



A simple electrochemical biosensor for highly sensitive and specific detection of microRNA based on mismatched catalytic hairpin assembly

Ye Zhang^{1,a}, Yurong Yan^{a,1}, Wenhong Chen^b, Wei Cheng^c, Shengqiang Li^a, Xiaojuan Ding^a, Dandan Li^a, Hong Wang^a, Huangxian Ju^{a,d}, Shijia Ding^{a,*}

^a Key Laboratory of Clinical Laboratory Diagnostics (Ministry of Education), College of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, China

^b Department of Clinical Laboratory, Children's Hospital of Chongqing Medical University, Chongqing 400014, China

^c The Center for Clinical Molecular Medical detection, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

^d State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, China

ARTICLE INFO

Article history:

Received 14 November 2014

Received in revised form

9 January 2015

Accepted 12 January 2015

Available online 12 January 2015

Keywords:

MicroRNA

Catalytic hairpin assembly

Mismatched base pairs

Differential pulse voltammetry

Electrochemical biosensor

ABSTRACT

MicroRNAs (miRNAs) play vital regulatory roles in cancer development and a variety of diseases, which make them become promising biomarkers. Here, a simple electrochemical biosensor was developed for highly sensitive and specific detection of target miRNA using mismatched catalytic hairpin assembly (CHA). The target miRNA triggered the toehold strand displacement assembly of two hairpin substrates, which led to the cyclic reuse of the target miRNA and the CHA products. Compared with the traditional CHA, mismatched CHA could decrease the nonspecific CHA products, which reduced the background signal significantly. Under the optimal experimental conditions and using differential pulse voltammetry, the established biosensor could detect target miRNA down to 0.6 pM ($S/N=3$) with a linear range from 1 pM to 25 nM, and discriminate target miRNA from mismatched miRNA with a high selectivity. It was also applied to the determination of miRNA spiked into human total RNA samples. Thus, this biosensing strategy might become a potential alternative tool for detection of miRNA in biomedical research and early clinical diagnosis.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

MicroRNAs (miRNAs) belong to a class of small (~22 nucleotides), endogenous, non-coding RNA molecules. These highly conserved single-stranded RNAs modulate gene expression by binding to target mRNAs, leading to translation blockade or target transcripts degradation (Zhou et al., 2010; Chowdhury et al., 2013; Wahlestedt, 2013). Such regulatory mechanisms enable miRNAs to mediate tumor metastasis (Nicoloso et al., 2009), stem-cell differentiation and renewal (Gangaraju and Lin 2009), and viral replication (Cullen 2009). These characteristics make miRNAs become promising biomarker candidates for early cancer diagnosis. Therefore, it's imperative to develop a simple and sensitive assay for miRNA expression analysis in clinical cases.

Due to their small size, vulnerable degradability, highly

homologous sequences, and relatively low expression levels in cells, detection of miRNAs is quite challenging (Cissell and Deo, 2009; Koshiol et al., 2010; Leshkowitz et al., 2013; Duan et al., 2013). Conventional methods suffer from some defects in practical applications. For instance, northern blotting has a relatively low sensitivity with time-consuming operation (Vá ló czi et al., 2004; Pall et al., 2007; Varallyay et al., 2008), real-time PCR is apt to generate false positive with hampered efficiency resulting from short primer in amplification process (Shi and Chiang, 2005; Lao et al., 2006; Markou et al., 2008), and microarrays requires extremely precise instrument which is unable to avoid cross-hybridization (Lee et al., 2008; Lagos-Quintana et al., 2011). Compared with these methods, the electrochemical biosensor attracts much attention owing to its great advantages, such as low-cost, small-size, simplicity of construction, ease of use, high sensitivity and selectivity (Liu et al., 2005; Cash et al., 2009; Ma et al., 2011; Lei and Ju, 2012).

So far, a variety of amplification strategies have been established for sensitive detection of miRNA, such as nanomaterials (Li

* Corresponding author. Fax: +86 23 68485786.

E-mail addresses: dingshijia@163.com, dingshijia@cqmu.edu.cn (S. Ding).

¹ These authors contributed equally to this work.

et al., 2014), hybridization chain reaction (Ge et al., 2014), and exonuclease III assisted signal amplification (Liu et al., 2013) and so on. Although the amplification strategies can greatly enhance the sensitivity of detection, these strategies have their disadvantages, including expensive reagents, poor stability, and vulnerable to external environment influence. Recently, catalytic hairpin assembly (CHA), developed from DNA nanostructure organization, has received particular interest owing to its excellent property of signal amplification (Yin et al., 2008; Li et al., 2011, 2012; Liao et al., 2014). Based on different technology platforms, such as fluorescence, colorimetry, and electrochemistry, miscellaneous CHAs have been developed (Zheng et al., 2012a, 2012b; Liu et al., 2013). As an enzyme-free amplification method, CHA overcomes the disadvantages of enzymatic amplification, such as complex operation, specific reaction conditions and the reaction-time dependent enzyme activity. Although CHA has a lot of advantages, a great background signal, caused by the nonspecific CHA products in the absence of target, is the most trouble problem (Chen et al., 2013; Jiang et al., 2014; Bhadra and Ellington, 2014), which may counteract the specificity of CHA-based signal amplification methodologies and compromise their analytical performance.

Aiming at improving the analytical performance of CHA-based biosensing strategies for miRNA detection, this work introduced mismatched base pairs into the breathing sites of the hairpin substrates, which could reduce nonspecific CHA products in the absence of target. A simple and specific electrochemical miRNA biosensor was developed based on enzyme-free mismatched CHA for the first time. The lower background leakage of mismatched CHA would ensure high sensitivity and specificity of the designed strategy. The designed platform exhibited excellent analytical performance towards miRNA determination and might become a potential alternative tool for detection of miRNA in biomedical research and early clinical diagnosis.

