

Enzymatic amplification detection of DNA based on “molecular beacon” biosensors

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Abstract

We described a novel electrochemical DNA biosensor based on molecular beacon (MB) probe and enzymatic amplification protocol. The MB modified with a thiol at its 5' end and a biotin at its 3' end was immobilized on the gold electrode through mixed self-assembly process. Hybridization events between MB and target DNA cause the conformational change of the MB, triggering the attached biotin group on the electrode surface. Following the specific interaction between the conformation-triggered biotin and streptavidin-horseradish peroxidase (HRP), subsequent quantification of DNA was realized by electrochemical detection of enzymatic product in the presence of substrate. The detection limit is obtained as low as 0.1 nM. The presented DNA biosensor has good selectivity, being able to differentiate between a complementary target DNA sequence and one containing G–G single-base mismatches.

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1. Introduction

The first fluorescent molecular beacon (MB) was initially described in 1996 as nucleic acid probe able to undergo spontaneous conformational change following hybridization with the complementary nucleic acid target (Tyagi and Kramer, 1996). Since then, molecular beacons (MBs) have become a class of DNA probes that were widely used in chemistry, biology, biotechnology and medical sciences for biomolecular recognition (Fang et al., 2000; Tsourkas and Bao, 2003; Li et al., 2003; Heyduk and Heyduk, 2002; Diana et al., 2003; Tyagi et al., 1998; Bernacchi and Mely, 2001; Benoit et al., 2001; Grégoire et al., 1999; Antonella et al., 2007; Jacqueline et al., 1999; Wang et al., 2002; Tsourkas et al., 2002), due to their many advantages.

Most of the early works on MB were based on optical detection. The fluorescence signal turns on after the DNA target

binds to the immobilized MB on the substrates (Du et al., 2003, 2005; Swearingen et al., 2005). Recently, electrochemical DNA biosensors based on MB have received particular attention due to their high sensitivity and selectivity, simple instrumentation, low production cost, and the fact that they are fast, accurate, compact, and portable. Typical MB-based electrochemical strategies are based on a current change, which is caused by the change of distance between the electroactive reporter and electrode surface. (Patolsky et al., 2002; Wang et al., 2003; Liu et al., 2005; Georgopoulou et al., 2000; Immoos et al., 2004; Xiao et al., 2005; Radi et al., 2006). However, such kind of “signal off” electrochemical biosensors has a disadvantage lies in the fact of the relatively higher frequency for producing false responses. Many approaches have been employed to overcome the shortcoming of this kind of protocols (Wu et al., 2007a,b; Bockisch et al., 2005).

Here, we demonstrated a novel electrochemical DNA biosensor based on MB and enzymatic amplification protocol. The principle is based on an electrochemical “signal off and on”. Hairpin probes modified with a thiol at its 5' end and a biotin at its 3' end were immobilized on the gold electrode

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through mixing self-assembly in the presence of mercaptopropionic acid (MPA). Hybridization events between the immobilized MB and target DNA cause the conformational change of the MB, triggering the attached biotin group on the electrode surface. Following the specific interaction between the conformation-triggered biotin and streptavidin-horseradish peroxidase (HRP) quantification of DNA would be realized by electrochemical detection of the enzymatic product (See Fig. 1a).

2. Experimental

2.1. Apparatus

Voltammograms were recorded at a CHI660 electrochemical work station (Shanghai Chenhua Instruments, Shanghai) with a three-electrode system consisting of a gold working electrode (GCE) of a diameter of 2 mm, a Ag/AgCl reference electrode and a platinum wire auxiliary electrode in a 25 mL beaker.

