analytical chemistry

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Ultrasensitive Electrochemical Detection of MicroRNA Based on an Arched Probe Mediated Isothermal Exponential Amplification

Yanyan Yu,[†] Zuanguang Chen,*[,†] Lijuan Shi,[†] Fan Yang,[‡] Jianbin Pan,[§] Beibei Zhang,[†] and Duanping Sun[†]

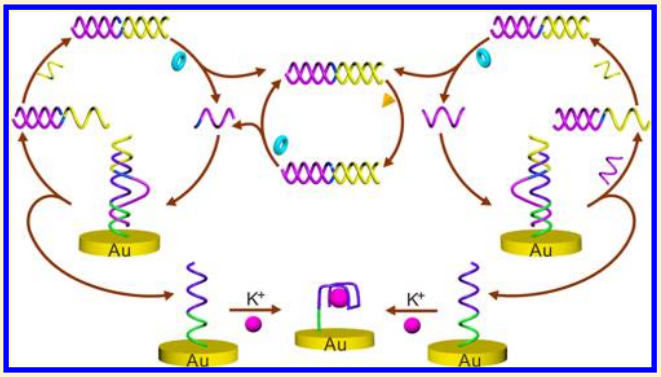
[†]School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, Guangdong 510006, China

[‡]School of Laboratory Medicine, Hubei University of Chinese Medicine, Wuhan, Hubei 430065, China

[§]State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing, Jiangsu 210093, China

Supporting Information

ABSTRACT: In this work, a simple and label-free electrochemical biosensor is developed for microRNA (miRNA) detection on the basis of an arched probe mediated isothermal exponential amplification reaction (EXPAR). The arched probe assembled on the electrode surface consists of two strands that are partially complementary to each other at both ends. The target can hybridize with the complementary sequence of the arched structure, leading to the cleavage of the probe. The strand fixed on the surface of the electrode self-assembles, in the presence of hemin, to G-quadruplex unit, yielding electrochemical signals. The other strand liberated into the solution triggers the EXPAR to recycle and regenerate targets. This method exhibits ultrahigh sensitivity toward miRNA with detection limits of 5.36 fM and a detection range of 3 orders of magnitude. The biosensor is capable of discriminating a single-nucleotide difference between concomitant miRNA and performs well in analyzing crude extractions from cancer cell lines.



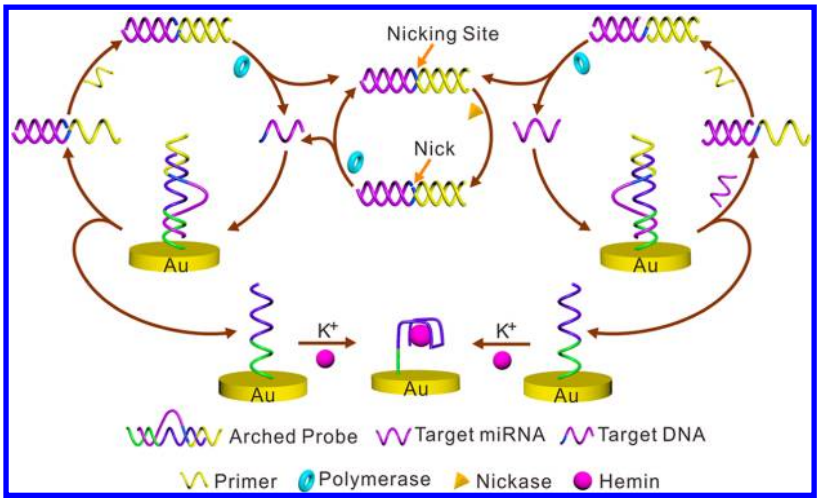
MicroRNAs (miRNAs) are abundant 22 nucleotide (nt) regulatory RNAs, derived from endogenous short hairpin transcripts, that are thought to function primarily as antisense regulators of other RNAs.[1] In general, miRNA, especially in animals, regulates protein synthesis post-transcriptionally by base pairing to target miRNAs. Abundant studies have revealed that various diseases, such as human cancers, cardiovascular diseases, and viral infections are directly associated with miRNA expression profiles.[2−4] Hence, effective detection and quantitation of miRNA is of great significance for biomedical research, early clinical diagnosis, pathogenesis of diseases, and therapeutic intervention.[5−7] To meet the urgent demands for miRNA expression analysis, several analytical approaches have been developed up to now. Northern blotting methods[8,9] and microassay-based techniques[10,11] are widely utilized in miRNA quantification. Unfortunately, the sensitivity and specificity are not satisfactory attributed to the intrinsic characteristics of miRNA, such as small size, sequence homology among family members, and low abundance.[12] Recently, various amplification strategies have been proposed for miRNA detection, including modification of the invader assay,[13] ribozyme amplification,[14] real-time polymerase chain reaction (RT-PCR),[15] rolling circle amplification,[16,17] and nanoparticle-based approaches.[18,19] Among all the amplification methods, RT-PCR paved a powerful way to ultrasensitive and accurate quantitation of miRNA. Nevertheless, the requirement of precise control of temperature cycling hindered the versatile applications, and the short length of miRNA made the PCR design rather sophisticated, especially in some complex clinical samples.[20] As an alternative amplification technique, the isothermal exponential amplification reaction (EXPAR) has gained great attention in profiling low-abundance miRNA because of its high sensitivity, low cost, and good tolerance to the inhibitory components in the clinical samples.[21,22] By combination of polymerase strand extension and single-strand nicking, EXPAR has the intrinsic merits of isothermal nature, high amplification efficiency, and rapid amplification kinetics, which can provide 10^6-10^9-fold amplification within minutes under isothermal conditions.[23−25]