2. Experimental

2.1. Reagents

Diethylpyrocarbonate (DEPC) was purchased from Sangon Inc. (Shanghai, China). DNA oligonucleotides were synthesized and purified by Sangon Inc. Breast adenocarcinoma (MCF-7) total RNA was obtained from Ambion (TX, USA). The base sequences are listed in Table S1 (Supporting information). 6-Mercapto-1-hexanol (MCH), streptavidin-alkaline phosphatase (ST-AP), α -naphthyl phosphate (α -NP), bovine serum albumin (BSA) and salmon sperm DNA were purchased from Sigma-Aldrich (St. Louis, MO, USA). MiRNA-21 was obtained from Takara (Dalian, China). All other reagents were of analytical reagent grade. All the consumable items were treated by DEPC and sterilized three times. TNAK buffer as the hybridization buffer (pH 7.5) contained 20 mM Tris, 140 mM NaCl, 5.0 mM KCl. Tris-HCl buffer as washing buffer (pH 7.40) contained 20 mM Tris, 0.10 M NaCl, 5.0 mM MgCl₂ and 0.05% Tween-20. Diethanolamine (DEA) buffer (pH 9.6) contained 0.1 M DEA, 1 M MgCl₂ and 100 mM KCl.

2.2. Apparatus

Electrochemical characterizations including differential pulse voltammetry (DPV), square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) measurements were carried out on a CHI660D electrochemical workstation (Shanghai Chenhua Instruments Co. Ltd., China) with a conventional three-electrode system composed of platinum wire as auxiliary, Ag/AgCl electrode as reference and a 3-mm diameter gold electrode as working electrode. The SPR device Biacore XTM analytical system

and the bare gold sensor chip were used (Biacore AB, Uppsala, Sweden). There are two independent flow cells on each sensor chip (Fc1, Fc2). All SPR experiments were conducted at a sample flow rate of 5 $\mu\text{L min}^{-1}$ and at an operating temperature of 25 °C.

2.3. Preparation of probes

Hairpin probe 1 (H1) and hairpin probe 2 (H2) were designed based on the principle of the enzyme-free strand-displacement nucleic acid circuits system. All DNA oligonucleotides were denatured at 95 °C for 5 min and cooled down 5 °C per minute to the room temperature. Then the probes were stored at 4 °C for further use.

2.4. Preparation of electrochemical biosensor

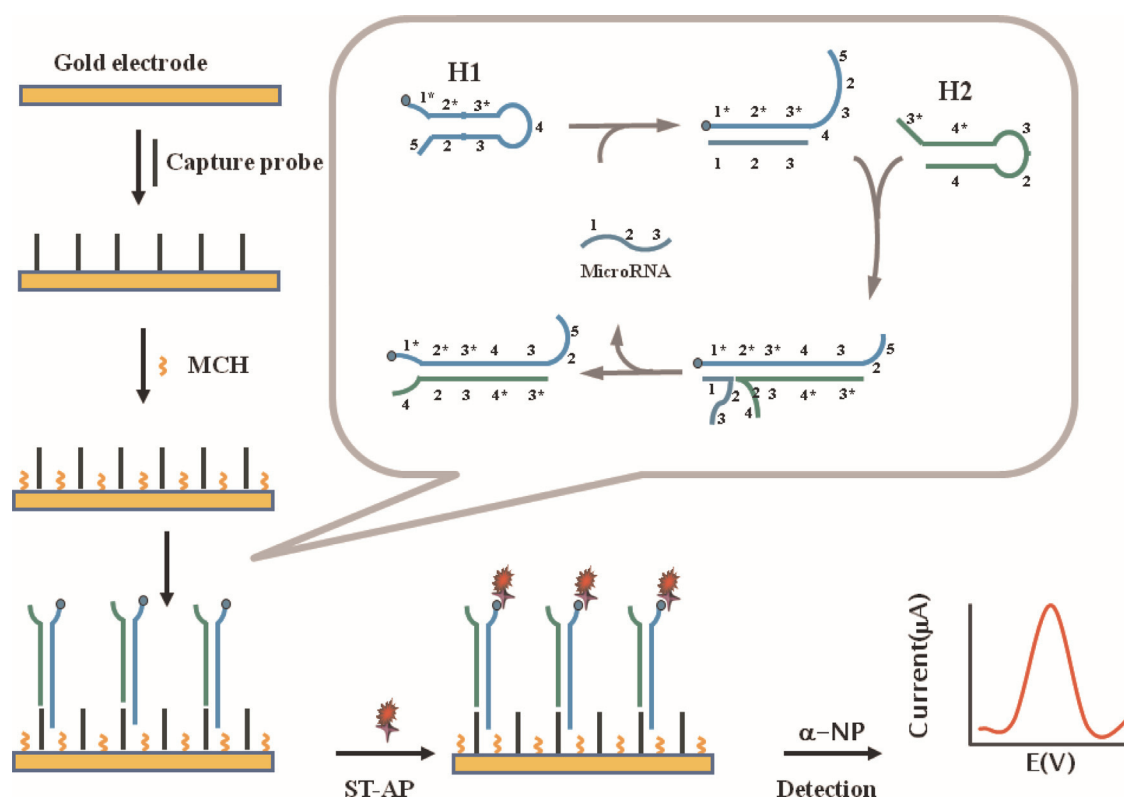
The bare gold electrode was polished with 50 nm alumina slurries and ultrasonically treated in ultrapure water for a few minutes, followed by soaking in piranha solution (H₂SO₄:H₂O₂ = 3:1) for 10 min to eliminate other substances. The pretreated gold electrode was rinsed with ultrapure water and allowed to dry at room temperature. 10 μL of 0.2 μM thiolated capture probe was dropped on the pretreated gold electrode surface and incubated overnight at 4 °C. After washed with the buffer, the electrode was immersed into 100 μL of 1 mM MCH for 1 h to obtain well-aligned DNA monolayer which would occupy the left bare sites. The electrode was further rinsed with the washing buffer and treated in salmon sperm DNA and 1% BSA for 30 min to block the non-specific binding sites on its surface to obtain the electrochemical DNA biosensor (Qian and He 2009; Cheng et al., 2012; Zhu et al., 2014).

