0) at the LNA probe-modified GCE with increasing scan rate from inner to outer: 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 mV s^{-1}. Inset shows plot of reduction peak current vs scan rate. (B) Cyclic voltammograms after incubation of 20 μM MB in 20 mM Tris-HCl buffer (pH 7.0) at the probe-modified GCE before (a) and after (b) hybridization with complementary target.

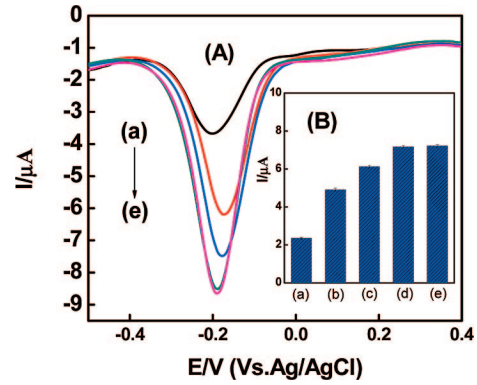

Figure 5. (A) Differential pulse voltammetry of 20 μM MB at a 4-ABSA/GCE (a) and as the redox indicator for (e) an LNA probe-modified GCE electrode and after hybridization with the (d) noncomplementary sequence, (c) one-base-mismatch sequence, and (b) complementary target sequence. (B) shows the bar graph of the peak current of MB when the LNA probe hybridized with different gene fragments. Error bars, ± relative standard deviation of three independent experiments. For DPV, the initial potential was 0.4 V; the final potential was −0.5 V; the amplitude was 0.05 V; the pulse width was 0.05 s; the pulse cycle was 0.2 s; the sample interval was 0.0167 V; and the standing time was 2 s. Buffer solution: 20 mM Tris-HCl buffer (pH 7.0) containing 20 mM NaCl.

Experimental Section. 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 −0.21V decreased in proportion to the amount of the target sequence used. Electrochemical responses of the complementary target with different concentrations also can be quantitatively analyzed. Under the constant MB, the response of DNA hybridization between the LNA probe and increasing

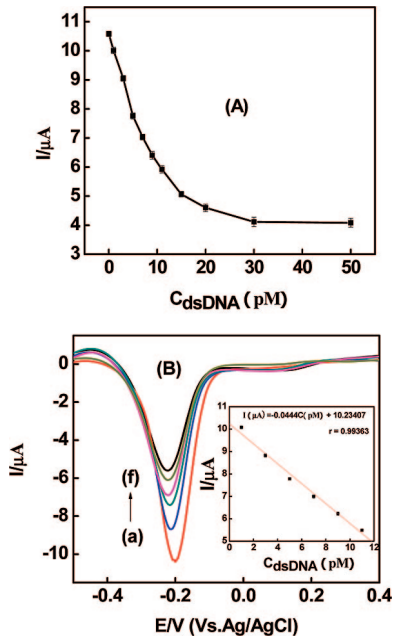

Figure 6. (A) Response of the capture probe with increasing concentration of target oligonucleotides. (B) Differential pulse voltammograms of MB accumulated on the LNA after its hybridization with different concentrations of the target sequence. Target concentration ($\times 10^{-12}$ M): (a) 1.0; (b) 3.0; (c) 5.0; (d) 7.0; (e) 9.0; (f) 11.0. Inset shows the plot of the peak current of MB as a function of the target concentration. Error bars, relative standard deviation of three independent experiments. Other experimental conditions are as in Figure 5.

concentration levels of complementary target was displayed in Figure 6A. The response for the reduction of MB after hybridization with the target DNA decreased with target concentration up to 50 pM and then tended to remain constant, which indicated that all the immobilized probes on the electrode were involved in hybridization. The formation duplex caused the decrease of the MB signal, and the interaction of guanine base with MB was prevented.

The average current response shows excellent correlation with the amount of the complementary oligonucleotides in the range of 1.0×10^{-12}—1.1×10^{-11} M (see Figure 6B). The regression equation is

$$I(\mu\text{A}) = -0.0444C_{\text{dsDNA}}(\text{pM}) + 10.234 \quad R = 0.9936$$

A detection limit of 9.4×10^{-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 7.0×10^{-12} M target DNA is 7.26% ($n = 8$).

Detection of Electrophoresis and Electrochemistry of PCR Products. Figure 7A shows the result of electrophoresis of PCR products. The PCR amplification products from a positive real sample and K562 cell (see Figure 7A, lane 4 and lane 5) show the light brands in the 1.5% agarose gel; however, the PCR amplification products from negative real sample (see Figure 7A, lane 3) do not present any brands in the gel. The light brand in lane 2 represented the PCR products amplified by β-actin primers. The purpose of addition of β-actin primers is to certify the quality of the cDNA template. It can be known that the quality of the cDNA template obtained from Fujian Institute of Hematology is

