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Design of a sandwich-mode amperometric biosensor for detection of PML/RARα fusion gene using locked nucleic acids on gold electrode

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ABSTRACT

In this study, a novel DNA electrochemical probe (locked nucleic acid, LNA) was designed and involved in constructing an electrochemical DNA biosensor for detection of promyelocytic leukemia/retinoic acid receptor alpha (PML/RARα) fusion gene in acute promyelocytic leukemia for the first time. This biosensor was based on a 'sandwich' sensing mode, which involved a pair of LNA probes (capture probe immobilized at electrode surface and biotinyl reporter probe as an affinity tag for streptavidin–horseradish peroxidase (streptavidin–HRP). Since biotin can be connected with streptavidin–HRP, this biosensor offered an enzymatically amplified electrochemical current signal for the detection of target DNA. In the simple hybridization system, DNA fragment with its complementary DNA fragment was evidenced by amperometric detection, with a detection limit of 74 fM and a linear response range of 0.1–10 pM for synthetic PML/RARα fusion gene in acute promyelocytic leukemia (APL). Otherwise, the biosensor showed an excellent specificity to distinguish the complementary sequence and different mismatch sequences. The new pattern also exhibited high sensitivity and selectivity in mixed hybridization system.

1. Introduction

Acute promyelocytic leukemia (APL) which is a subtype of acute myeloid leukemia (AML) comprising 5–15% of all AMLs is characterized by selective expansion of immature myeloid precursors that are blocked at the promyelocytic stage (Franco et al., 2002; Raymond et al., 1993).

In APL, the reciprocal translocation *t*(15;17)(*q*22;21), which involves the retinoic acid receptor alpha (RARα) and promyelocytic leukemia (PML) genes, results in the expression of the promyelocytic leukemia/retinoic acid receptor alpha (PML/RARα) fusion protein (Borrow et al., 1990; Rowley et al., 1977). This single gene rearrangement plays an important role in leukemogenesis through antagonizing retinoic acid signalling and the regulatory pathways mediated by APL (Andres et al., 2003). Thus, the availability of genotype-specific therapy for PML/RARα acute promyelocytic leukemia requires a precise diagnosis of the disease.

Recently, the monitoring methods of clinical diagnosis and prognosis about fusion gene of APL have included chromosome analysis (Soo et al., 2006), flow cytometry (FCM) (Tirado et al., 2003), fluorescence in situ hybridization (FISH) (Amare et al., 2001), real-time quantitative reverse transcription PCR (RT-PCR) (Han et al., 2007). But there are some limitations in these methods, such as time-consuming, poor precision and expensiveness. Thus, it is necessary to develop a new rapid and effective method to detect PML/RARα.

In these years, electrochemical DNA biosensors are widely recognized to be a promising detection mode for the rapid and inexpensive diagnosis of genetic diseases and other biological analysis due to their prominent advantages such as simple, portable, rapid, precise, sensitive and inexpensive (Bagni et al., 2006; Lee et al., 2008). The typical electrochemical DNA biosensor is composed of a solid electrode immobilized with short single-stranded DNA probes and redox-active hybridization indicators. The interaction between DNA probes with complementary sequences assays, which has a great influence on the performance of electrochemical DNA biosensors, depends primarily on the selection of the probes and the conditions of the hybridization. Thus, how to design the DNA electrochemical probe is the key point for the construction of the electrochemical DNA biosensor. Most of typical short DNA electrochemical probes show poor stability, specificity and sensitivity.

As a novel oligonucleotide derivative, locked nucleic acid (LNA) described in 1998 (Koshkin et al., 1998a,b) has attracted people's

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widespread attention and been applied to the study of gene in the past few years. LNA nucleotides contain a methylene bridge between the 2′-oxygen and the 4′-carbon of the ribose moiety. This bridge can effectively lock the furanose ring in the C′3-endo conformation, reducing the conformational flexibility of the ribose and increasing the local organization of the phosphate backbone (Koshkin et al., 1998a,b; Wang et al., 1999). This results in a very high affinity of LNA oligomers for DNA and RNA complementary sequences, with each LNA substitution increasing the melting temperatures (Tm) by as much as 3.0–9.6 °C (Bondensgaard et al., 2000; Wengel, 1999; Yang et al., 2007). In addition, an oligonucleotide with such a modification is more resistant to nuclease digestion (Vester et al., 2006; Wahlestedt et al., 2000). Furthermore, LNAs have shown the peculiar properties, such as low toxicity (Wahlestedt et al., 2000), enhanced triplex formation (Obika et al., 2000, 2001; Torigoe et al., 2001), synthesis by standard methods (Koshkin et al., 1998a) and availability from a commercial supplier. Due to these advantages, our laboratory has reported some researches demonstrating LNAs have great potential to improve capability of nucleic acid recognition through the typical intercalator-based DNA sensors mode (Chen et al., 2008; Lin et al., 2009, 2010).