CHA amplification system consisted of hairpin probes (H1 and H2), TNAK buffer, RNAase inhibitor, and DEPC-treated water. The final concentration of RNAase inhibitor was 1 U μL^{-1} . After 0.5 h amplification reaction, the products hybridized with the capture probes immobilized on the gold electrode for 0.5 h at 37 °C. Following washed by DEA buffer containing 0.05% Tween-20, 10 μL of 0.9 $\mu\text{g mL}^{-1}$ ST-AP was dipped onto the electrochemical biosensor at 37 °C for 30 min, and washed thoroughly with DEA buffer containing 0.05% Tween-20. The DPV measurement was performed in DEA buffer containing 1 mg mL⁻¹ of α -NP substrate with modulation time of 0.05 s, interval time of 0.017 s, step potential scan from 0.0 to +0.6 V.

3. Results and discussion

3.1. Design of the electrochemical sensor

The principle of the fabricated electrochemical biosensing platform for detection of miRNA is depicted in Scheme 1. The lengths of the hairpin stem determine the overall stability of the CHA circuit. Upon increasing of the base pair number of hairpin stem, the overall stability of hairpin is increased. As the overall stability increases, the background leakage decreases. The catalytic reaction rate is based on the length of the toehold. H1 unfolding rate favors the longest toehold as possible, on the contrary, the target dissociation rate favors the shortest toehold as possible. In order to keep the balance of the overall stability and catalytic rate, the 8 nt is chosen as the toehold, and 14 nt as the hairpin stem. At the 3'-end of H1, the domain 5 which only contain 7 nt is added. In the presence of target miRNA, domain 2 of the H1 is exposed, and the capture probe immobilized on the gold electrode can hybridize with the H1-H2 complexes through the domain 2 and domain 5. Through the hybridization interaction of H1 and H2, target miRNA is displaced by H2 and released into next circulation. After washed



Scheme 1. Schematic representation of miRNA electrochemical detection based on mismatched catalytic hairpin assembly amplification.

in DEA buffer and reacting with ST-AP, AP catalyzes the irreversible conversion of substrate α -NP to an electroactive product for generating an amplified electrochemical signal, which is quantified by differential pulse voltammetry (DPV).

3.2. Probe design principle and validity of the mismatched CHA amplification

The breath sites of hairpin were located at the ends of the hairpin stem. When the hairpin breathed, the ends of the stem could unfold occasionally. In the CHA amplification, this kind of unfolded stem could cause the non-specific binding between H1 and H2. To investigate the effects of the four breath sites on the background signal, we introduced two consecutive mismatched base pairs at the end of the stem and the adjacent region (Fig. 1A).

The validations of distinct mismatched CHA were monitored by electrochemical signal (Fig. 1B). Compared with the traditional hairpin substrates, the mismatched base pairs were introduced at the H2D2M2 (where H2 refers to the hairpin substrate, D2 refers to the region, M2 refers to the mismatched number) leading to a significant decrease of background signal, which had little effect on the signal in the presence of target. On the contrary, H1D1M2 and H2D3M2 not only had a little effect on the background signal, but also had some influence on the target signal, which did not improve the signal-to-noise ratio. Moreover, H1D4M2 may prevent the uncatalyzed reaction effectively and block the opening of H1 in the presence of target miRNA, which could decline the signal-to-noise ratio evidently. These results indicated that H2D2M2 was the best mismatched site and the signal-to-noise was promoted to 33.6 in H2D2M2, which would lead to a wider dynamic linear range and more sensitive and specific detection of miRNA.

Surface plasmon resonance was also used to further confirm mismatched CHA amplification process. The SPR experimental results indicated that the H2D2M2 suppressed background noise without compromising circuit performance (Fig. 1C and D), which

were in good agreement with those obtained from electrochemical measurements.

3.3. Characterization of electrode surface modification

EIS and SWV measurements were adopted to characterize the established electrochemical biosensor. The EIS curves were obtained in 0.4 M KCl containing 0.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ and the semicircle diameter was equal to electron-transfer resistance (R_{et}) (Fig. 2A). In 0.4 M KCl containing 0.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$, bare electrode exhibited an almost straight line (curve a) reflecting the outstanding electrochemical conductivity. When the thiolated capture DNA was self-assembled onto the bare electrode via Au-thiol binding, the R_{et} increased (curve b). This was because that the negatively charged phosphate backbone of the oligonucleotides produced an electrostatic repulsion force to $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$. However, after MCH and BSA were immobilized on the electrode surface, the R_{et} increased obviously (curve c), which was attributed to that the biomacromolecules could obstruct the electron transfer. Afterwards, the remaining capture DNA hybridized with the H1–H2D2M2 complexes without the participation of target miRNA, the R_{et} slightly increased (curve d). With participation of target miRNA, the R_{et} increased significantly (curve e), which indicated that only a few of H1 and H2D2M2 can initiate reaction in the absence of the target, and in the presence of target miRNA catalysis, a large amount of H1–H2D2M2 complexes were obtained. These results were in a good agreement with those obtained from SWV measurements (Fig. 2B), in which the peak currents varied upon the assembly and binding processes. Both results of EIS and SWV proved that the biosensor worked indeed as described in the principle scheme.