Electrochemical methods, with high portability and affordability, are particularly attractive for nucleic acid detection. Various signal amplification strategies have been proposed to achieve ultrasensitive electrochemical nucleic acid detection, such as the nanoparticle amplified assay,[26,27] quantum dots mediated detection,[28] and enzyme-based detection.[29,30]

Received: April 24, 2014
Accepted: July 23, 2014

   dx.doi.org/10.1021/ac501505a | *Anal. Chem.* XXXX, XXX, XXX−XXX

Scheme 1. Schematic Illustration of the Arched Probe Mediated EXPAR Strategy Based on Polymerase and Nicking Endonuclease for the miRNA Assay



Although these methods enabled low detection limit, the labeling process is laborious or expensive. Thus, it is desirable to design a convenient and efficient amplification system for label-free and sensitive miRNA detection.

In the present work, we developed an ultrasensitive and label-free electrochemical miRNA biosensor based on an arched probe mediated EXPAR. MiR-21, as a potential cancer biomarker with elevated expression levels in numerous tumor tissues, was chosen as the target miRNA. The two strands of the arched probe, which were partially hybridized with each other, contained a target-complementary region and G-quadruplex-forming region, respectively. After hybridization of target miRNA with the complementary strand, the arched probe cleaved, leading to the initiation of the amplification cycles and the release of the G-quadruplex-forming region. By combination of EXPAR and electrochemical detection, this new strategy allowed ultrasensitive quantification of miRNA with a detection limit of 5.36 fM. The approach proposed also performed well when applied to crude extractions from cell lines.

EXPERIMENTAL SECTION

Chemicals and Materials. DNA sequences were synthesized from Sangon Biotech. Co., Ltd. (Shanghai, China). MiRNAs were synthesized by TaKaRa Bio Inc. (Dalian, China). The Bst DNA polymerase, Large Fragment, Nb.BbvCI nicking endonuclease, deoxynucleotide solution mixture (dNTPs), and CutSmart Buffer were purchased from New England Biolabs (Beijing, China). Diethypyrocarbonate (DEPC) treated water and TE buffer were purchased from Sangon Biotech. Co., Ltd. (Shanghai, China). Hemin, tris(2-carboxyethy) phosphine hydrochloride (TCEP), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt (HEPES), and dimethyl sulfoxide (DMSO) were obtained from Aladin Chemistry Co. Ltd. (Shanghai, China). 6-Mercapto-1-hexanol (MCH) was from J&K Scientific Ltd. (Guangzhou, China). One mM MCH solution was made by dilution of the MCH with DNA hybridization buffer (10 mM HEPES, 500 mM NaCl, pH 7.4). A stock solution of hemin (10 mM) was prepared in DMSO and stored in the dark at −20 °C. DEPC-treated water was used throughout. All DNA sequences were diluted in 1× TE buffer (pH 8.0) to give stock solutions of 100 μM. Invasive breast ductal carcinoma (MCF-7) cells were kindly provided by Ph.D. candidate Lu Jing from Department of Pharmacology and Toxicology in Sun Yat-sen University (Guangzhou, China). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, L-glutamine, fugizone, and fetal bovine serum (FBS) were all purchased from Gibco (Gibco Invitrogen Corporation, USA). Sequences of the oligonucleotides were designed with the help of online software (from the Web site of Integrated DNA Technologies (IDT)), as listed in Table S-1(Supporting Information).