However, high background signals caused by nonspecific binding of intercalators to unhybridized ssDNA and lower sensitivity restricted the detection range and specificity of the typical intercalator-based DNA sensors (Millan and Mikkelsen, 1993; Millan et al., 1994; Wang et al., 2008). In order to solve the above-mentioned problems, electrochemistry combining with enzyme-labeled bioelectrical catalytic reaction was applied in our study and the typical 'sandwich' electrochemical DNA sensing mode was constructed (Zhang et al., 2006). Through dual hybridization, the specificity of this biosensor and the signal to noise ratio can be significantly improved.

In the study, a novel DNA electrochemical probe (locked nucleic acid, LNA) was designed and involved in building a sandwich-type electrochemical DNA biosensor for detection of PML/RARα fusion gene in APL. The sensing mode was shown in Scheme 1. Through Au–S bonding, the capture probe was assembled on the surface of gold electrodes. In the present of target DNA, the capture probe and the reporter probe franked the target sequence to form the 'sandwich' sensing mode. Streptavidin–HRP connected with biotin labeled at the end of reporter probe via streptavidin–biotin affinity binding would offer an enzymatically amplified electrochemical current signal for detection of target DNA. Under the optimal condition of the hybridization, the biosensor worked both in simple and mixed hybridization systems and showed excellent selectivity and specificity for the discrimination between fully complementary and mismatch sequences. The result of our study laid a good basis of further detection research in practical samples.

2. Experimental

2.1. Self-assembled monolayers (SAMs) at gold electrode surfaces

Gold electrodes (AuEs) were cleaned following the reported protocol (Zhang et al., 2007). Gold electrodes (2 mm diameter) were first polished using 1.0 μm, 0.3 μm and 0.05 μm Al_2O_3 suspension followed by ultrasonic cleaning in ethanol and Milli-Q water. Then electrodes were electrochemically treated in fresh 0.5 M H_2SO_4 solution (Fan et al., 2003). Finally, the electrochemically cleaned gold electrodes were washed with a great amount of Milli-Q water and dried under a nitrogen stream. Then, electrodes were immediately used for DNA immobilization. The cleaned electrodes were incubated in the immobilization buffer which contained 1 μM capture probes modified with thiolate for 2 h at room temperature. After that, the SH–DNA modified electrodes were further treated with 1 mM mercaptohexanol (MCH) for 1 h to obtain well-aligned DNA monolayers. Prior to the hybridization reaction, the electrodes were slightly rinsed with a solution of 50 mM NaOH in 0.1 M phosphate buffer to be activated (Zhang et al., 2008).

2.2. Hybridization with target DNA

The synthetic targets were first mixed with the biotinylated reporter probe (100 nM) in the hybridization buffer and heated to 90 °C for 10 min and then cooled in the ice bath before use. The sensor surface modified capture probes was incubated in the hybridization buffer which contained the biotinylated reporter probes and target DNA at 63 °C for 1 h. The sensor was rinsed with the washing buffer and then incubated with 3 μL of streptavidin–HRP (0.5 U/ml) for 15 min at room temperature. After removing any remaining HRP adsorped nonspecifically, the 'sandwich' structure was formed and subjected to electrochemical measurement.