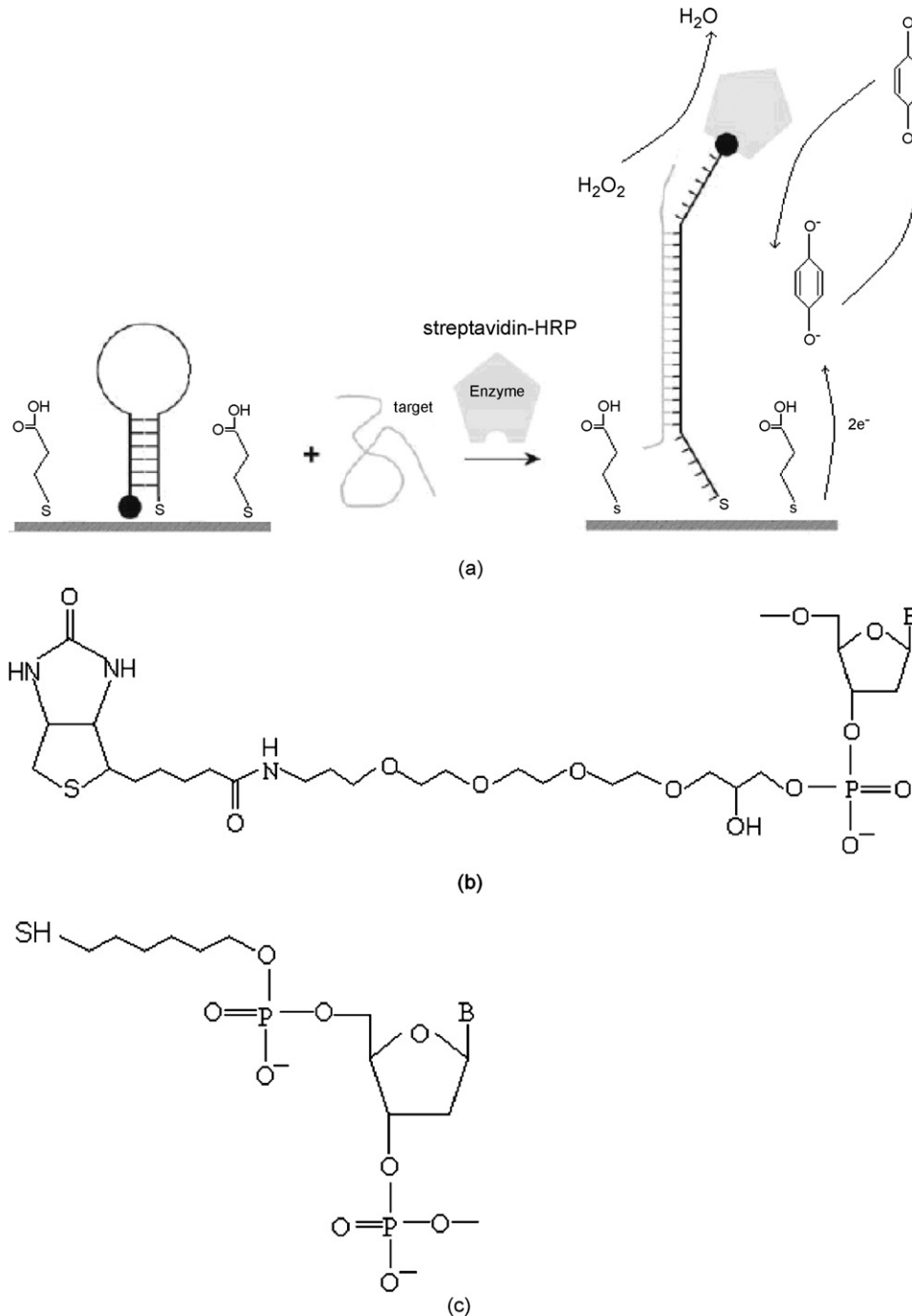


Fig. 1. (a) The procedure for DNA assay, (b) 3'-Biotin modification of the MB, (c) 5'-SH modification of the MB.

Table 1
DNA hairpin probe and its targets

Name	Sequence
H1 (probe)	5'-(C6Thiol)ACACGCTCATCAAGCTTAA-CTCATAGTGAGCGTGT(-3'C6) (biotin)-3'
T1 (H1 complement)	5'-ACGCTCACTATGAGTTAAAGCTTG-3'
T1M1 (single mismatch of H1)	5'-ACGCTGACTATGAGTTAAAGCTTG-3'

2.2. Materials

The oligonucleotide sequences for both probe and targets are shown in Table 1. All oligonucleotides were purchased from Takara Biotechnology Co., Ltd. (Dalian, China) and were used as received. Oligonucleotide probe was functionalized at the 5' end with a thiol group and at the 3' end with a biotin group (Fig. 1b and c). Albumin bovine serum (BSA) was supplied by Dingguo Biological Products (Beijing). 3-Mercaptopropionic acid and streptavidin labeled horseradish peroxidase (HRP) were products of Sigma and used without further purification. All H₂O used in the preparation of buffers and for rinse solutions had a resistivity of 18.2 MΩ, as produced by a Barnstead Nanopure system. Hydroquinone, H₂O₂ and other reagents were all of analytical reagent grade. The buffer of 0.01 M sodium phosphate-buffered saline (PBS) was used as incubating and washing buffer of pH 7.4.