Biosensor Fabrication. The gold electrode (3 mm in diameter) was cleaned by immersion in a freshly prepared piranha solution (a 3:1 v/v mixture of concentrated H_2SO_4 and 30% H_2O_2) for 20 min, followed by a thorough rinse with ultrapure water. *CAUTION: Piranha solution reacts violently with organic materials; it must be handled with extreme care.* Then, the electrode was polished with 0.05 μm alumina slurry to obtain a mirror surface, followed by sonication in ethanol and ultrapure water for 5 min each to remove residual alumina powder. The electrode was then electrochemically cleaned over the potential between −0.2 and 1.5 V in H_2SO_4 (0.5 M) at a scan rate of 100 mV s^{-1} until a stable cyclic voltammogram was obtained. The gold electrode was rinsed with a copious amount of ultrapure water before it was blow-dried with nitrogen.

The arched duplex probe was prepared by mixing Strand 1 and Strand 2 in hybridization buffer (10 mM HEPES, 500 mM NaCl, pH 7.4). The solution was incubated at 90 °C for 5 min and then was allowed to cool to room temperature for at least 2 h. The probe was incubated with 1 mM TCEP for 1 h to reduce disulfide bonds before being assembled onto the electrode. The pretreated gold electrode was subjected to the above probe solution at 4 °C for 12 h and then was thoroughly rinsed with hybridization buffer. After drying with nitrogen, the electrode was subsequently immersed in 1 mM MCH solution for 1 h to remove the nonspecific DNA adsorption. Then, the electrode surface was rinsed and dried.

Preparation of Cell Extracts. MCF-7 cells were routinely cultured in traditional cell culture dishes in a humidified atmosphere at 37 °C with 5% CO_2, using DMEM supplemented with 10% FBS, 100 μg mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Total RNA of MCF-7 cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA quantity

dx.doi.org/10.1021/ac501505a | Anal. Chem. XXXX, XXX, XXX−XXX

was determined by measuring the absorbance at 260 nm with a NanoDrop ND-1000 UV−vis Spectrophotometer (NanoDrop Technologies).

Arched Probe-Based EXPAR. The probe modified gold electrode was incubated into 50 μL of CutSmart Buffer containing 32 U Bst DNA polymerase, 20 U nicking endonuclease, 400 μM dNTPs, 2 μM primer, and different concentrations of target DNA for 60 min at 30 °C. The resulting electrode was thoroughly rinsed before being subjected to 0.1 mM hemin in 20 mM HEPES buffer (pH 8.0, 50 mM KCl, 200 mM NaCl, 1% DMSO) for 30 min to induce the liberated Strand 2 on the electrode to fold into a G-quadruplex−hemin complex.

Electrochemical Measurement and Apparatus. Electrochemical experiments were conducted on a RST5200F electrochemical workstation (Suzhou Risetest Instrument Co., Ltd., Suzhou, China). A three-electrode system, consisting of bare or modified gold electrode, an Ag/AgCl reference electrode, and a platinum plate counter electrode, was employed in all electrochemical measurements. The differential pulse voltammetry (DPV) was performed with the potential window from −0.15 to −0.5 V in 20 mM HEPES buffer (pH 8.0, 50 mM KCl, and 200 mM NaCl), which was degassed with nitrogen for 15 min to avoid the interference from the reduction of oxygen. The electrochemical impedance spectroscopy (EIS) experiments were recorded under the stable open circuit potential (OCP) in 5 mM $Fe(CN)_6^{3-/4-}$ solution containing 500 mM KCl with the frequency ranging from 0.1 Hz to 100 kHz and 0.007 V for the amplitude of alternating current.

