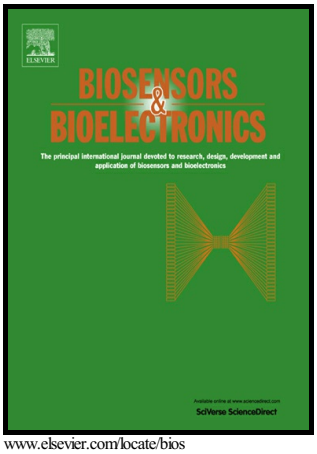

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2'-Fluoro ribonucleic acid modified DNA dual-probe sensing strategy for enzyme-amplified electrochemical detection of double-strand DNA of PML/RARα related fusion gene

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Abstract:

In the study, a novel sensing strategy based on dual-probe mode, which involved two groups of 2'-fluoro ribonucleic acid (2'-F RNA) modified probes, was designed for the detection of synthetic target double-strand DNA (dsDNA) of PML/RARα fusion genes in APL. And each pair of probes contained a thiolated capture probe (C1 or C2) immobilized on one of electrode surfaces in the dual-channel electrochemical biosensor and a biotinylated reporter probe (R1 or R2). The two groups of 2'-F RNA modified probes were separately complementary with the corresponding strand (Sa or Sb) from target dsDNA in order to prevent renaturation of target dsDNA. Through flanking target dsDNA, two "sandwitch" complexes (C1/Sa/R1 and C2/Sb/R2) were separately shaped by capture probes (C1 and C2) and free reporter probes (R1 and R2) in hybridization solution on the surfaces of different electrodes after the thermal denaturation. The biotin-modified enzyme which produced the measurable electrochemical current signal was localized to the surface by affinity binding between biotin with streptavidin. Under the optimal condition, the biosensor was able to detect 84 fM target dsDNA and showed a good specificity in PBS hybridization solution. Otherwise, the investigations of the specificity and sensitivity of the biosensor were carried out further in the mixed hybridization solution containing different kinds of mismatch sequences as interference background. It can be seen that under a certain interference background, the method still exhibited excellent selectivity and specificity for the discrimination between the fully-complementary and the mismatch sequences. The results of our research laid a good basis of further detection research in practical samples.

Key words: 2'-Fluoro ribonucleic acid; Dual-probe sensing strategy; Enzyme-amplified electrochemical detection; Double-strand DNA; PML/RARα

1. Introduction

The acute promyelocytic leukemia (APL), as a cancer of the white blood cells, is a subtype of acute myelogenous leukemias (AML) (Mandelli et al., 2002; Warrell et al., 1993). In APL, over 95 % of the cases are characterized by a translocation t(15:17), which fuses the retinoic acid receptor alpha (RARα) gene with the promyelocytic leukemia (PML) gene, resulting 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 essential role in leukemogenesis through antagonizing retinoic acid signaling and the regulatory pathways mediated by APL (Laurenzana et al., 2006). Thus, the availability of genotype-specific therapy for PML/RARα acute promyelocytic leukemia requires a precise diagnosis of the disease. Current methods of clinical diagnosis about this translocation include flow cytometry (FCM) (Kern et al., 2010), fluorescence in situ hybridization (FISH) (Choughule et al., 2009), chromosome analysis (Tirado et al., 2003) and real-time quantitative reverse transcription PCR techniques (RT-PCR) (Koshy et al., 2012), however, time-consuming purification of nucleic acid, costly equipment and reagents and tedious procedure prevent their utilization in detection of the specific marker gene associated with APL. Hence, it's urgently needed to develop a fast, effective, and convenient strategy with high selectivity and sensitivity to detect PML/RARα fusion gene of APL.

During the past decade, a variety of studies have demonstrated the DNA analysis using fluorescence (Ye et al., 2016; Zhu et al., 2017), surface plasmon resonance (Ghrera et al., 2016), electrochemiluminescence (Feng et al., 2017), quartz-crystal microbalance (Prakrankamanant et al., 2013; Wang et al., 2013), and electrochemical biosensors (Chao et al., 2016; Huang et al., 2017; Li et al., 2016). Among these,

electrochemical biosensors have been attracting a great deal of attention due to their cost effectiveness, fast response time, simplicity of the process and viability of equipment miniaturization. The typical electrochemical DNA biosensor (E-DNA biosensor) contains a solid electrode immobilized with single kind of DNA probe. The reorganization interaction between DNA probes with target sequences, which has a great influence on the performance of E-DNA biosensors, depends primarily on the design of DNA probes and the optimization of hybridization conditions. The specificity and stability of the binding between target sequences and support-bound probe molecules preserve an important role to improve the fidelity and reproducibility of E-DNA biosensors. Thus, how to design the DNA probe is the key point for the construction of the electrochemical DNA biosensor. Our research group has reported several E-DNA biosensors for the detection of the synthetic single-stranded (ss-) PML/RARα fusion gene using different specific ssDNA probes (e.g., locked nucleic acid, LNA) with satisfactory results in recent years (Lei et al., 2013; Liu et al., 2011; Wang et al., 2011; Wang et al., 2012; Zhong et al., 2011).