3.4. Optimization of experiment conditions

To achieve optimal sensing performance, different experimental parameters were investigated. The signal-to-noise ratio was used to

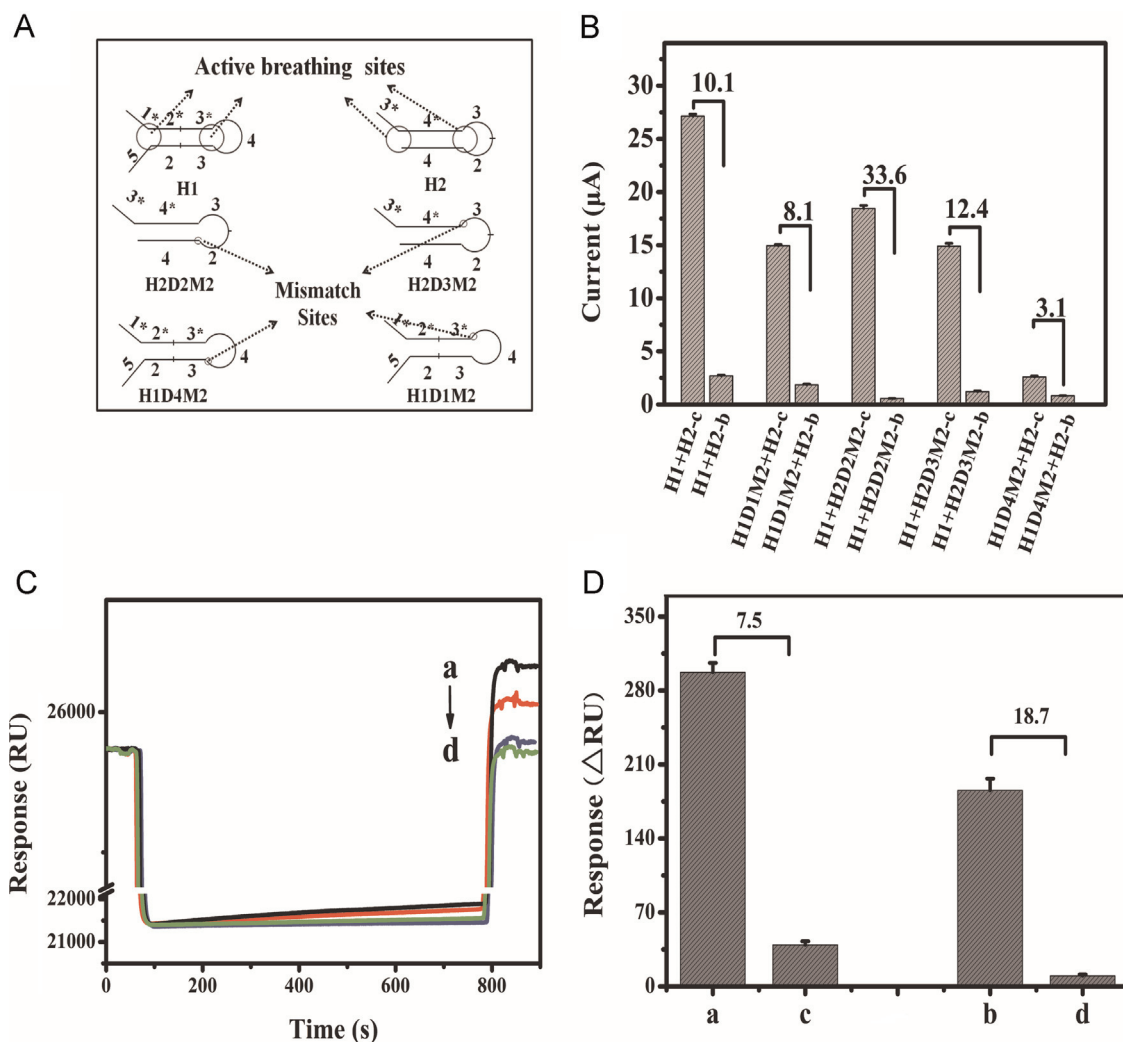


Fig. 1. (A) The four mismatch positions correspond to the revealed interactions that could initiate CHA reactions between H1 and H2. (B) Signal-to-background ratio for four different mismatches. c denotes a target concentration of 25 nM, b denotes an experiment conducted without target. Signal-to-noise ratios were calculated from the linear portion of each electrochemical curve. (C) SPR sensorgram and (D) signal of hybridization with H1–H2 complexes with target (a), H1–H2D2M2 complexes with target (b), H1–H2 complexes without target (c), H1–H2D2M2 without target (d).

evaluate the performance of the electrochemical biosensor. The mismatched CHA process could be strongly influenced by both the mismatched number and concentration of H2D2M2. The signal-to-

noise ratio increased as the increasing mismatched number of the H2D2M2 up to 2, but the signal-to-noise ratio decreased with the further increasing of mismatched number, indicating that the more