2.3. Electrochemical measurement

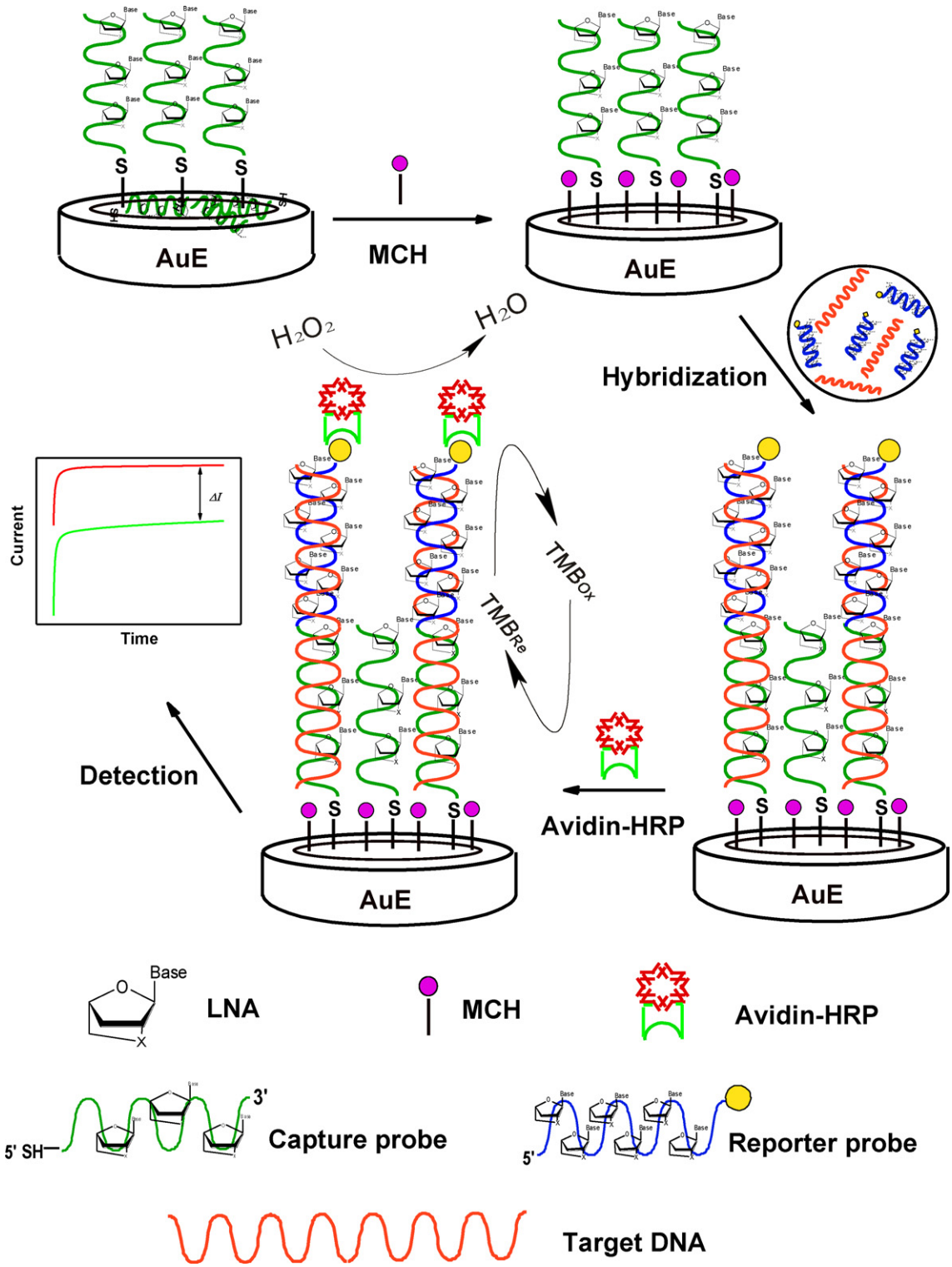
The electrochemical signal of the enzymatically produced 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was measured by cyclic voltammetry (CV) and amperometric *i–t* curve detection. CV was carried out at a scan rate of 100 mV/s. Amperometric detection was performed with a fixed potential of 100 mV and the steady state was usually reached and recorded within 100 s.

3. Results and discussion

3.1. Electrochemical studies of sandwich-mode electrochemical LNA biosensor preparing process

In order to characterize the preparing process of the electrochemical biosensor, different stages of sandwich-mode electrochemical LNA biosensor preparation were tested by cyclic voltammetry in 10 mM $K_3Fe(CN)_6$ solution. From CVs shown in Fig. 1(A), it was clearly observed that the reversible redox peaks of $[Fe(CN)_6]^{3-}$ had a highest peak current on the bare AuE surface (curve (a)), and self-assembly of the thiolated single-stranded LNA (ssLNA) capture probe monolayer on the surface of AuE induced that the decrease of the reversible peaks significantly (curve (c)). The results indicated that a negative-charge interface was formed at the surface of AuE, and the electrostatic repulsion between $[Fe(CN)_6]^{3-}$ anion and the negatively charged DNA backbones considerably retarded the electron transfer kinetics at the electrode interface. When incubating the ssLNA capture probe modified AuE in MCH solution, the insulating property decreased (curve (b)) since the short alkyl backbone molecule MCH formed a self-assembled monolayer that contained many pinholes and the redox was sufficiently small to freely penetrate through these pinholes (Porter et al., 1987). After exposing ssLNA capture probe/MCH/AuE in the hybridization buffer containing the target sequences and biotinylated reporter probes, the 'sandwich' sensing mode was formed and the number of negative charges increased accordingly. The decrease of current in Fig. 1(A), curve (d) meant that electron transfer on the electrode surface was blocked further. After the modification of streptavidin–HRP to capture probe/target/reporter probe complex at the electrode surface, the peak of the current curve almost completely disappeared (Fig. 1(A), curve (e)). The associated bulky streptavidin–HRP molecules provided an effective barrier to electron transfer of the redox species in solution.