2.3. Protocol of DNA quantification

The gold electrode (2 mm diameter) was first polished using rough alumina slurry. It was then polished to a mirror finish subsequently with 0.3 and 0.05 mm alumina slurry. After it was thoroughly sonicated with ethanol and ultra pure water, gold electrodes were then scanned between 0 and +1.3 V until a steady-state current–voltage curve was obtained followed by applying a potential of −0.6 V for 600 s. The self-assembly process was performed by dropping a mixture solution of hairpin oligonucleotide (H1) and MPA on gold electrodes in a humidified chamber. Ten hours later, the electrode was thoroughly rinsed with hot PBS solution (50 °C) to remove any nonbonding MPA and DNA. It is mentioned that rinsing the gold electrode after assembly is an important step. Otherwise, some mercaptopropionic acid and probe molecules will attach to the substrate by nonspecific adsorption causing a large background signal and staleness of the sensor. The concentration of the hairpin oligonucleotide used was 1 μM and the mixing molar ratio of DNA probe to MPA was 1:5. The buffer used for self-assemble procedure is 0.01 M PBS solution containing 1 M NaCl. It is worthy to mention that the hairpin oligonucleotide used should be treated subsequently at 70 °C and ice-cold water bath for 30 and 10 min, respectively before mixing with MPA. And the procedure should be repeated again to escape the forming of dipolymer. Hybridization reaction between the immobilized hairpin probes and DNA targets was performed by dropping 10 μL of either their full complement (T1), or one containing a single base mismatch (T1M1), or PBS solution on electrodes. The hybridization pro-

cedure was conducted at 37.5 °C for 2 h in a humidified chamber. The biotin groups labeled on MBs were then triggered while hybridization occurred. After being rinsed with PBS solution for 15 min, these electrodes were transferred to a beaker containing 1% BSA solution (prepared in PBS) to react for 1 h to block the active sites of these electrodes. Twenty microlitres of 1:100 diluted streptavidin-HRP containing 1% BSA was dropped on electrodes' surface and these electrodes were incubated 35 min at 4 °C in the humidified chamber. After being rinsed with PBS solution for 25 min, electrodes were soaked in pH 7.4 PBS at 4 °C prior to quantification. The aforementioned protocol is shown in Fig. 1a.

2.4. Procedure of electrochemical quantification

Electrochemical quantification was performed with a CHI660 electrochemical work station (Shanghai Chenhua Instruments, Shanghai). A three-electrode configuration was employed, consisting of the gold electrode (GE) with a diameter of 2 mm serving as a working electrode, a Ag/AgCl reference electrode and a platinum wire auxiliary electrode in a 5 mL PBS solution containing 1 mM hydroquinone. Amperometric measurements were performed by applying a working potential of −0.15 V under unceasing stirring until transient currents were allowed to decay to a steady-state value. Then 10 μL of 3% H₂O₂ were added quickly to the solution. The sharply increasing of the reduction current implies that the streptavidin-HRP have been captured on the electrode. The cyclic voltammetry measurements were carried out in the potential range from −0.5 to 0.5 V at a scan rate of 100 mV/s after adding of H₂O₂ and no solution agitation was necessary during the run.

3. Results and discussion

3.1. Electrochemical behaviors of the DNA sensor

Fig. 1a schematically illustrates the principle of new electrochemical biosensing of DNA based on a MB and enzymatic amplification protocol. It involves a DNA hybridization reaction between the immobilized MB and target DNA, capturing the streptavidin-HRP tracers by the triggered biotin and electrochemical detection of the enzymatic products. One can see the self-assembled MB on the electrode surface retains a hairpin structure prior to its hybridization. The biotin, which is labeled on one end of the MB, is located in the close proximity to the gold electrode surface, thus unable to interact with the streptavidin-HRP tracers because of stereo-hindrance. The hybridization reaction between the immobilized MB and the target DNA induces the conformation change of MB and triggers the biotin from the electrode surface. Following the specific interaction between the triggered biotin and streptavidin-HRP, the subsequent quantification of DNA was realized by electrochemical detection of enzymatic product in the presence of substrate.