RESULTS AND DISCUSSION

Principle of the Sensor for miRNA Detection. This newly designed electrochemical biosensor based on an arched probe mediated EXPAR was illustrated in Scheme 1. The arched probe consisted of two strands (Strand 1 and Strand 2, as shown in Table S-1, Supporting Information), which were partially complementary at both ends. The two sequences were designed to act as blockers to each other. Strand 2 prevented the hybridization of primer with Strand 1. Meanwhile, Strand 1 minimized the formation of any nonspecific hemin-containing G-quadruplex from Strand 2. The recognition site of the nicking endonuclease was located in the loop region of Strand 2 and was unsuitable to bind with the enzyme. Target miRNA was complementary to the 5′ stem region and part of the loop region of Strand 1. The separation of one hybridized domain through the formation of a target−substrate complex resulted in the thermal melting of the remaining duplex.[31−33] After cleavage of the arched probe, the free Strand 2 self-assembled, in the presence of hemin, to the G-quadruplex unit on the surface of the electrode, giving electrochemical signals.[34,35] On the other hand, Strand 1 hybridized with the target was released to the solution and initiated a series of cyclic chain amplification reactions. Once the engaging primer annealed with the complementary region of Strand 1, the polymerase initiated the polymerization, which regenerated the target miRNA and synthesized a DNA duplex. As a result, the displaced miRNA was free to bind to another probe and triggered a new cycle for recycling the target and forming a DNA duplex as well. The DNA duplex generated above activated the recognition site of the nicking enzyme. After the nicking endonuclease nicked at the DNA duplex, the polymerization started and the primer part got extended again. At the same time, a single strand DNA will be generated and released, whose sequence contained the same part of the target miRNA except that the ribonucleotides and uridine were replaced with deoxyribonucleotides and thymine, respectively. Thus, nicking, extension, and strand displacement repeated continuously, generating more target DNA sequences. Hybridization of the released target DNA with the arched probe initiated another series of cyclic amplification recycle processes. Through the miRNA recycle circle and the target DNA regenerate circle, many more probes can be activated, resulting in an enhanced electrochemical response.

Electrochemical Characterization of the Arched Probe Mediated EXPAR. The stepwise modification process of the electrode was monitored by EIS. Figure 1 showed the Nyquist

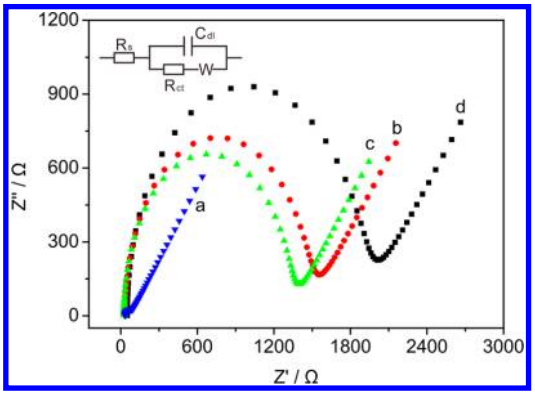


Figure 1. EIS spectra of bare gold electrode (a), after arched probe immobilization (b), further treatment by target miRNA (c), and the formation of G-quadruplex−hemin complex (d). Inset shows the equivalent circuit: the solution resistance (R_s), the double layer capacitance (C_{dl}), the charge-transfer resistance (R_{ct}), and the Warburg impedance (diffusion) element (W).

plots of electrode modification at different stages. For the bare electrode, the EIS exhibited a very small semicircular domain (curve a, 52.70 Ω). After self-assembly of DNA probes and MCH on the gold electrode, a noticeable increase in charge-transfer resistance (R_{ct}) was observed (curve b, 1518.33 Ω), which can be ascribed to the repellence to $[Fe(CN)_6]^{3-/4-}$ from negative charges on the phosphate backbone of the immobilized duplex probes on the surface. After treatment with target miRNA, R_{ct} decreased to 1372.45 Ω (curve c), as the duplex probe has cleaved into single-stranded oligomer, leading to the decrease of the negative charge of the electrode interface. Finally, after G-quadruplex−hemin formed, the electrochemical impedance dramatically increased, 1957.83 Ω for R_{ct} (curve d), as the G-quadruplex−hemin complexes made the interface on the electrode surface more orderly and reinforced the electrostatic repulsion. The stepwise alteration of the electrochemical impedance confirmed the self-assembly of the arched probe on the electrode surface as well as its viability to hybridize with target and fold into G-quadruplex.