The performance of the sandwich-mode electrochemical LNA biosensor was further evaluated by electrochemical impedance spectroscopy (EIS) in a solution of 0.1 M KCl containing 10 mM



Scheme 1. The preparing process for the fabrication of sandwich-mode electrochemical LNA biosensor.

$[Fe(CN)_6]^{3-/4-}$ at a potential of 0.08 V (versus Ag/AgCl) in the frequency range of 0.1–10^5 Hz. Fig. 1(B) showed the nyquist plots of impedance spectra at different stages of the electrochemical biosensor preparation. The bare AuE exhibited the smallest semicircle at high frequency region (curve (a)) implying a fast electron-transfer process which was diffusion-controlled. When AuE was modified with thiolated LNA capture probe with negative charges on its phosphate backbone, the Ret increased to 11,800 Ω (curve (c)). This should be attributed to the fact that self-assembled negatively charged capture probe monolayer developed an electrostatic repulsive force to $[Fe(CN)_6]^{3-/4-}$. After MCH was employed to remove the nonspecifically adsorbed probe, the Ret further decreased to 10,250 Ω (curve (b)). This was mainly because that the less densely packed monolayer of MCH resulted in a relatively slow electron-transfer. When the hybridization was performed, LNA/DNA duplex was modified on the surface of AuE. At this time,

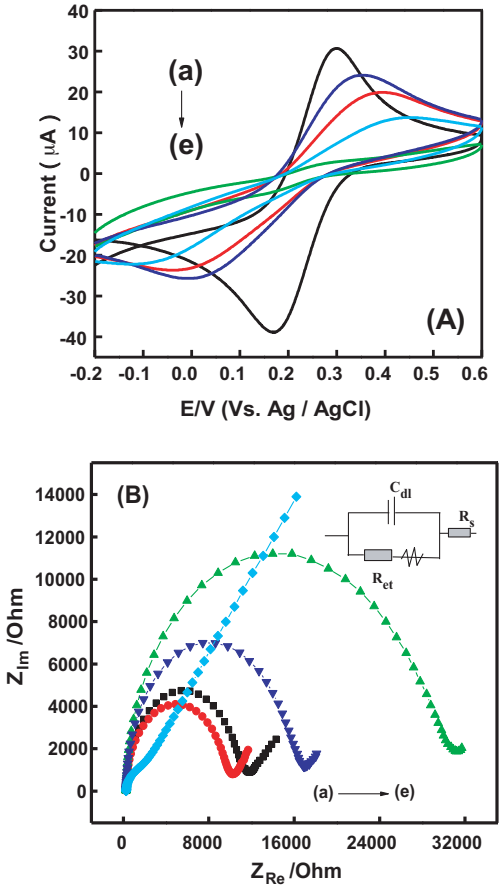


Fig. 1. (A) Cyclic voltammograms of 10 mM $K_3Fe(CN)_6$ in 0.1 M KCl solution at scan rate of 100 mV/s for a bare AuE (a), LNA capture probe/MCH/AuE (b), LNA capture probe/AuE (c), LNA capture probe/target DNA/LNA reporter probe/AuE (d) and LNA capture probe/target DNA/LNA reporter probe/avidin–HRP/AuE (e) and (B) impedance spectra (Nyquist plot) of bare AuE (a), LNA capture probe/MCH/AuE (b), LNA capture probe/AuE (c), LNA capture probe/target DNA/LNA reporter probe/AuE (d) and LNA capture probe/target DNA/LNA reporter probe/avidin–HRP/AuE (e) in the presence of 10 mM $[Fe(CN)6]^{4-/3-}$. The biased potential was 0.08 V (versus Ag/AgCl) in the frequency range of 0.1–10^5 Hz and the amplitude was 5.0 mV.

the monolayer of DNA on the electrode surface became denser, and the negative charges on the electrode surface increased. Thus the electron-transfer resistance was enhanced further, the value of Ret increased to 16,940 Ω (curve (d)). After the affinity conjugation of streptavidin–HRP on ssLNA capture probe/MCH/target DNA/ssLNA reporter probe/AuE, the electron transfer was blocked by the bulky protein molecule. So there was an increase of Ret (31,110 Ω, curve (e)). These results were in consistent with the cyclic voltammogram shown in Fig. 1(A).