Fig. 2A presents the typical cyclic voltammograms of bare gold electrode (curve a), and the H1 probes and MPA modified gold electrode (curve b) in 1 mM hydroquinone solution.

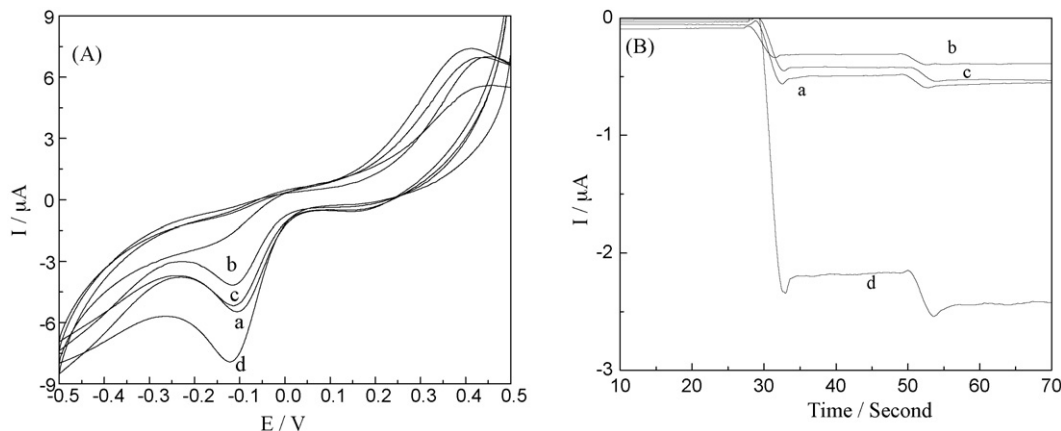


Fig. 2. (A) Cyclic voltammograms of different electrodes in PBS solution containing 1 mM hydroquinone. (a) Bare gold electrode; (b) H1 probe and MPA modified gold electrode, the electrode was incubated 2 h in PBS in the absence of DNA target; (c) H1 probe and MPA modified gold electrode, the electrode was incubated 2 h in the presence of 100 nM of one base mismatch target DNA (TIM1); (d) H1 probe and MPA modified gold electrode, the electrode was incubated 2 h in the presence of 100 nM of Target DNA (T1). (B) Chronoamperometric curves of the different electrodes in the successive addition of, amperometric measurements were performed by applying a working potential of -0.15 V under unceasing stirring until transient currents were allowed to decay to a steady-state value.

A well-defined reduction peak at -0.1 V was observed with the bare gold electrode (curve a). When gold electrode was modified with H1 probes and MPA, the reduction peak decreased (curve b). It indicated the gold electrode surface was blocked by the immobilized H1 probes and MPA. The reduction peak currents increased significantly after the electrodes were incubated with one-mismatch target DNA (TIM1, curve c) and complementary DNA (curve d) (Dequaire and Heller, 2002). Such increments would benefit from the DNA hybridization events, which cause the conformation change of MB and trigger the biotin groups. The streptavidin-HRP was captured to the electrode surface resulting an enzymatic amplification and enhanced reduction peak current. One can see the reduction peak current of the biosensor in the presence of target DNA (curve d) is higher than that of the biosensor in the presence of one-mismatch target DNA (curve c). Such difference may come from its different hybridization efficiency of MB with target DNA and one mismatch target DNA. Above results also indicate the biosensor may be used to differentiate the target DNA and one-mismatch target DNA.

To evaluate the binding specificity of the H1 probes on the gold electrode, a control experiment with the buffer solution was conducted. Fig. 2B displays typical chronoamperometric curves of biosensors for successive additions of the same amounts of H_2O_2 under optimized experimental conditions (Caruana and Heller, 1999). One was exposed to T1 (Fig. 2b, curve d), and the other was exposed to buffer solution (Fig. 2b, curve b). Similar results were also obtained compared with the cyclic voltammograms. As shown in the inset figure, for H1-T1, the reduction current increase attributing to the amplification process of hydroquinone in the presence of enzyme and H_2O_2 enhanced by a factor of 10. To test for single mismatch discrimination, we employed a TIM1 which has one G–G mismatch compared with the complementary sequence T1. An 8-fold weaker current signal occurs for the single mismatched target compared with the full complement (Fig. 2b, curve c). These results indicated that

the biosensor exhibited a rapid and sensitive response for single mismatch discrimination.