DPV was utilized to further verify the feasibility of the designed amplification process. As depicted in Figure 2, a well-defined reduction peak around −0.32 V can be observed in the presence of 50 pM miRNA with an amplification process (curve e), which can be contributed to the electrochemical reduction of hemin incorporated in the G-quadruplex.[36,37] In contrast, the DPV peak from 50 pM target without amplification (curve b) was just a little higher than that from the blank control (curve a) whose electrochemical response can be ascribed to the

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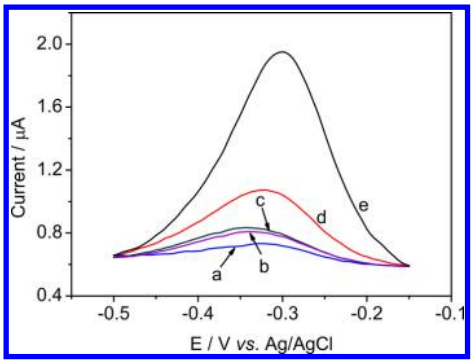

Figure 2. Differential pulse voltammograms of blank control (a), 50 pM miRNA without enzyme (b), with nicking endonuclease (c), or polymerase alone (d) and with both enzymes (e).

nonspecific adsorption of hemin on the electrode surface. The greatly enhanced current in curve e affirmed that much more target had been generated after EXPAR. As expected, the arched probe mediated EXPAR was characterized not only by target miRNA recycled through strand displacement but also by another target DNA regenerated reaction during subsequent cyclic reactions. For the proof-of-concept experiment, we carried out a series of experiments differing in enzymes. If only nicking endonuclease was added (curve c), no obvious improvement in current was obtained, compared to that without enzymes (curve b). The addition of polymerase alone enlarged the DPV peak significantly (curve d), indicating that the initiation of this reaction was strictly dependent on the polymerase and only polymerase can start the miRNA circular strand displacement reaction, whereas the exponential signal amplification greatly relied on the nicking endonuclease, as the peak current in the presence of both enzymes (curve e) was much higher (3 times) than that with polymerase alone (curve d). Without nicking endonuclease, the polymerase triggered a linear amplification process, but the exponential amplification process can be triggered with both enzymes. As the recycle reaction for DNA target was more efficient than that for miRNA target, the exponential process can generate a great amount of targets and enhance the electrochemical response significantly.

Optimization of the Arched Probe Mediated EXPAR. First, the immobilization concentration of duplex DNA probe was investigated to verify the effect of assembly density on the electrode performance. Different concentrations of duplex probes (from 0 to 2.0 μM) were used for the response toward 50 pM target miRNA. The result in Figure 3A showed that the immobilization concentration of 1.0 μM gave the best performance. The higher immobilization concentration of the probe can increase the DNA density on the electrode surface, whereas it gave rise to a greater steric hindrance effect as well, which may restrict the hybridization efficiency of the target and the effective folding of the quadruplex.[27] This factor may interpret the relatively lower electrochemical response at the higher concentration of the probe (2.0 μM). Thus, 1.0 μM was chosen as the optimized immobilization concentration for the following miRNA detection.

We further investigated the effects of reaction time on the EXPAR. The electrode modified with duplex probe was incubated in the amplification solution for a certain period of time ranging from 0 to 100 min. As demonstrated in Figure 3B, with the incubation time increased gradually, the peak currents rose accordingly. Meanwhile, the currents started to plateau after 60 min. The enhanced signal within 60 min validated the feasibility of the amplification process, while the plateau phenomenon indicated the cleavage of almost all the duplex DNA probes, liberating all available G-quadruplex-forming oligomers. Therefore, a reaction time of 60 min was chosen for further EXPAR.

Detection Performance of the Assay. To investigate the sensitivity of the proposed method, target miRNA with different concentrations was measured. To obtain the best performance, all samples were incubated at 30 °C for 60 min before being subjected to DPV measurements. It can be observed in Figure 4A that the DPV peak currents increased along with the concentration of target miRNA from 0−10 nM. The highly dependent relationship between the amount of target and the value of peak current verified the detection principle that more original miRNA will result in a greater amount of targets to anneal with the probe. As a result, elevated amounts of G-quadruplex-forming sequences were liberated to incorporate more hemin, leading to higher peak current. Figure 4B showed the variation of the peak current as a function of the concentration of target miRNA. For each concentration of miRNA, the electrochemical measurements were repeated for three times independently. As illustrated in the inset, the average peak currents showed a good linear correlation with the logarithm of miRNA concentration ranging from 20 fM to 50 pM. According to the 3σ method, we achieved a detection limit of 5.36 fM for miRNA. Notably, the sensitivity of our biosensor was superior to that of the hairpin probe-based circular exponential amplification with fluorescence assay,[23] the