Furthermore, the target DNA derived from clinical blood samples usually is native double-stranded (ds-) fragments (e.g., double-stranded DNA or DNA/RNA complexes) which can be more stable in vitro. The existing double-stranded configuration hampers the hybridization between target sequences and probes which are anchored onto the transducer surface. To address this issue, several different pretreatment methods are used to denature the dsDNA fragments (e.g., thermal denaturation, chemical reagent treatment) to obtain the ss-DNA target fragments. Nevertheless, the reannealing of two separate strands from target dsDNA would impede the identification between the DNA probes and the corresponding complementary target strand during subsequent probe-target hybridization.

Therefore, the improvement of the hybridization efficiency between probe and capturing target dsDNA without any complex pretreatment process is particularly important on constructing a novel sensing strategy in order to solving the above-mentioned problem. In our research, a novel sensing strategy based on dual-probe detection was designed. This sensing strategy was based on two kinds of DNA probes which were complementary to the two strands from target dsDNA, respectively. The two kinds of DNA probes modified onto the two different electrodes could specifically recognize the corresponding complementary sequences of target dsDNA in the same hybridizaiton system after thermal denaturation. Consequently, the biosensor based on dual-probe format can effectively reduce the influence of reannealing of the two separate strands of target dsDNA and improve the efficiency of probe-target hybridization. A dual-probe E-DNA (DE-DNA) biosensor has been reported to detect the dsDNA of PML/RARα fusion gene based on "Y" junction structure probe and restriction endonuclease assisted cyclic enzymatic amplification by our group (Wang et al., 2015).

Although the aforementioned E-DNA biosensor platforms constructed by our research group were reported to improve the specificity of the biosensor with LNA probes (Wang et al., 2011) and directly detect the target dsDNA with dual-probe strategy based on restriction endonuclease assisted cyclic enzymatic amplification (Wang et al., 2015), there still more or less existed some problems such as high cost of the LNA probe and requirement of complicated procedure which would limit the massive application in real sample.

In order to develop an economic and facile sensing strategy with the prominent specificity and sensitivity, the two kinds of oligonucleotide probes modified with 2'-fluoro ribonucleic acid (2'-F RNA) monomers were firstly introduced into the

E-DNA biosensor. 2'-F RNA monomer is a RNA mimic in which the 2' position of the pentofuranose moiety is replaced with a fluoro group. The oligonucleotides with 2'-modifications with electronegative substituents prefer RNA-like-3’-endo sugar conformations which should largely improve the oligonucleotide binding affinity (Kawasaki et al., 1993). The 2'-F-RNA-modified oligonucleotides show high enzymatic resistance both in vitro and in vivo (Ono et al., 1997; Muhonen et al., 2007) and have been used as antisense (Alahari et al., 1998) and can retain the ability to silence gene expression in human cells (Allerson et al., 2005). Certainly, the most important point for the performance of the E-DNA biosensor designed in this study is that the introduction of 2'-F RNA residues into synthetic oligonucleotides increases the thermal melting points of the duplexes by about 1 to 2 °C for each fluoro modification (Sabahi et al., 2001). According to the property about thermal stability of 2'-F RNA residues, it was indicated that 2'-fluoro backbone-modified oligonucleotides possess extraordinarily high affinities for complementary sequences and forcefully suggested that 2'-F RNA residues had great potential to improve capability of nucleic acid recognition via optimization of the hybridization temperature. So 2'-F RNA monomers were attractive candidate probes with extraordinary specificity in the application involving a sensing strategy based on dual-probe detection.

In the present work, to test the feasibility of the 2'-F RNA-modified probe as recognition element for constructing an E-DNA sensor that provides a package solution for target dsDNA detection in real sample, we utilized the “sandwich” mode E-DNA biosensor reported in our previous research (Wang et al., 2011) and designed a novel sensing strategy based on dual-probe mode, which involved two groups of 2'-F RNA-modified probes (two thiolated capture probes (C1/C2) immobilized on two

different separate Au electrodes and two biotinylated reporter probes (R1/R2)), for the detection of synthetic target dsDNA of PML/RARα fusion genes in APL. The sensing strategy is shown in Scheme 1. Through Au-S bonds, capture probes (C1 and C2), which were complementary to partial segments of the two corresponding strands in target dsDNA respectively, were assembled on the surfaces of two separate Au electrodes (channel 1 and channel 2) for the sake of detection of the two corresponding complementary strands in target dsDNA at the same time. Then, the two modified electrodes were simultaneously incubated into the hybridization system containing target dsDNA (S3) and reporter probes (R1 and R2) after the thermal denaturation. In the present of target dsDNA, capture probes and reporter probes would franked the corresponding complementary strand from target dsDNA sequence to form two independent "sandwich" complexes on different Au electrodes. Streptavidin-HRP connected with biotin labeled at the end of reporter probes via streptavidin-biotin affinity binding would offer different enzymatically amplified electrochemical current signals in the two channels of dual-probe E-DNA biosensor for detection of target dsDNA. If the hybridization system was in the absence of target dsDNA that could participate in formation of the "sandwich" complexes with capture probes and reporter probes, biotins could not be modified onto the surfaces of Au electrodes via hybridization. Less streptavidin-HRP could be fixed onto the electrode surface to catalyze TMB substrate. Therefore, the current signals collected from the two channels of the dual-probe E-DNA biosensor were very weak. The change of the current signals could be used as an important criterion for determining the existence of the target dsDNA in the solution. Through dual-probe (C1/S3a/R1 and C2/S3b/R2) hybridization strategy, the specificity of biosensor for detection of dsDNA and the signal to noise ratio of the E-DNA biosensor can be significantly improved.