3.2. Optimization of experimental conditions

3.2.1. Effect of modifying surface density of MB

In current study, H1 probe was immobilized on the gold electrode surface by self-assembling in the presence of MPA. Previous studies have demonstrated that the use of blocking agents during the self-assembling bimolecular (such as thiol DNA) on the gold substrate would reduce its nonspecific adsorption and maintain its original structure. (Du et al., 2005; Pena et al., 2002; Gearheart et al., 2001; Sauthier et al., 2002; Lin et al., 2002). Therefore, we prepared the modified gold electrode by self-assemble of H1 probe and MPA mixing in different molar ratios. When the immobilization procedure was conducted without adding MPA, the response was relatively higher than that in the presence of MPA. It seems that more H1 probes are immobilized on the gold substrates under this condition. However, the background also increased when performed experiments without adding of MPA. We assume that MPA plays three key roles in the experiment. First, the use of MPA potentially provides sufficient space between hairpin probes such that hairpin loops were formed and hybridization could occur since the short carbon chain of MPA would not interfere with the hybridization reaction. Second, noncovalent interactions between the DNA backbone and the gold electrode caused by interaction between nitrogen-containing nucleotide bases and gold would be reduced by MPA efficiently. Third, the presence of MPA also prevents the biotin group labeled on H1 probes which unreleased by hybridizing reaction from reacting with streptavidin-HRP directly to some extent. Fourth, protein molecules including streptavidin usually show nonspecific adsorption on the gold substrate through hydrophobic and electrostatic interaction. Such interactions could be mitigated via a hydrophilic modification of the gold surface such as self-