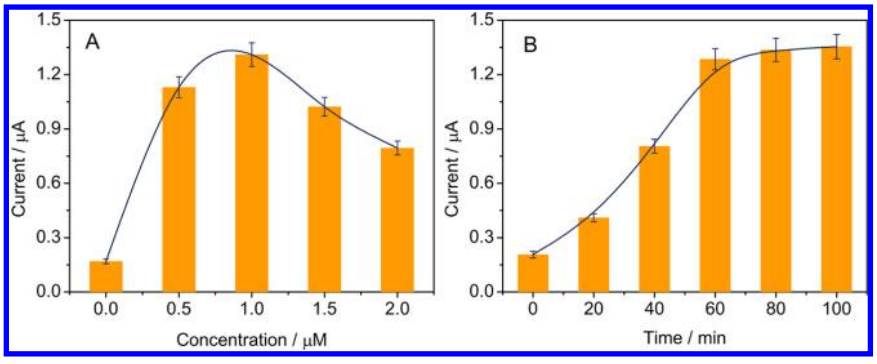

Figure 3. Effects of the self-assembling concentrations of probe (A) and the reaction time of EXPAR (B) on the performance of the biosensor for detection of 50 pM target miRNA. The data depicted the averages of three experiments, and the error bars represented the standard deviation of three trials.

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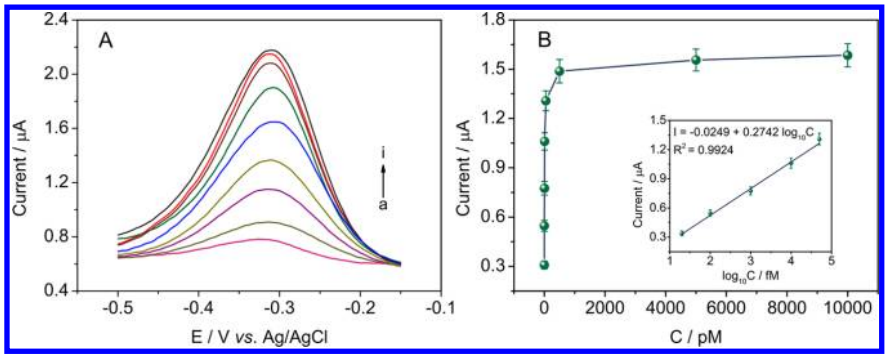

Figure 4. (A) Differential pulse voltammograms in response to different concentrations of target miRNA (a–i: 0 fM, 20 fM, 100 fM, 1 pM, 10 pM, 50 pM, 500 pM, 5 nM, and 10 nM). (B) Variation of the peak current as a function of miRNA concentration. Inset: the linear relationship of currents versus the logarithm of target concentration in the range from 20 fM to 50 pM. Error bars showed the standard deviation of three experiments.

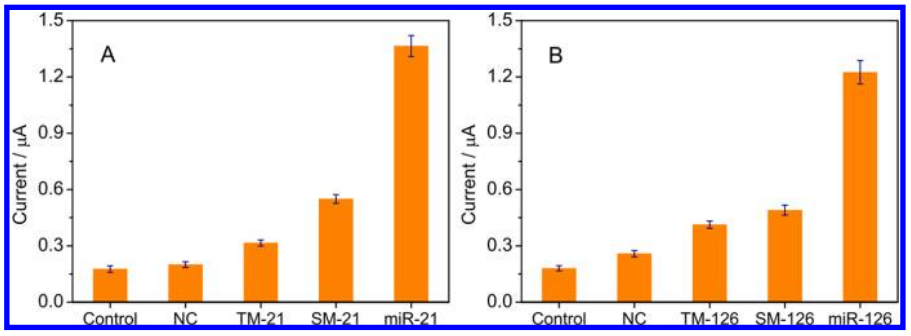

Figure 5. Specificity investigations of the biosensor for target miR-21 (A) and target miR-126 (B). The concentration of each sequence was 50 pM except for the bank control. Error bars were calculated from three independent experiments.