Scheme 1

2. Experimental

The details of the experimental section were shown in Supporting Information.

3. Results and discussion

3.1 Feasibility of the dual-probe E-DNA biosensor

In order to insure the specificity of the designed dual detection probes, the thermodynamic parameters of all oligonucleotides used in the research were calculated using integrated DNA technologies (ttp://mfold.rit.albany.edu) (Zuker, 2003). Two groups of detection probes (C1/R1 and C2/R2) were designed based on the melting temperatures in our system according to the target dsDNA sequence (Shown in Supporting Information, Table S2). The detection probes (C1/R1 and C2/R2) were designed based on the melting temperatures in our system according to the target dsDNA sequence (Shown in Table S2). Each group contained two probes: a sulfhydryl capture probe and a biotinylated reporter probe. From Table S2, we can see that the melting temperatures for the C1-R1 (3.4 °C), C1-R2 (28 °C), C2-R1 (16.2 °C) and C2-R2 (-8.3 °C) were all so far below that for C1-S1 (84 °C), R1-S1 (81.4 °C), C2-S2 (83.8 °C) and R2-S2 (79 °C). Additionally, the melting temperatures of other double helix structures which might been formed in the same hybridization solution would also been calculated by the integrated DNA technologies (C1-S2 (25.3 °C), R1-S2 (41.2 °C), C2-S1 (28 °C) and R2-S1 (9.2 °C)), which were all far lower than that between the detection probes and the corresponding target ssDNA (C1-S1, R1-S1, C2-S2 and R2-S2). It has been demonstrated by simulation that the designed detection probes can avoid the interference among the nonspecific sequences in the same system and effectively identify the target sequence through the optimization of

hybridization condition, which is attributed to the unique probe with 2'-fluoro backbone modification. Furthermore, in order to characterize the preparation process of the electrochemical biosensor, the different stages of the dual-probe E-DNA biosensor preparation were tested by cyclic voltammetry and electrochemical impedance spectroscopy (EIS), respectively (Shown in Supporting Information, Figure S1).

Electrochemical responses of the E-DNA biosensor were also investigated in the research (Fig. 1). Fig. 1 (A) shows the cyclic voltammograms (CVs) of different modified dual-probe electrodes tested in TMB substrate by dual-channel electrochemical analyzer. Compared two curves (Fig. 1 (A), curve (a) and (b)), there was a prominent catalytic reduction peak that appeared in the CV of DNA duplexes modified electrodes, respectively (C1/S3a/R1 and C2/S3b/R2). Additionally, we can find that the current magnitudes produced by the catalysis of streptavidin-HRP were much larger (Fig. 1 (B), channel 1: curve (b), 2700 nA and channel 2: curve (b), 2580 nA) than that of currents without enzymatic catalysis (Fig. 1 (B), channel 1: curve (a), 83 nA and channel 2: curve (a), 78 nA) via comparing with i-t curves. This result indicated that upon hybridization, streptavidin-HRP could conjugate with biotinylated reporter probes via affinity bond to be modified onto the surfaces of AuEs (HRP/R1/S3a/C1/AuE and HRP/R2/S3b/C2/AuE), and the reduction of H_2O_2 with the oxidized TMB at the electrode surface could be efficiently catalyzed along with the catalysis of HRP, resulting in significantly amplified electrochemical current signals (Das and Hecht, 2007; Fanjul-Bolado et al., 2004; Liu et al., 2016; Wang et al., 2011). In the absence of target dsDNA, no “sandwich” structure can be formed, resulting in very few HRP fixed onto the surface of the electrode. And the current responses were decreased nearly to the blank background at the electrode surface. According to the results, it’s

clear that the biosensor can nicely discriminate the existence of target dsDNA in the solution under optimized conditions.

Figure 1

3.2 Study of specificity and sensitivity of 2'-F RNA modified dual-probe E-DNA biosensor for detection dsDNA in PBS hybridization system without interferences

It represented an important problem how to ensure excellent recognition ability between dual detection probes and target dsDNA for 2'-F RNA modified dual-probe E-DNA biosensor. So the specificity of the E-DNA biosensor was studied via detecting several different kinds of dsDNA sequences (shown in Table S1 for details) including the complementary target dsDNA (S3), single-base mismatch dsDNA (S4), three-base mismatch dsDNA (S5), five-base mismatch dsDNA (S6), ten-base mismatch dsDNA (S7), PML partial dsDNA (S8) and RARα partial dsDNA (S9), respectively. Amperometric technique, which provided a rapid and direct measure of the current associated with the HRP catalyzed electrochemical process, was chosen to detect these dsDNA fragments in PBS hybridization solutions without other dsDNA interference sequences in turn.