3.2. Electrochemical responses of the enzyme-based LNA-modified biosensor

The catalytic nature of HRP was investigated by CV. Fig. 2(A) showed there were two pairs of redox peaks at the surface of bare electrode exposed in TMB substrate without HRP (curve (a)). After adding HRP into TMB, the prominent catalytic reduction peak was found in CV of bare electrode (Fig. 2(A), curve (b)). This reflected that along with the catalysis of HRP, TMB was oxidized into a colored compound by H_2O_2, and thousands of reduction reactions of H_2O_2 could be efficiently catalyzed, leading to significantly amplified electrochemical current signals (Volpe et al., 1998). The different responses of the capture probe (ss-LNA) or LNA capture probe/target DNA/LNA reporter probe modified biosensors were also studied under the same measurement condition. The streptavidin–HRP conjugate was used as the model protein since its adsorption could be electrochemically monitored. As shown in Fig. 2(B), we could find the current signals of LNA capture probe/target DNA/LNA reporter probe modified biosensor (Fig. 2(B), curve (b)) were more significant than that of ssLNA capture probe modified biosensor (Fig. 2(B), curve (a)). The reason was that upon hybridization, streptavidin–HRP could be modified on the surface of AuE and efficiently catalyze reduction reactions of H_2O_2 with the redox reaction of TMB, leading to significant amplify of current signal (Das and Hecht, 2007; Fanjul-Bolado et al., 2004). However, ssLNA capture probe modified biosensor did not contain the biotinylated reporter probe and target DNA, HRP could not be fixed onto the surface of electrode firmly. After washing nonspecific absorption, there were few HRP on the electrode. So the catalytic reduction reaction was not obvious, leading to weak reduction current signal. Accordingly, the sandwich-mode biosensor can discriminate ss-LNA and LNA/DNA complex very well.

3.3. Study on specificity and sensitivity of sandwich-mode electrochemical LNA biosensor in simple hybridization system

Detection of target DNA in simple hybridization system was investigated by amperometric technique, which provided a rapid and direct measure of the current associated with the HRP catalyzed electrochemical process. As the redox site of HRP was shielded within insulating peptide backbones, HRP did not directly exchange electrons with the electrode (Liu et al., 2008). So as an electron shuttle, TMB, which was a small redox molecule and can diffuse in and out of the redox site of the macromolecule, was employed. And the catalytic reduction of H_2O_2 coupling with the redox reaction of TMB was carried out on the electrode surface (Das and Hecht, 2007; Fanjul-Bolado et al., 2004). The potential was held at the catalytic reduction potential for H_2O_2 (100 mV). A decay curve for current (I) vs time (t) was observed instantly after the onset of the potential, which rapidly became steady-state current within 100 s (Fig. 3). The specificity of this assay was studied by using the LNA probes as the probe to hybridize with various DNA sequences (complementary S_5, one-base mismatch S_6, three-bases mismatch S_7, five-bases mismatch S_8, ten-bases mismatch S_9, PML partial sequence S_{10} and RARα partial sequence S_{11}). For comparison, we also tested the same sensing mode immobilizing a DNA sequence without modifying locked nucleic acids as probe (DNA probe) on the surface of AuE under the same condition. Fig. 3(A) presented a large amperometric signal (1786 nA, curve (a)) obtained after hybridization with complementary DNA, which was more than an order of magnitude compared with background amperometric current (125 nA, curve (h)). It indicated that LNA probes showed the excellent affinity for complementary target. The amperometric signal obtained after hybridization with oligonucleotide containing a single-base mismatch decreased significantly (1042 nA, curve (b)), which indicated that the complete hybridization was not accomplished due to the base mismatch. In addition, as expected, with the increasing of mismatch base number, the values of amperometric signal became smaller. When the number of mismatch base was greater than or equal to 5, there were no significant differences of currents for capture probe and the hybridization with mismatch sequences of LNA probes (see Fig. 3(A) curve (h) versus curve (d), curve (e), curve (f), curve (g)), since no successful hybridization occurred due to the mismatch bases between the capture probe and these mismatch sequences. Comparison between Fig. 3(A) and (B), we can find the main distinguish was that the differential value of current signals obtained for LNA probes hybridizing, respectively, with complementary sequence and single-base mismatch sequence was 2 times larger than that obtained for DNA probes. This result indicated that LNA probe exhibited an enhanced discriminatory power