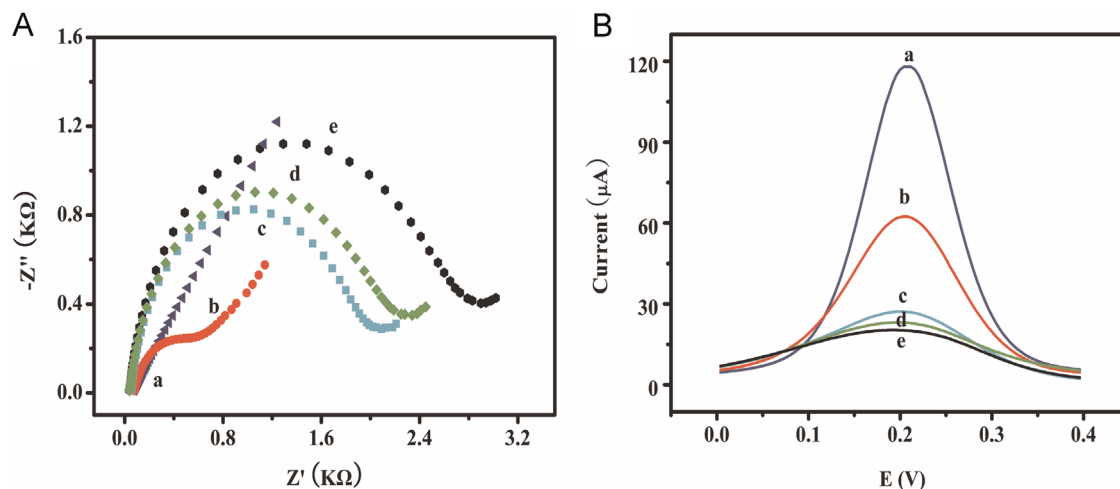


Fig. 2. EIS (A) and SWV (B) in 0.4 M KCl containing 0.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ at bare electrode (a), capture DNA modified electrode (b), MCH and BSA immobilized on the electrode surface (c), capture DNA modified electrode after hybridized with H1–H2 complexes without (d) and with target miRNA (e), respectively.

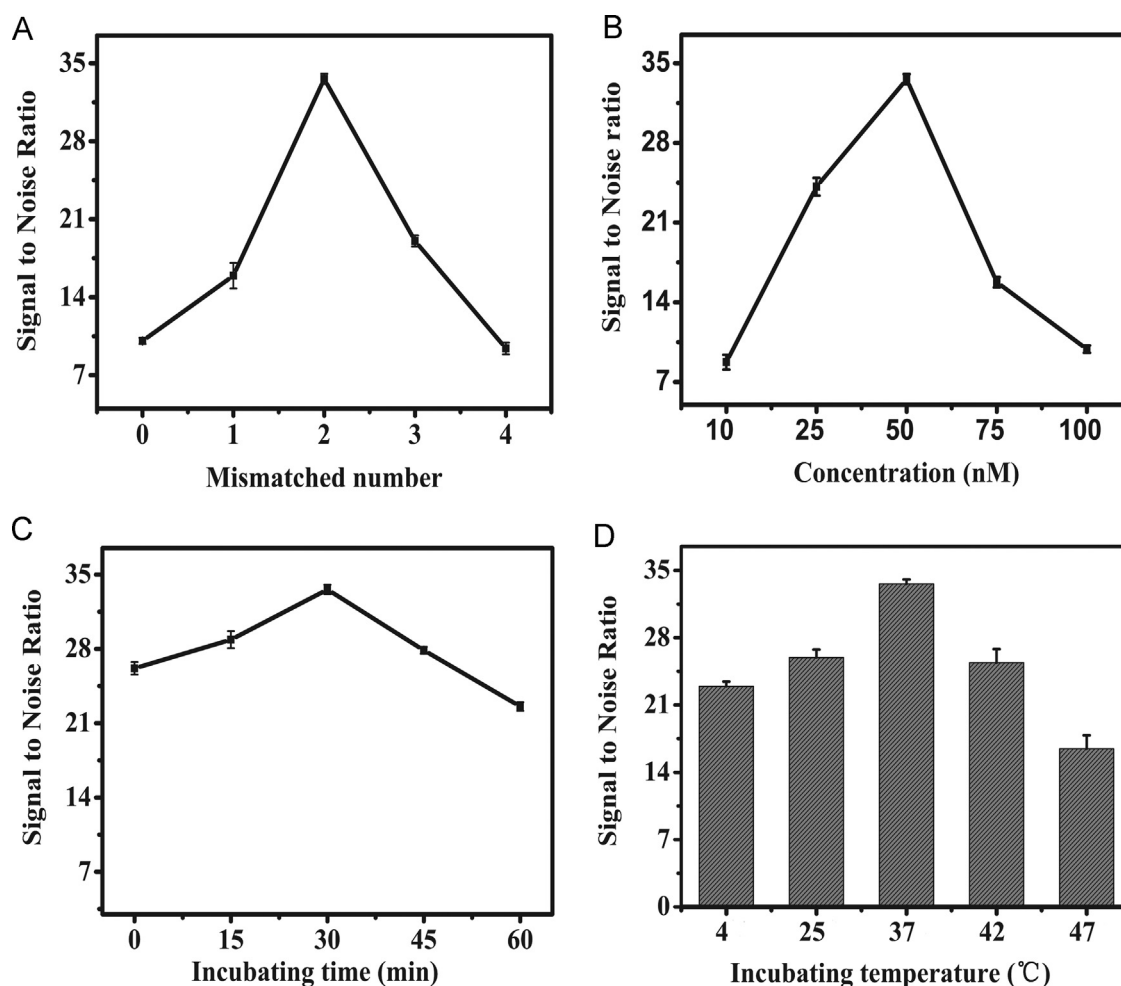


Fig. 3. Optimizations of experimental parameters: (A) evaluation of the effect of mismatched number, (B) evaluation of the effect of concentration of H2D2M2, (C) evaluation of the effect of incubating time on the signal-to-noise ratio, and (D) evaluation of the effect of incubating temperature on the signal-to-noise ratio. When one parameter changed, the others were under their optimal conditions.

mismatched number not only decreased the background but also significantly prevented the target miRNA catalyzed reaction (Fig. 3A). Therefore, double mismatched number was chosen as the optimal mismatched number of H2D2M2. With the increasing concentration of H2D2M2, the signal-to-noise ratio also increased and tended to decrease at 50 nM, indicating that the higher concentration of H2D2M2 increased the target miRNA catalyzed reaction and the nonspecific CHA products (Fig. 3B).