For comparison, the same sensing mode using the DNA detection probes which have the same sequences without any 2'-F RNA monomer modification (unmodified DNA detection probe) was also investigated under the same condition. Fig. 2 presented a larger sum of current signals ($\sum I$) (8190 nA) obtained after hybridization with complementary dsDNA, which was more than an order of magnitude greater as compared with $\sum I$ of background (160 nA). However, the values of $\sum I$ obtained after hybridization with oligonucleotides containing single-base mismatch or three-base mismatch sequences revealed a significant downward trend (5850 nA and 3100 nA),

which indicated that the complete hybridization was not accomplished due to the mismatch bases. In addition, comparing the results from 2'-F RNA modified detection probes and unmodified DNA detection probes, we found that the differential values of $\sum I$ obtained for 2'-F RNA modified detection probes after hybridizing respectively with complementary sequence and single-base mismatch sequence was about 2.5 times larger than that obtained for unmodified DNA detection probes (Fig. 2). So it was demonstrated that the 2'-F RNA modified detection probes showed the excellent ability of identification between complementary target dsDNA and single-base mismatch dsDNA. Meanwhile, as expected, with the increasing of mismatch base number, the values of $\sum I$ obtained became smaller. When the number of mismatch base was greater than or equal to 5, there were no significant differences of current signals for background and the hybridization with mismatch sequences of 2'-F RNA modified capture probes, since no successful hybridization occurred due to the mismatch bases between the capture probe and these mismatch sequences. This result indicated that 2'-F RNA modified detection probes exhibited an enhanced discriminatory power for the complementary and mismatch target sequence, outperforming the unmodified DNA detection probe. These results could be explained by the fact that 2'-modification with electronegative substituent fluoro confer a unique 3’-endo sugar conformation to 2'-F RNA modified detection probes, which preorganization will lead A-form probe-target duplex structures and enhanced binding affinity.

Figure 2

Electrochemical responses of the complementary target dsDNA with different concentration from dual channels were also quantitatively analyzed in PBS hybridization solution without other interferences. Fig. 3 showed i-t curves of

C1/S3a/R1 (A: channel 1) and C2/S3b/R2 (B: channel 2) in dual-probe E-DNA biosensor hybridized with target dsDNA at a series of concentrations from dual channels. The E-DNA biosensor was challenged with the target dsDNA of a series of concentrations range from 0.1 pM to 10 nM, spanning a response region of 5 orders of magnitude (Fig. 3 (C)). It was worthwhile to point out that this dual-probe detection mode used for dsDNA detection achieved even higher gain than the E-DNA biosensors based single-probe (C1/A1 or C2/A2, Fig. 3 (C)). Under the optimal condition of the hybridization, while detecting the complementary DNA standard concentration range of target dsDNA from 0.5 pM to 15 pM, there was a good linear relationship between$\sum I$ and the complementary dsDNA concentration with the detection limit of 84 fM (Fig. 3 (D)).

Figure 3

3.3 Study of specificity and sensitivity of 2'-F RNA modified dual-probe E-DNA biosensor for detection dsDNA in simulated interference system

According to the above studies and the previous researches reported (Wang et al., 2011), the developed E-DNA biosensor possessed outstanding discrimination capability when the number of mismatch bases was greater than or equal to 5. This recognization capability of the biosensor would meet the requirement of real sample measurement. So we decided to design the simulated real sample test in the mixed hybridization system containing different kinds of mismatch sequences (S_6, S_7, S_8 and S_9) as interference background. First, the effect of increasing mismatch types on the specificity of the E-DNA biosensor was investigated in different combination of mismatch targets as interference background (in Fig. S4, different interference backgrounds: S_6 (a), $S_6 + S_7$ (b), $S_6 + S_7 + S_8$ (c), $S_6 + S_7 + S_8 + S_9$ (d)).

Comparing PBS hybridization solution without interferences with the simulated interference system, we can find that the results tested in mixed interference hybridization solution were basically identical with the results obtained in PBS hybridization solution without interferences. 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. Additionally, the specificity of the E-DNA biosensor was also investigated about the effect of interference backgrounds consisting of mismatch sequences with different concentrations in the research. The composition of mixed hybridization solution was shown in Table S4. The results were as follows: when the range of total interference sequences concentration was 0.2 - 20 nM, the values of $\sum I$ from the hybridization with complementary sequences (1 nM) showed no obvious difference with increasing of interference sequence concentration and kept a big difference with the current values from interference background. However, when the total concentration of interference background was up to 200 nM, the value of $\sum I$ obtained from the hybridization with complementary sequences significantly decreased and was close to that of interference background (Fig. S4).