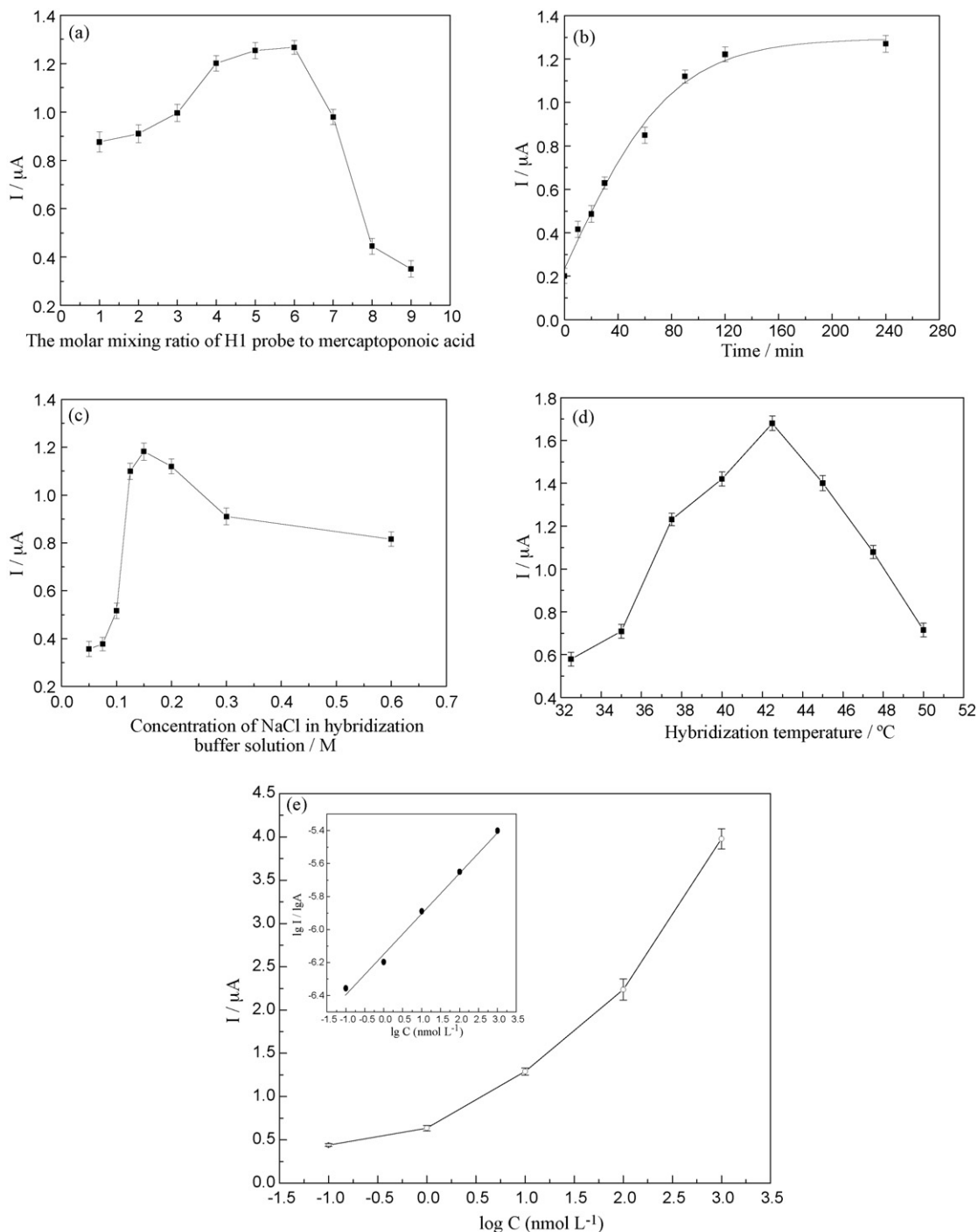


Fig. 3. Optimization of conditions (a) the molar mixing ratio of H1 probe to mercaptopropionic acid. (b) Hybridization time with T1. (c) Concentration of NaCl in hybridization buffer solution. (d) Hybridization temperature. 10 nM T1 were used as target oligonucleotides. (e) Current responses of the electrode on concentration of T1.

assembled monolayer of MPA. Experiments were conducted by using different molar ratios of H1 probes to MPA (Fig. 3a). As shown in the figure, less MPA results in high background responses, and thus a small difference in signal between pre- and post-hybridization. On the other hand, much MPA results in a low probe density on the gold electrode surface, which leads to a low response after hybridization. A ratio of 1:5 gave the

relatively better performance. Additionally, the signal to noise ratio in this condition is much higher than that of without adding MPA. However, the best result would be obtained with a ratio of 1:10 in a previous report (Du et al., 2005). It seems different blocking agent adopted may affect biosensors' performance with different optimized ratios. And the blocking agent adopted in the reference is 3-mercaptopropyl-1-propanol.

3.2.2. Hybridization kinetics of the hairpin probe on the gold electrode

The kinetics of hairpin invasion by the target was analyzed by monitoring the reduction currents as a function of hybridization time. Fig. 3b displays the effect of hybridization time on the reduction current increases. We found a 50% maximal relative reduction currents increase was obtained in approximately 30 min of incubation with T1, and steady state was reached in less than 2 h. To ensure the complete reaction of hybridization with target DNA, 2 h of hybridization time was selected throughout our experiments.

3.2.3. Effect of the concentration of NaCl in hybridization buffer solution

Saline concentration poses an important role during the procedure of hybridization. The effects of the concentration of NaCl in hybridization buffer solution on the response signal were investigated, and the results are presented in Fig. 3c. It can be seen that the response increases up to 0.15 M of NaCl, and then it decrease at higher concentration. Higher or lower concentration of NaCl in hybridization buffer leads to a decrease of the signal because of poor hybridization efficiency. The reason to cause the decrease of signal at higher concentration of NaCl may be attributed to the difficulty of H1 probe loop structure release, which also results in poor efficiency of hybridization. Thus, 0.15 M NaCl was selected as the optimal saline concentration in hybridization reaction. It is worthy of mention that the concentration of NaCl in self assemble procedure is high up to 1 M. The relatively higher concentration of NaCl provides a better circumvent for developing the efficiency of self-assemble of the hairpin probes on gold substrates.

3.2.4. Effect of the temperature in hybridization procedure

Experiments indicated that the hybridization temperature also acts as a very important parameter (Fig. 3d). Melting temperature (T_m) of the H1 probe, H1-T1, H1-T1M1 were about 70, 55 and 52 °C under conditions of hybridization as each oligonucleotides' concentration was 1 μ M. Some studies discovered that the immobilization of molecular beacon on solid substrates resulted in difficulty of its conformational change. Therefore, relatively lower temperature leads to poor efficiency of hybridization. On the other hand, relatively higher temperature leads to dissociation of H1-T1 also decreasing the efficiency of hybridization. 37.5 °C was used throughout our experiments to ensure relatively higher hybridization efficiency while lower concentrations of T1 and T1M1 were employed.