In order to enhance detection sensitivity, the incubating time and temperature were also optimized. The effect of CHA incubating time on the signal-to-noise ratio was investigated in Fig. 3C. As anticipated, the signal-to-noise ratio increased with the increment of incubating time and reached the maximum at 30 min, indicating the signal produced by target miRNA catalyzed reaction products reached the maximum at 30 min and kept steady status, but the signal caused by nonspecific products increased with the increase of incubating time. The incubating temperature was also examined from 4 to 47 °C (Fig. 3D), and the maximum signal-to-noise ratio was achieved at 37 °C. At low temperature, H1 and H2D2M2 could not get a sufficient collision probability that significantly reduced the formation of H1–H2D2M2 complexes; at high temperature, non-stable hairpins would lead a large number of nonspecific products. Thus 37 °C was selected for the optimal incubating temperature.

3.5. Analytical performance of miRNA detection

Under the optimal experimental conditions, the dynamic range and sensitivity of the proposed electrochemical sensor was confirmed. As shown in Fig. 4A, The DPV peak current increased with the increase of target miRNA concentration. The calibration plots showed a good linear relationship between the peak currents and the logarithm of target miRNA concentrations in the range from 1 pM to 25 nM (Fig. 4B), with detection limit of 0.6 pM from three times the standard deviation corresponding to the blank sample detection. The resulting linear equation was $i_p(A) = 3.64E-6 \log C_{miRNA} + 1.2E-5$ with a correlation coefficient of 0.9973. In order to elucidate the mismatched CHA effect on amplification efficiency, the DPV assays were performed using the traditional H2 instead of H2D2M2 under the same condition. As a result, the linear ranges from 25 pM to 25 nM was achieved for miRNA with detection limit of 10 pM (Fig. 4C and D), which provided an effective evidence for the sensitivity improvement of proposed mismatched CHA amplification. To further highlight the merits of the designed miRNA biosensor, the analytical properties were compared with those of other biosensors based on the CHA amplification. Characteristics including the linear range and LOD were summarized in Table S2 (Supporting information). It proved that the mismatched CHA strategy in this work hold the feature of the lowest detection limit among the reported biosensors based on the traditional CHA strategy. Thus, this biosensor could be applied

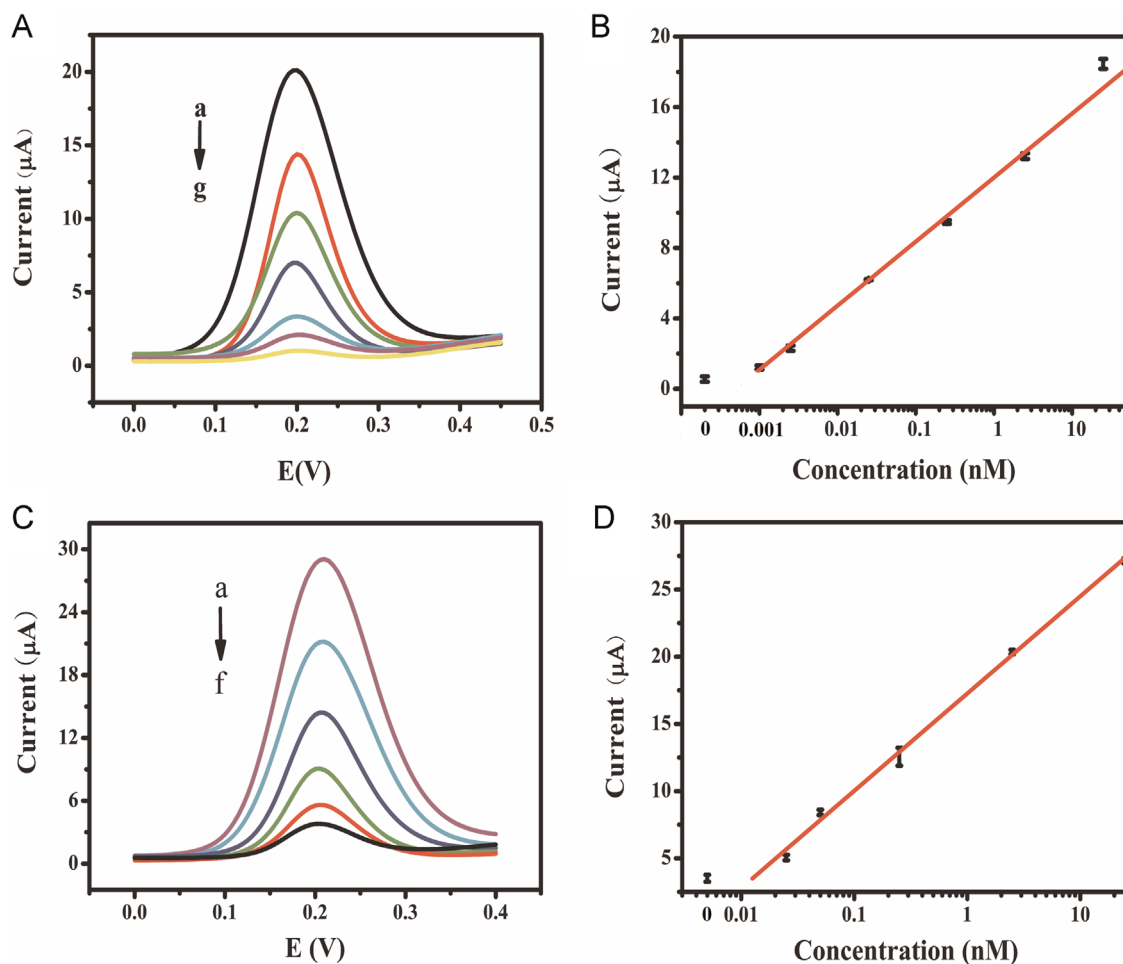


Fig. 4. (A) DPV responses and (B) the corresponding calibration curve to 25, 2.5, 0.25, 0.025, 0.0025, 0.001 and 0 nM of target miRNA (from a to g) using mismatched CHA amplification. (C) DPV responses and (D) the corresponding calibration curve to 25, 2.5, 0.25, 0.025 and 0 nM of target miRNA using traditional CHA amplification.

to quantification of miRNA with a wide linear range and low detection concentration.