According to the above-mentioned results, the sensitivity of the DNA biosensor was further investigated under the simulated interference system which contained four kinds of mismatch sequences in 20 nM as interference background. The current *vs.* time signals from dual channel were shown in Fig. 4 (A) and (B). Under the optimal condition of the hybridization, while detecting the complementary DNA concentration range of target dsDNA from 50 pM to 5 nM (Fig. 4 (C)), there was a good linear relationship between the values of $\sum I$ and the logarithmic function of complementary target dsDNA concentration, the limit of detection (LOD) was 9.2 pM (estimated

using 3σ/S, where σ is the standard deviation of the interference background solution and S is the sensitivity), demonstrating that this novel E-DNA biosensor has wider linear range and limit of detection with the same order of magnitude compared with the E-DNA biosensor based on endonuclease assisted electrochemical signal amplification (Wang et al., 2015). It also can be seen that the E-DNA biosensor possessed certain anti-interference ability and can hybridize with completely complementary dsDNA sequences well under a certain interference background. It provided a good basis of further detection research in practical samples.

Figure 4

4. Conclusion

In summary, a novel dual-probe electrochemical sensing strategy based on 2'-F RNA-modified probes for detection of dsDNA of PML/RARα related fusion gene was designed in this study in order to prevent the reannealing of the two separate strands from target dsDNA and enhance the hybridization efficiency between detection probes and target DNA. Owing to the unique conformation of 2'-F RNA-modified probe and dual-probe detection mode, under the optimal hybridization condition, the biosensor exhibited excellent selectivity and specificity for the discrimination between the fully-complementary and the mismatch sequences and could recognize completely complementary sequences with high sensitivity, both in the PBS hybridization solution without other interferences and in the simulated interference system. The experimental results show that 2'-F RNA-modified probe would be an ideal candidate for constructing a novel E-DNA biosensor for target dsDNA detection in real sample. In addition, it has been demonstrated that the concept in this research is in connection with simulation study of the simulated 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.

Supporting Information Available: Details of the apparatus, chemicals and some experiments are provided.

5. Acknowledgment

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Captions

Scheme 1 Schematic illustration of sandwich-type dual-probe electrochemical DNA biosensor based on 2'-fluoro RNA monomers modified probes for detection of synthetic PML/RARα fusion gene dsDNA.

Fig. 1 Cyclic voltammograms (A) and i-t curves (B) of TMB substrate at different modified dual-channel electrodes (Channel 1 and Channel 2): (a) C1/AuE and C2/AuE; (b) HRP/R1/S3a/C1/AuE and HRP/R2/S3b/C2/AuE.

Fig. 2 Sum of current signals ($\sum I$) corresponding to the respective different hybridizations with 2'-F RNA modified probes (green) and unmodified DNA probe (red): after hybridization without any sequence (blank) (a); after hybridization with the complementary target dsDNA sequence (S3) (b); after hybridization with the single-base mismatch dsDNA sequence (S4) (c); after hybridization with the three-bases mismatch dsDNA sequence (S5) (d); after hybridization with the five-bases mismatch dsDNA sequence (S6) (e); after hybridization with the ten-bases mismatch dsDNA sequence (S7) (f); after hybridization with PML dsDNA sequence (S8) (g); after hybridization with RARα dsDNA sequence (S9) (h).

Fig. 3 i-t curves of C1/S3a/R1 (A: channel 1) and C2/S3b/R2 (B: channel 2) in sandwich-type dual-probe electrochemical sensing strategy based on 2'-F RNA modified DNA probes hybridized with synthetic target dsDNA at a series of concentrations from dual channels. (From (a) to (g): 0 pM, 0.1 pM, 1 pM, 10 pM, 100 pM, 1000 pM and 10000 pM); (C) Comparison of sum of current value ($\sum$ I) for dual-probe electrochemical DNA biosensor modified with two groups of 2'-F RNA modified probes (C1/S3a/R1 + C2/S3b/R2, green) or with single group of 2'-F RNA modified probes (C1/S3a/R1, red or C2/S3b/R2, blue) after hybridization with

different concentration of target dsDNA species under the same condition; (D) Linear relationship between ∑ I and target dsDNA concentration (From 0.5 pM to 15 pM). Error bars represent the standard deviations of measurements (n=3).

Fig. 4 Dual-channel current-time curves (A: channel 1 and B: channel 2) of hybridization with different concentrations of the target dsDNA in hybridization solution mixed interference mismatch sequences. (target dsDNA concentrations: from (a) to (f): 0 pM, 50 pM, 100 pM, 500 pM, 1 nM and 5 nM)) (C) The logarithmic plot of target dsDNA concentrations versus ∑ *I* (target dsDNA concentrations: from 0.05 nM to 5 nM).