3.3. Reusability of the biosensor

One of the valuable features to evaluate a biosensor is its reusability. We tested the possibility to reuse the DNA sensor by regenerating the original state of the hairpin probes. Specifically, after the first quantification process, the electrode was immersed in 8 M urea solution containing 0.1 mg/mL biotin. Urea denatured the activity of proteins which have been captured on the surface of the gold electrode. The high concentration of biotin also provides an effective mean to displace down the

streptavidin-HRP from H1 probes. After continuous stirring for 30 min at 50 °C, the electrode was transferred to a beaker containing ice-cold PBS solution to refold the probe hairpins followed by washing with PBS solution. The regenerated electrode was then treated with the same procedure mentioned in previous sections. As expected, the reduction current increase of the biosensor decreases with repeated regenerative cycles by a factor of 42%. But the ratio of responses to backgrounds is almost as the same as biosensors used in the first time. One thought the heating and rinsing procedures might cause the probe molecule loss from the gold electrode and incompletely refolding of hairpin probes. The sensor's lifetime is also especially important for evaluating the performance of sensors. The activity of the sensor we advanced could retain its 90.12% original value after stored for 2 weeks in refrigerator.

3.4. Analytical performance of biosensor

Fig. 3e shows the relative reduction current increase of biosensors vs. the logarithm of concentration of perfectly matched target T1 range from 0.1 to 1000 nM with a detection limit of 0.1 nM as estimated by 3σ rule (MacDougall et al., 1980). Low concentration of target caused to weak reduction current increase. The increase of reduction current was about 0.2 μ A when experiments were performed without adding of perfectly matched target using the modified gold electrodes. For the unmodified bare gold electrode, the increase of reduction current was about 0.3 μ A. It seems that the background response is mainly caused by direct reduction of hydroquinone at gold electrode in the presence of H_2O_2 . This undesired feature of the gold electrode limits the sensitivity of the special protocol for DNA quantification. Yet compared with the fluorescence method reported in reference which adopted the same hairpin probe and target DNA (H3 and T3 in reference (Du et al., 2005) the fluorescence intensity is proportional to the concentration of T3 in the range from 1.18 to 2.6 μ M), the novel electrochemical protocol based on enzyme amplification reaction of this paper promoted the sensitivity and extended the range of detection by magnitudes. The inset figure shows the logarithm of the reduction current increase is proportional to the logarithm of the concentration of perfectly matched target T1 in the range from 0.1 to 1000 nM with a coefficient of variation of 0.9964. The performance of the biosensors is worthy of mention considering the diameter of the gold electrode used in the experiment was just 2 mm. Eight electrodes were treated in the same procedure to test the reproducibility of the method. Experiments indicated that the RSD of the method is 9.95% for determination of 10 nM T1 under the same conditions.

4. Conclusion

The present study reported a novel DNA electrochemical biosensor based on conformational change of molecular beacon. This special protocol greatly simplified the procedure of DNA quantification and single nucleotide polymorphism studying. It holds great promise for clinical applications. However, the relatively higher detection limit is not satisfied. Further study

concentrated in enhancing the novel biosensor's sensitivity is under procedure.