3.6. Specificity and reproducibility of the proposed sensor

The specificity of the proposed electrochemical sensor was evaluated by detecting five different kinds of control miRNA

sequences, including complementary target (mir-21), single-base mismatched strand (SM), double-base mismatched strand (DM), non-complementary mismatched strand (NC) and mir-222 at the same condition. As shown in Fig. 5A, the DPV currents for 250 pM of DM, 250 pM of NC and 250 pM of mir-222 were all similar to that of the blank. However, in the presence of target miRNA at only 25 pM concentration, there was a remarkable increase in the DPV

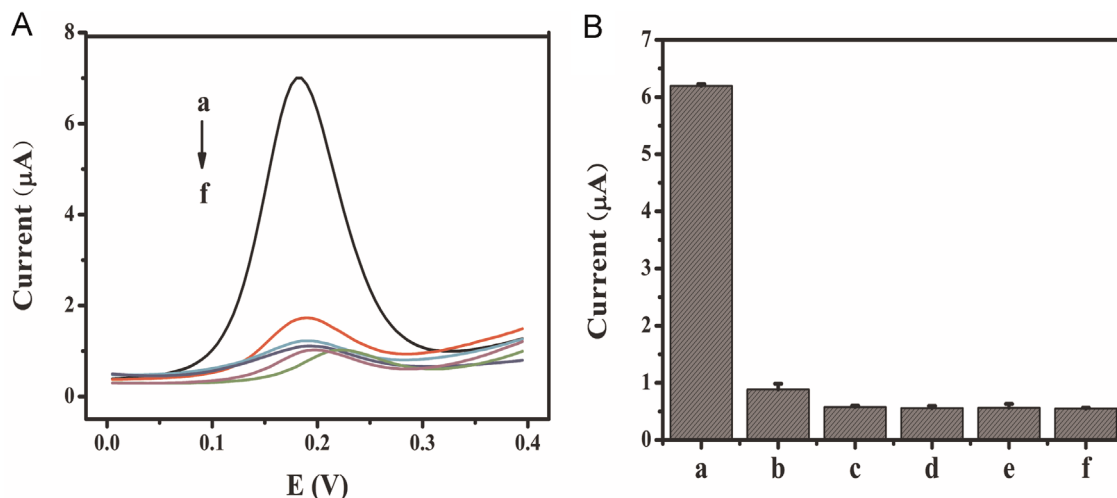


Fig. 5. (A) Typical DPV curves and (B) DPV peak currents respectively respond to 25 pM of target miRNA (a), 250 pM of SM (b), 250 pM of DM (c), 250 pM of NC (d), 250 pM of mir-222 (e), and blank (f). The error bars represent the standard deviations in three different measurements for each concentration.

current, which was about 7 times as that of SM and 10 times as that of DM and NC (Fig. 5B). In addition, the reproducibility of prepared biosensor was further investigated by measuring the target miRNA at 1 pM to 25 nM with five replicates, respectively. The coefficients of variation for both concentrations were about 1.7% and 4.8%. Therefore, the electrochemical sensor was able to effectively detect the target miRNA with high specificity and good reproducibility by the mismatched CHA and had great potential for clinical analysis.

3.7. Real sample analysis

To demonstrate the capability of the proposed method in real sample analysis, we performed the miRNA assay using MCF-7 total RNA. The RNA sample was diluted to 200 ng μL^{-1} with DEPC treated water, and 1 μL of the total RNA sample (200 ng in total) was used for the measurement. The electrochemical signal produced by 200 ng of total RNA can be distinguished obviously from that by the blank. According to the simultaneously constructed calibration curve (Supporting Information, Fig. S1), the amount of mir-21 in the total RNA sample was estimated to be 13.8 amol per 200 ng (RSD=3.4%, $n=5$). For further evaluation of its performance in real sample analysis, 20 amol mir-21 was spiked into 200 ng of MCF-7 total RNA for the assay, and the amount of mir-21 in the spiked sample was estimated to be 36 amol (RSD=2.6%, $n=5$) with a recovery ratio of 106.5%, suggesting that the analytical performance of the biosensor was not comprised in complex mixtures. These results demonstrated that the designed biosensing method provided a potential tool for detection of miRNA in biological samples.

4. Conclusion

In summary, this work has demonstrated an innovative and simple electrochemical sensor for highly sensitive and specific detection of target miRNA by taking advantages of the mismatched CHA amplification. The developed electrochemical biosensor based on the mismatched CHA amplification substantially decreased the background reaction signal, which improved the sensitivity for detection of target miRNA down to 0.6 pM only within 1.5 h, and a good performance in real sample analysis. It showed a wide dynamic detection range, high sensitivity, excellent specificity and good reproducibility, and the amplification process was performed without the participation of enzymes. Therefore, the proposed electrochemical sensor would become a potential alternative platform for detection of miRNA in biomedical research and early clinical diagnosis.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (81371904 and 81101638), the Natural Science Foundation Project of CQ (CSTC2013jjB10019 and cstc2014kjc-qncr0012) and Application Development Plan Project of Chongqing (cstc2014yykfB10003).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2015.01.026>.