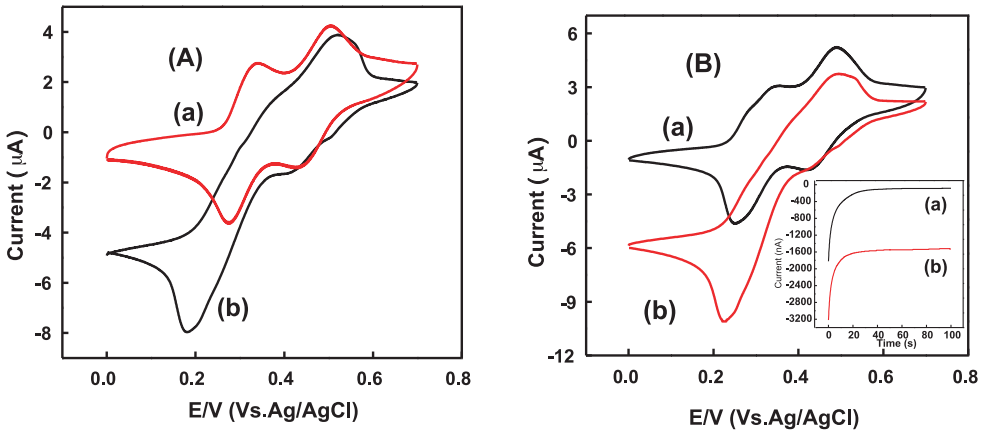


Fig. 2. (A) Cyclic votammograms of bare AuE in 1 mL TMB substrate (a) and in 1 mL TMB substrate containing 3 μL avidin–HRP (b) and (B) cyclic votammograms of ssLNA modified electrode (a) and LNA–DNA modified electrode after dripping 3 μL avidin–HRP in 1 mL TMB substrate (b).

for the complementary and single-base mismatch target sequence, outperforming the DNA probe.

Electrochemical responses of the complementary target with different concentration in simple hybridization system were also quantitatively analyzed. The sensor was challenged with the target DNA of a series of concentration across the range of 100 fM to 10 nM, spanning a response region of 6 orders of magnitude. The amperometric signal was found to be a non-linear logarithmic function related to the target concentration (Fig. 3(C)), and the detection limit was 74 fM with a linear response range of 0.1–10 pM for synthetic PML/RARα fusion gene in APL. The detection limit of 74 fM for the target DNA can be estimated using 3σ (where σ is the standard deviation of the blank solution, $n = 3$). The reproducibility of the biosensor for detection of 5.0 pM target DNA is 9.50% ($n = 3$) (Fig. S2). It was worthwhile to point out that this novel biosensor achieved even higher gain than the previously reported intercalator-based