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References

- Antonella, C., Massimo, L.C., Massimo, Z., Laura, F., Cristian, B., Giovanna, B., 2007. *Bioconjug. Chem.* 18, 318–322.
- Bernacchi, S., Mely, Y., 2001. *Nucl. Acids Res.* 29, e62.
- Benoit, D., Michel, C., Albert, J.L., 2001. *Nat. Biotechnol.* 19, 365–370.
- Bockisch, B., Grunwaldt, T., Spillner, E., Bredehorst, R., 2005. *Nucl. Acids Res.* 33, e101.
- Caruana, D.J., Heller, A., 1999. *J. Am. Chem. Soc.* 121, 769–774.
- Diana, P., Bratu, B.-J., Cha, M.M., Mhlanga, F.R.K., Sanjay, T., 2003. *Proc. Natl. Acad. Sci.* 100, 13308–13313.
- Du, H., Disney, M.D., Miller, B.L., Krauss, T.D., 2003. *J. Am. Chem. Soc.* 125, 4012–4013.
- Du, H., Strohsahl, C.M., Camera, J., Miller, B.L., Krauss, T.D., 2005. *J. Am. Chem. Soc.* 127, 7932–7940.
- Dequaire, M., Heller, A., 2002. *Anal. Chem.* 74, 4370–4377.
- Fang, X., Li, J., Perlette, J., Wang, K., Tan, W., 2000. *Anal. Chem.* 72, 747A–753A.
- Grégoire, B., Sanjay, T., Albert, L., Fred, R.K., 1999. *Proc. Natl. Acad. Sci.* 96, 6171–6176.
- Georgopoulou, A.S., Mingos, D.M.P., White, A.J.P., Williams, D.J., Horrocks, B.R., Houlton, A., 2000. *J. Chem. Soc. Dalton Trans.* 17, 2969–2974.
- Gearheart, L.A., Ploehn, H.J., Murphy, C.J., 2001. *J. Phys. Chem. B.* 105, 12609–12615.
- Heyduk, T., Heyduk, E., 2002. *Nat. Biotechnol.* 20, 171–176.
- Immoos, C.E., Lee, S.J., Grinstaff, M.W., 2004. *J. Am. Chem. Soc.* 126, 10814–10815.
- Jacqueline, A.M., Vet, A.R., Majithia, S.A.E., Marras, S., Tyagi, S.D., Bernard, J.P., Fred, R.K., 1999. *Proc. Natl. Acad. Sci.* 96, 6394–6399.
- Li, J.J., Geyer, R., Tan, W., 2003. *Nucl. Acids Res.* 28, e52.
- Liu, J., Tian, S., Tiefenauer, L., Nielsen, P.E., Knoll, W., 2005. *Anal. Chem.* 77, 2756–2761.
- Lin, Z., Strother, T., Cai, W., Cao, X., Smith, L.M., Hamers, R.J., 2002. *Langmuir* 18, 788–796.
- MacDougall, D., Crummett, W.B., et al., 1980. *Anal. Chem.* 52, 2242–2249.
- Patolsky, F., Weizmann, Y., Willner, I., 2002. *J. Am. Chem. Soc.* 124, 770–772.
- Pena, S.R., Raina, S., Goodrich, G.P., Fedoroff, N.V., Keating, C.D., 2002. *J. Am. Chem. Soc.* 124, 7314–7323.
- Radi, A.E., Sanchez, J.L.A., Baldrice, Eva., O'Sullivan, C.K., 2006. *J. Am. Chem. Soc.* 128, 117–124.
- Swearingen, C.B., Wernette, D.P., Cropek, D.M., Lu, Y., Sweedler, J.V., Bohn, P.W., 2005. *Anal. Chem.* 77, 442–448.
- Sauthier, M.L., Carroll, R.L., Gorman, C.B., Franzen, S., 2002. *Langmuir* 18, 1825–1830.
- Tyagi, S., Kramer, F.R., 1996. *Nat. Biotechnol.* 14, 303–308.
- Tsourkas, A., Bao, G., 2003. *Brief Funct. Genomic. Proteomic.* 1, 372–384.
- Tyagi, S., Bratu, D.P., Kramer, F.R., 1998. *Nat. Biotechnol.* 16, 49–53.
- Tsourkas, A., Behlke, M.A., Bao, G., 2002. *Nucl. Acids Res.* 30, 4208–4215.
- Wang, H., Li, J., Liu, H.P., Liu, Q.J., Mei, Q., Wang, Y.J., Zhu, J.J., He, N.Y., Lu, Z.H., 2002. *Nucl. Acids Res.* 30, e61.
- Wang, J., Li, J., Baca, A.J., Hu, J., Zhou, F., Yan, W., Pang, D.W., 2003. *Anal. Chem.* 75, 3941–3945.
- Wu, Z.S., Guo, M.M., Zhang, S.B., Chen, C.R., Jiang, J.H., Shen, G.L., Yu, R.Q., 2007a. *Anal. Chem.* 79, 2933–2939.
- Wu, Z.S., Jiang, J.H., Shen, G.L., Yu, R.Q., 2007b. *Hum. Mutat.* 0, 1–8.
- Xiao, Y., Lubin, A.A., Heeger, A.J., Plaxco, K.W., 2005. *Angew. Chem. Int. Ed.* 44, 5456–5459.