Scheme 1

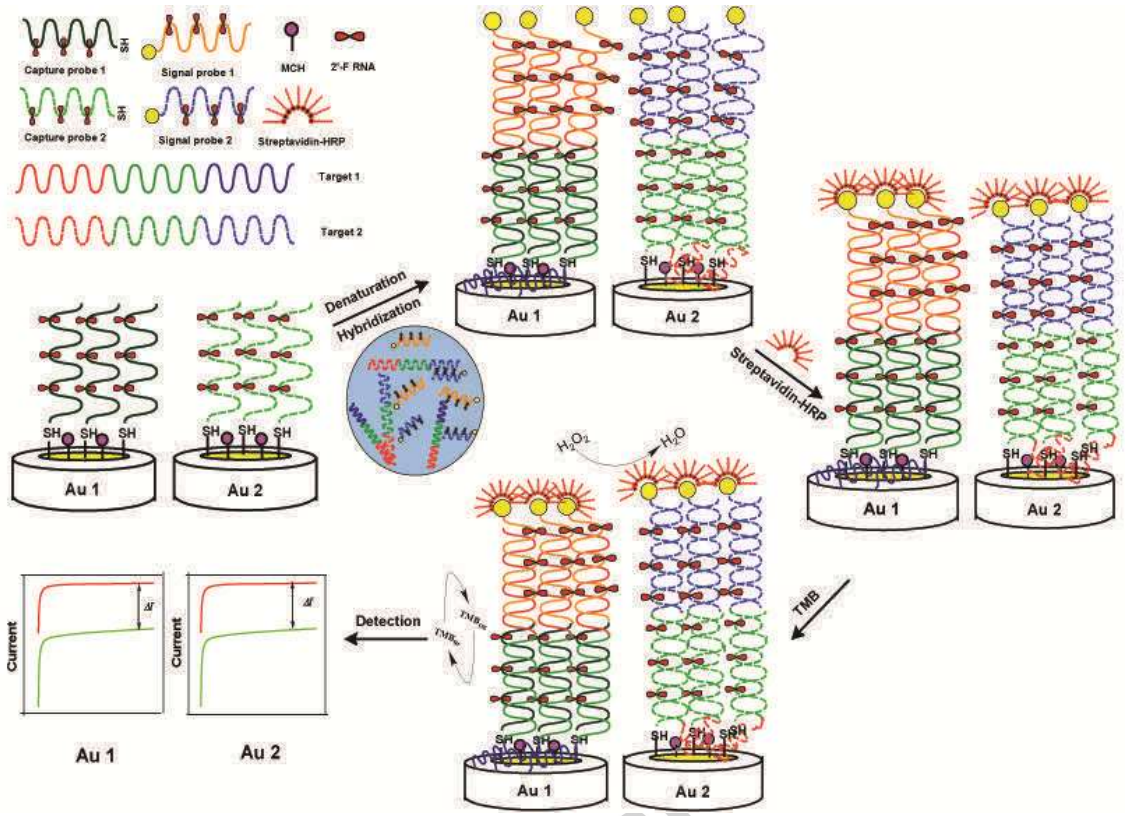
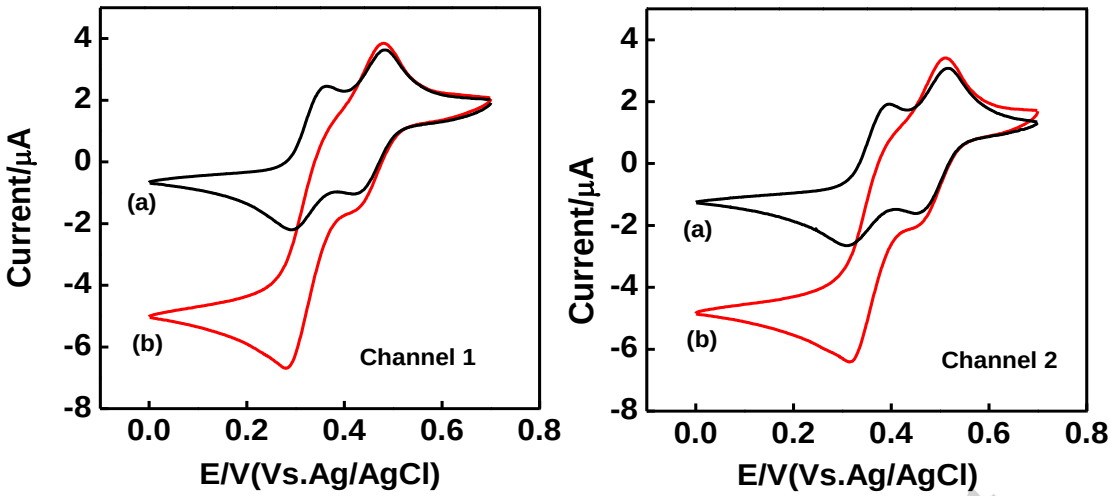
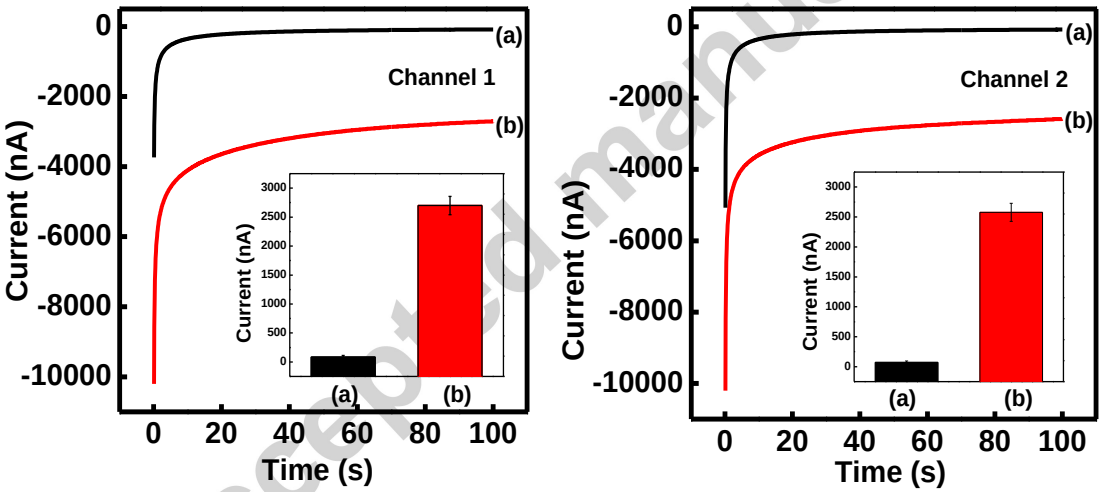