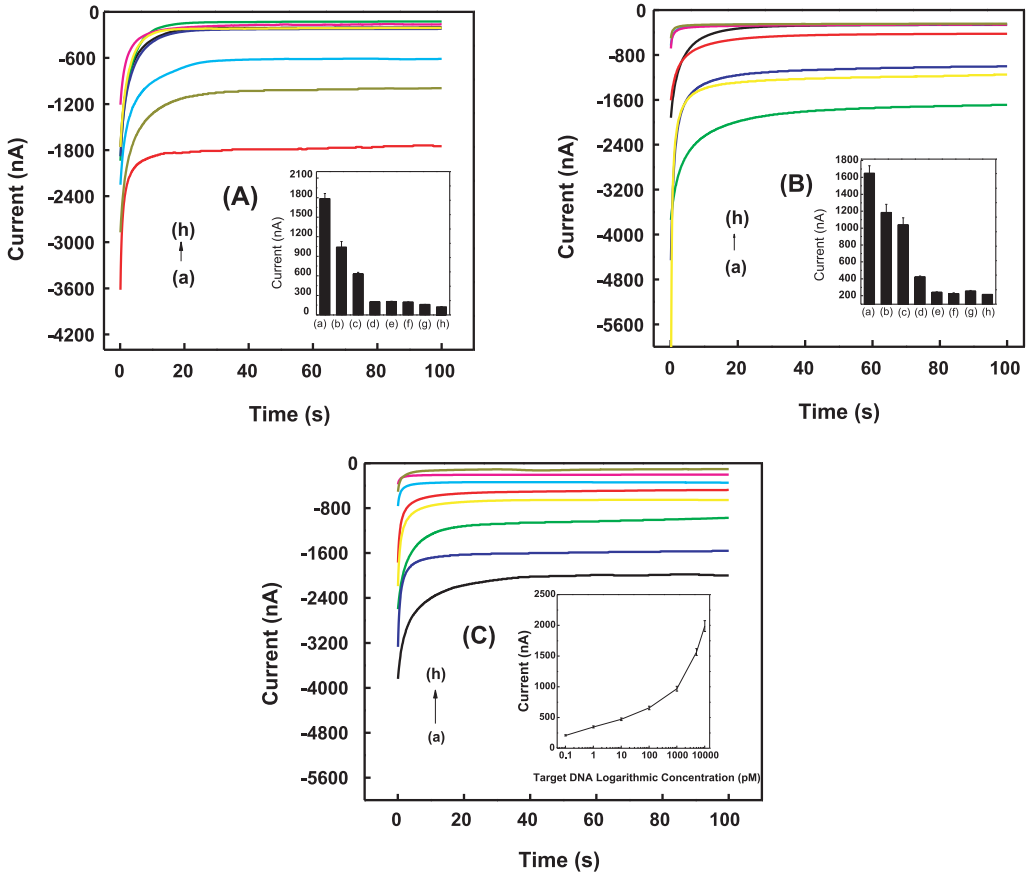


Fig. 3. Current–time curves in respective different hybridizations with LNA probe (A) and DNA probe (B): after hybridization with S_5 (a); S_6 (b); S_7 (c); S_8 (d); S_9 (e); S_{10} (f); S_{11} (g) and the capture probe sequence (h). Inserts show the bar graphs of the peak currents when the biosensor hybridized with different gene fragments. (C) Amperometric measurements for sandwich-mode electrochemical LNA biosensor hybridized with synthetic target DNA at a series of concentrations (from (a) to (h): 10 nM, 5 nM, 1 nM, 100 pM, 10 pM, 1 pM, 0.1 pM and 0 pM). Insert shows logarithmic plot of currents versus target DNA concentration. Error bars show the standard deviations of measurements taken from at least three independent experiments. The base solution for hybridization measurement was TMB substrate.