References

- Bhadra, S., Ellington, A.D., 2014. *Nucleic Acids Res.* 42, e58.
- Chowdhury, D., Choi, Y.E., Brault, M.E., 2013. *Nat. Rev. Mol. Cell Biol.* 14, 181–189.
- Cullen, B.R., 2009. *Nature* 457, 421–425.
- Cissell, K.A., Deo, S.K., 2009. *Anal. Bioanal. Chem.* 394, 1109–1116.
- Cash, K.J., Heeger, A.J., Plaxco, K.W., Xiao, Y., 2009. *Anal. Chem.* 81, 656–661.
- Chen, X., Briggs, N., McLain, J.R., Ellington, A.D., 2013. *PANS* 110, 5386–5391.
- Cheng, W., Ding, S.J., Li, Q., Yu, T.X., Yin, Y.B., Ju, H.X., Ren, G.S., 2012. *Biosens. Bioelectron.* 36, 12–17.
- Duan, R., Zuo, X., Wang, S., Quan, X., Chen, D., Chen, Z., Jiang, L., Fan, C., Xia, F., 2013. *J. Am. Chem. Soc.* 135, 4604–4607.
- Ge, Z.L., Lin, M.H., Wang, P., Pei, H., Yan, J., Shi, J.Y., Huang, Q., He, D.N., Fan, C.H., Zuo, X.L., 2014. *Anal. Chem.* 86, 2124–2130.
- Gangaraju, V.K., Lin, H., 2009. *Nat. Rev. Mol. Cell Biol.* 10, 116–125.
- Jiang, Y.S., Bhadra, S., Li, B.L., Ellington, A.D., 2014. *Angew. Chem. Int. Ed.* 126, 1876–1879.
- Koshiol, J., Wang, E., Zhao, Y., Marincola, F., Landi, M.T., 2010. *Cancer Epidemiol. Biomark. Prev.* 19, 907–911.
- Lee, I., Ajay, S.S., Chen, H., Maruyama, A., Wang, N., McInnis, M.G., Athey, B.D., 2008. *Nucleic Acids Res.* 36, e27.
- Leshkowitz, D., Horn-Saban, S., Parmet, Y., Feldmesser, E., 2013. *RNA* 19, 527–538.
- Lao, K., Xu, N.L., Yeung, V., Chen, C., Livak, K.J., Straus, N.A., 2006. *Biochem. Biophys. Res. Commun.* 343, 85–89.
- Lagos-Quintana, M., Rauhut, R., Lendeckel, W., Tuschl, T., 2011. *Science* 294, 853–858.
- Lei, J.P., Ju, H.X., 2012. *Chem. Soc. Rev.* 41, 2122–2134.
- Liao, Y.H., Huang, R., Ma, Z.K., Wu, Y.X., Zhou, X.M., Xing, D., 2014. *Anal. Chem.* 86, 4596–4604.
- Liu, S.F., Wang, Y., Ming, J.J., Lin, Y., Cheng, C.B., Li, F., 2013. *Biosens. Bioelectron.* 49, 472–477.
- Liu, G., Lee, T.M.H., Wang, J., 2005. *J. Am. Chem. Soc.* 127, 38–39.
- Li, B.L., Jiang, Y., Chen, X., Ellington, A.D., 2012. *J. Am. Chem. Soc.* 134, 13918–13921.
- Li, B.L., Ellington, A.D., Chen, X., 2011. *Nucleic Acids Res.* 39, e110.
- Li, F.Y., Peng, J., Wang, J.J., Tang, H., Tan, L., Xie, Q.J., Yao, S.Z., 2014. *Biosens. Bioelectron.* 54, 158–164.
- Liu, S.F., Wang, C.F., Zhang, C.X., Wang, Y., Tang, B., 2013. *Anal. Chem.* 84, 2282–2288.
- Markou, A., Tsaroucha, E.G., Kaklamanis, L., Fotinou, M., Georgoulas, V., Lianidou, E. S., 2008. *Clin. Chem.* 54, 1696–1704.
- Ma, C., Wang, W., Yang, Q., Shi, C., Cao, L., 2011. *Biosens. Bioelectron.* 26, 3309–3312.
- Nicoloso, M.S., Spizzo, R., Shimizu, M., Rossi, S., Calin, G.A., 2009. *Nat. Rev. Cancer* 9, 293–302.
- Pall, G.S., Codony-Servat, C., Byrne, J., Ritchie, L., Hamilton, A., 2007. *Nucleic Acids Res.* 35, e60.
- Qian, H., He, L., 2009. *Anal. Chem.* 81, 4536–4542.
- Shi, R., Chiang, V.L., 2005. *Biotechniques* 39, 519–525.
- Varallyay, E., Burgyan, J., Havelda, Z., 2008. *Nat. Protoc.* 3, 190–196.
- Vá ló czi, A., Hornyik, C., Varga, N., Burgyá n, J., Kauppinen, S., Havelda, Z., 2004. *Nucleic Acids Res.* 32, e175.
- Wahlestedt, C., 2013. *Nat. Rev. Drug Discov.* 12, 433–440.
- Yin, P., Choi, H.M.T., Calvert, C.R., Pierce, N.A., 2008. *Nature* 451, 318–323.
- Zhou, Y., Huang, Q., Gao, J., Lu, J., Shen, X., Fan, C., 2010. *Nucleic Acids Res.* 38, e156.
- Zheng, A.X., Wang, J.R., Li, J., Song, X.R., Chen, G.N., Yang, H.H., 2012a. *Biosens. Bioelectron.* 36, 217–221.
- Zheng, A.X., Li, J., Wang, J.R., Song, X.R., Chen, G.N., Yang, H.H., 2012b. *Chem. Commun.* 48, 3112–3114.
- Zhu, D., Yan, Y.R., Lei, P.H., Shen, B., Cheng, W., Ju, H.X., Ding, S.J., 2014. *Anal. Chim. Acta* 846, 44–50.