Fig. 1



(A)



(B)

Fig. 2

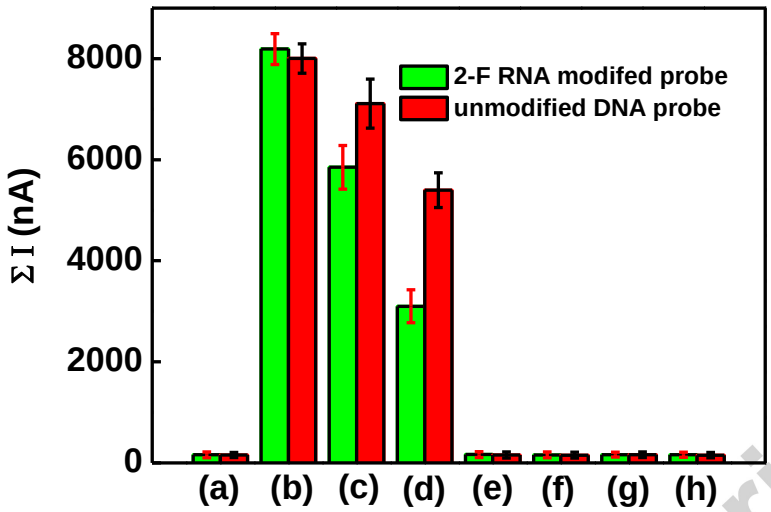


Fig. 3

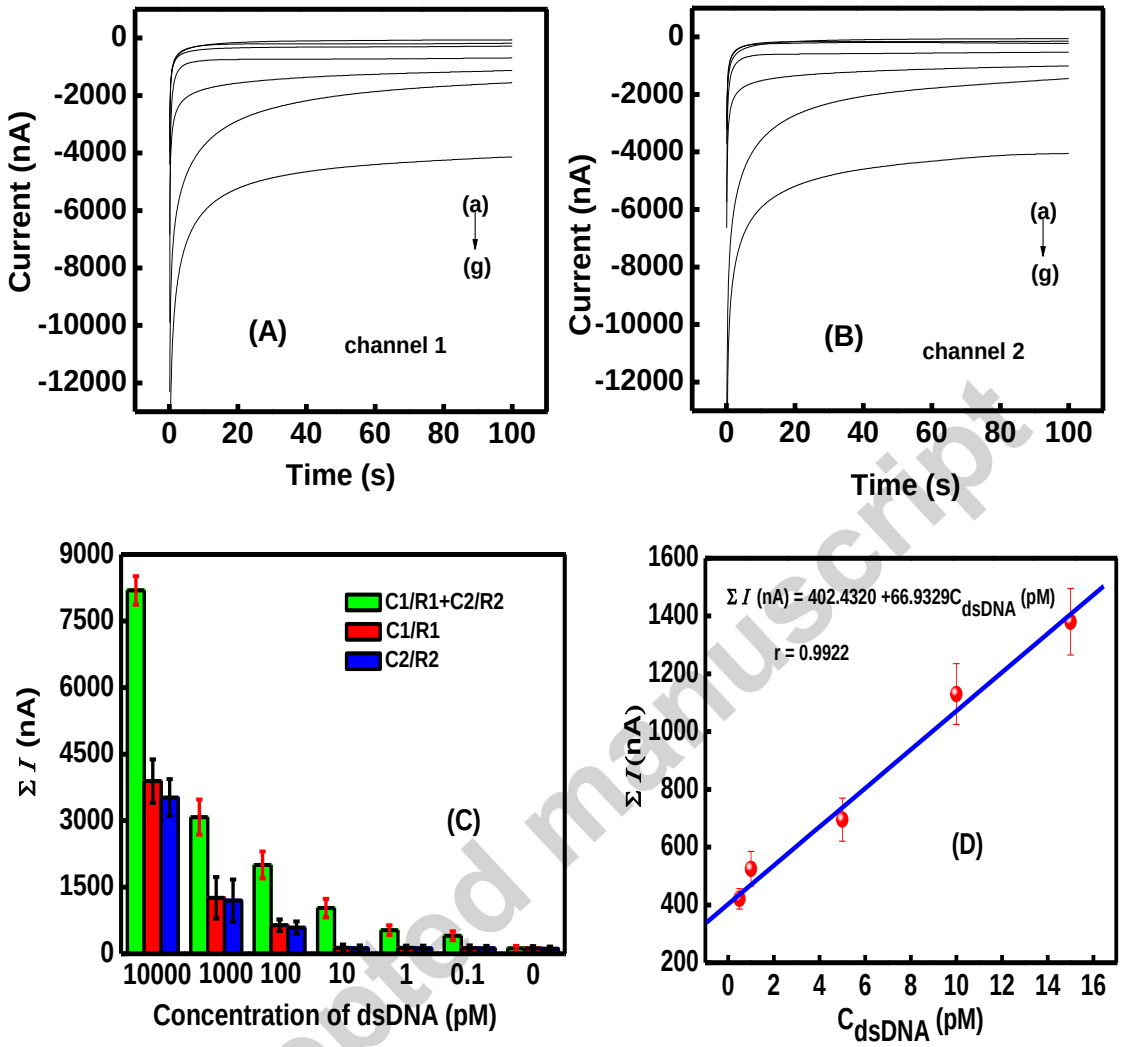
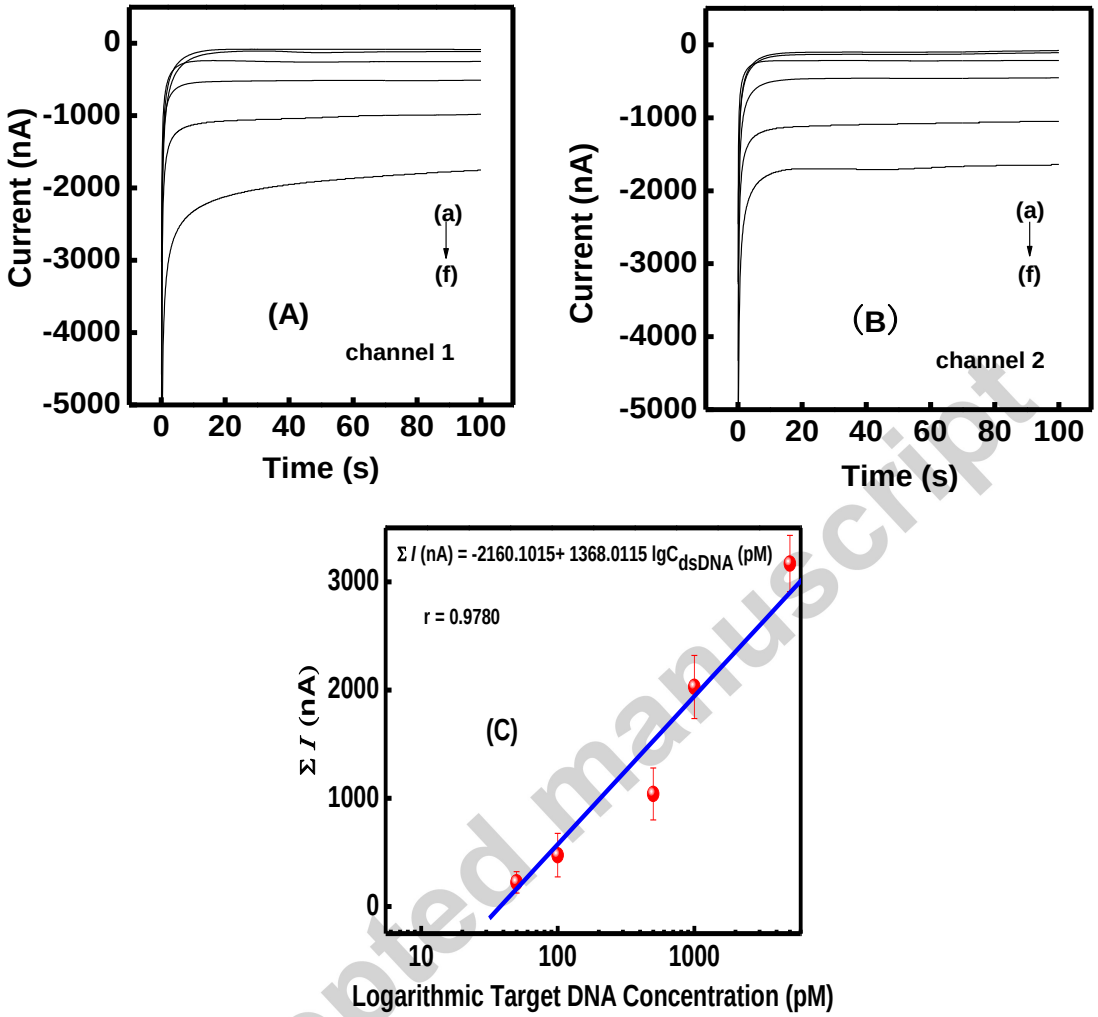


Fig. 4



Highlights

> The two kinds of oligonucleotide probes modified with 2'-fluoro RNA monomers were introduced into the E-DNA biosensor.

> A novel dual-probe E-DNA biosensor can highly specifically detect target dsDNA in the complicated background.

> The biosensor showed excellent selectivity and reproducibility with wider linear range and a low detection limit, and provided a promising potential in clinical applications.