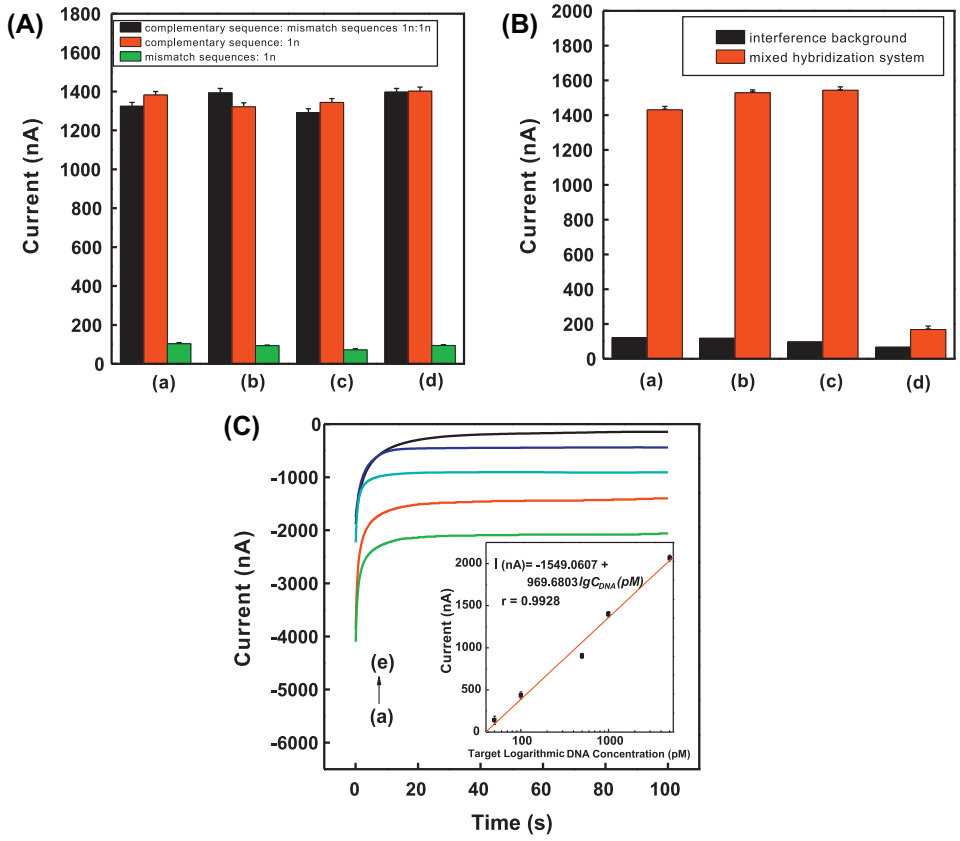


Fig. 4. (A) The effect of increasing mismatch types on specificity of sandwich-mode electrochemical LNA biosensor. Different interference background: S_8 (a), $S_8 + S_9$ (b), $S_8 + S_9 + S_{10}$ (c), $S_8 + S_9 + S_{10} + S_{11}$ (d). Different color barcharts show mixed hybridization solution containing 1 nM S_5 and 1 nM mismatch interference background (black), hybridization solution containing 1 nM S_5 (red), hybridization solution containing 1 nM mismatch interference background (green), (B) the effect of interference background concentration on specificity of sandwich-mode electrochemical LNA biosensor. Different color barcharts showed interference background (black), mixed hybridization solution containing 1 nM S_5 (red). Different interference background concentrations (from (a) to (d)) were 0.4 nM, 4 nM, 40 nM and 400 nM, respectively, and (C) current–time curves of hybridization with different concentrations of the target sequence in mixed hybridization solution containing four kinds of mismatch sequences in 40 nM as interference background. Target concentration (from (a) to (e): 5 nM, 1 nM, 500 pM, 100 pM, and 50 pM)). Insert shows the logarithmic plot of currents versus target DNA concentration (from 0.05 nM to 5 nM). Error bars show the standard deviations of measurements taken from at least three independent experiments. The base solution for hybridization measurement was TMB substrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

LNA-modified DNA sensors (Chen et al., 2008; Lin et al., 2009, 2010).

3.4. Study on specificity and sensitivity of sandwich-mode electrochemical LNA biosensor in mixed hybridization system

Via the homologous comparison analyzed by DNAMAN software, we could find the sequence in human genome, which had highest matching degree with 30 bp PML partial sequence in S_5, still existed ten mismatch bases. At the same time, the similarity degree of the rest 30 bp RARα partial sequence in S_5 was also analyzed, and the highest matching degree between this 30 bp sequence with human genome was up to 24 bp. When the number of mismatch base was greater than or equal to 5, the biosensor possessed good nucleic acid recognization. This recognization ability of the biosensor was satisfied with the requirement of real sample measurement. So we decided to design the simulation test in the mixed hybridization solution containing different kinds of mismatch sequences (S_8, S_9, S_{10} and S_{11}) as interference background.

In order to evaluate the applicability of the electrochemical DNA sensor in interference condition, we compared its performance in the mixed hybridization solutions containing equal amounts of complementary target and mismatch targets. The mismatch targets included S_8, S_9, S_{10} and S_{11}. With the experiment study in different combination of mismatch targets as interference background (in Fig. 4(A), different interference background: S_8 (a), $S_8 + S_9$ (b), $S_8 + S_9 + S_{10}$ (c), $S_8 + S_9 + S_{10} + S_{11}$ (d)), the effect of increasing mismatch types on specificity of the biosensor was investigated. From the results, we could find the biosensor still kept high selectivity to discriminate complementary sequence from the interference background, even from the mixed hybridization solution containing four different kinds of mismatch sequences as interference background.

In addition, the effect of different interference background concentrations on specificity of the biosensor was also investigated. The composition of mixed hybridization was shown in Table S2. The findings were as follows: when the range of total interference concentration was 0.4–40 nM, the values of amperometric signal from the hybridization with complementary sequences remained basically unchanged and kept a large difference-value with the current values from interference background. However, when the total concentration of interference background was up to 400 nM, the signal obtained from the hybridization with complementary sequences significantly decreased (Fig. 4(B)).

According to the above-mentioned results, the sensitivity of the DNA biosensor was further investigated under the mixed hybridization solution containing four kinds of mismatch sequences in 40 nM as interference background. Under the optimal condition of the hybridization, while detecting the complementary DNA standard concentration range of PML/RARα fusion gene from 0.05 nM to 5.0 nM, there was a good linear relationship between the current signal and the logarithmic function of complementary DNA concentration, the lower limit was 9.6 pM, which can be estimated

using 3σ (where σ is the standard deviation of the blank solution, $n = 3$). The reproducibility of the biosensor for detection of 0.5 nM target DNA is 7.91% ($n = 3$) (Fig. 4(C)). It could be seen that under a certain interference background, the electrochemical DNA biosensor still possessed good molecular recognizability and can hybrid with completely complementary sequences well. It provided a good basis of further detection research in practical samples.

4. Conclusion

In summary, a novel DNA electrochemical probe (locked nucleic acid, LNA) was designed and involved in building an electrochemical DNA biosensor for detection of PML/RARα fusion gene in APL in this study. Under the optimal condition of the hybridization, the biosensor exhibited excellent selectivity and specificity both in simple and mixed hybridization systems. While it has been demonstrated that the concept in this research is in connection with simulation study of interference hybridization system for detection of APL, further improvements will be achieved to detect quantitative target sequences from real samples directly and solve the actual problem of the early diagnosis and prognosis monitoring of APL and other diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.11.030.

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