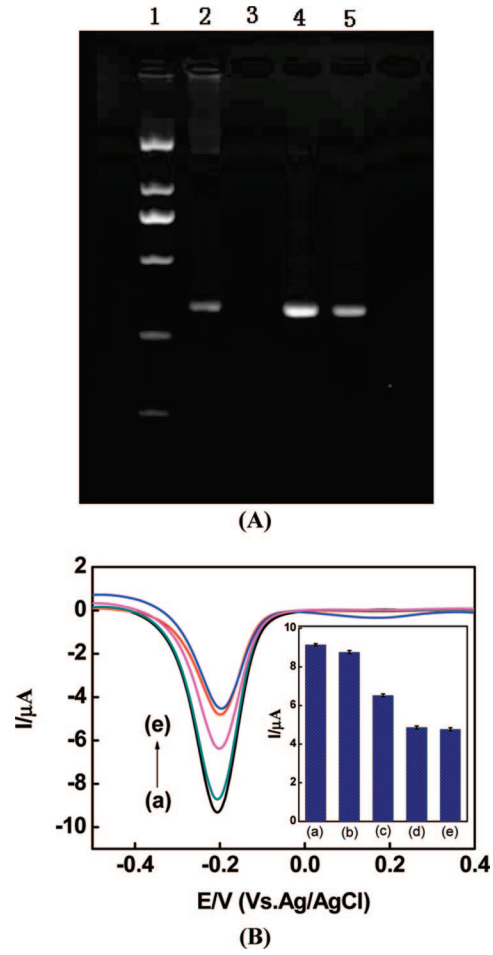

Figure 7. (A) Electrophoresis of PCR products. The lanes from left to right: (1) DL2000 DNA marker (the brands from up to down: 2000, 1000, 750, 500, 250, 100 bp); (2) β-actin amplification products; (3) negative real sample; (4) positive real sample; (5) K562 cell. (B) DPV responses using the reduction signal of MB for LNA-based probe modified GCE (a); LNA-based probe modified GCE after hybridization with negative real sample PCR products (b); LNA-based probe-modified GCE after hybridization with PCR blank buffer (c); LNA-based probe-modified GCE after hybridization with positive real sample PCR products (d); LNA-based probe-modified GCE after hybridization with K562cell PCR products (e). Inset B shows the bar graph of the peak current of MB when the LNA probe hybridized with different PCR products. Error bars, ± relative standard deviation of three independent experiments. Other conditions were the same as in Figure 5.

excellent. As we all know, the K562 cell is the positive cell strain of leukecythemia, which contains t(9;22)(q34;q11.2), and the PCR amplification band from the positive real sample consistent with the band from the K562 cell. Thus, we can extrapolate that the primers have amplified specific band to discriminate the positive real sample from the negative real sample well.

Figure 7B shows the DPV signals of MB obtained when the LNA probe for BCR/ABL was immobilized and hybridized with real samples in the hybridization step. The DPV signals of MB obtained from the probe-modified GCE gave a mean average of 9.14 μA with a RSD of 6.29%. The DPV signals of MB obtained from the hybridization of the probe with the positive and negative real samples gave a mean average of 4.86 μA with a RSD of 7.91% and 8.76 μA with a RSD of 8.45%, respectively. The signal of MB for the BCR/ABL probe-modified GCE was much higher than that

of the BCR/ABL probe-modified GCE after hybridization with a positive real sample. The obvious decreases in the magnitude of the MB signals were obtained with positive real samples. The decrease of DPV signals showed that the hybridization at the GCE surface occurred and MB could not interact with the bound guanine base of the hybrid. The decrease in the reduction signal of MB was attributed to the steric inhibition of the reducible groups of MB because of the formation of hybrid at the GCE surface. If the blood sample was negative, the negative real sample would not contain a target sequence complementary to the specific BCR/ABL probe. The hybridization between these negative real samples and the immobilized probe would not occur, so the signal of MB was nearly as high as the signal of probe (see Figure 7B). The results showed that the electrochemical DNA biosensor were in good agreement with those obtained from the gel electrophoresis.

CONCLUSIONS

An electrochemical DNA biosensor based on the covalent bonding of LNA-modified capture probe on the 4-ABSA-modified GCE was fabricated. The covalent immobilized capture probe could selectively hybridize with its target DNA to form dsDNA on the GCE surface. Since the PCl_5 approach produces a more uniform DNA immobilization with higher DNA surface density, covalent bonding could be the method for developing DNA biosensors with high sensitivity and better hybridization efficiency. The LNA-modified probe GCE was shown to be an effective biosensor for the detection of hybridization by using MB as the electrochemical indicator. This new method demonstrates its excellent specificity for single-base mismatch and complementary dsDNA after hybridization, and this probe has been used for assay of PCR real sample with satisfactory results. While the concept in this study has been demonstrated in connection with PCR real samples for the detection of CML, further improvements may be achieved to detect quantitative target sequences from real samples directly and solve the actual problem of the early diagnosis and prognosis moniter of CML and other diseases.

SUPPORTING INFORMATION AVAILABLE

Details of the apparatus, chemicals, and some experiments.This material is available free of charge via the Internet at http://pubs.acs.org.

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