exonuclease III-assisted target recycling amplification electrochemical strategy,[34] the bimolecular beacons molecular-based fluorescence method,[38] and the quenched staudinger-triggered probe-based rolling circle amplification assay[39] as well. The detailed comparison of our strategy with other methods was illustrated in Table S-2 (Supporting Information). The significant improvement of the sensitivity can be attributed to (1) the high amplification efficiency of EXPAR, which enabled the conversion of a small amount of miRNA to a large number of universal triggers, (2) the rationally designed arched probe, which can minimize the background signal, and (3) the integration of EXPAR with sensitive electrochemical detection. The results above testified that the biosensor we fabricated can ensure ultrasensitive electrochemical detection of miRNA.

A great challenge of the miRNA assay was to distinguish the concomitant miRNA with high similarity. To investigate the specificity of the detection method proposed, we performed a series of contrast experiments using four kinds of miRNA sequences, including perfect complementary target miR-21, single-base mismatched miR-21 (SM-21), two-base mismatched miR-21 (TM-21), noncomplementary sequence (NC), and a sample without target as a blank control. The results in Figure 5A showed that the peak currents from NC scarcely changed compared with the blank control. The signals for SM-21 and TM-21 were only about 40.4% and 23.5% of that for miR-21 at the same concentration, respectively. The good performance to discriminate perfect complementary target and the mismatched targets gave this detection strategy great potential for single nucleotide polymorphism analysis.

To investigate the generality of our design, we chose another kind of miRNA, the miR-126, a metastasis suppressor miRNA in human breast cancer,[40] to investigate the specificity of our method as well. To design a probe for miR-126, we just needed to alter the target relevant region of probe for miR-21 in accordance with miR-126. Figure 5B showed that the peak currents from single-base mismatched miR-21 (SM-126), two-base mismatched miR-21 (TM-126), NC, and blank control were all much smaller than that from miR-126. The above results proved that our strategy is theoretically suitable for other miRNAs. The universality of our arched probe mediated EXPAR strategy can be realized easily by altering the target concerning region of the duplex probe.

Real Sample Analysis. It has been reported that miR-21 has an elevated expression in tumorigenic breast cell lines compared with nontumorigenic ones.[41] Thus, accurate miR-21 detection in real samples will be of great help in clinical diagnosis. To verify the effectiveness of our assay in real biological samples analysis, we measured the level of miR-21 in total RNA extracts prepared from MCF-7 cells. The total RNA was adjusted to 0.1 $\mu g\ \mu L^{-1}$ with DEPC-treated water, and 1 μL of the total RNA sample (0.1 μg in total) was employed for measurement using the standard addition method with synthetic miR-21 as the standard. Aliquots of the diluted RNA sample (1 μL) were spiked with standard solutions containing synthetic miR-21 at concentrations of 20, 50, 500, 2500, and 5000 fM, respectively. Then, arched probe mediated EXPAR and electrochemical detection were conducted according to the optimized conditions as described. The results were shown in Figure S-1, Supporting Information. The content of miR-21 in total RNA extracted from MCF-7 cells was found to be 1.15 fmol μg^{-1}. We also evaluated the accuracy of the method by determining the recovery of miR-21 spiked in cell extracts. After 3 pM miR-21 was spiked into the total RNA solution, the amount of miR-21 was measured to be 0.255 fmol

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with a recovery ratio of 93.3%. These results demonstrated that our assay was competent to detect miR-21in real samples.

CONCLUSIONS

We developed an electrochemical platform using an arched probe mediated EXPAR, which enabled simple and ultrasensitive miRNA detection. With the assistance of polymerase and nicking endonuclease, both the miRNA recycling circle and the DNA target regenerating circles were triggered in the presence of target miRNA and thus realized the exponential amplification of the target. Through the formation of the G-quadruplex–hemin unit on the surface of electrode, we could get an electrochemical response. The protocol proposed was rather simple and rapid. It showed a broad dynamic range and could achieve ultrasensitive electrochemical detection of miRNA down to the femto-mole level. The content of miR-21 extracted from cancer cell lines was determined as well. As the need for detection of early molecular markers in cancer diagnosis is urgent, the success of this method in specific detection of expressing miR-21 in breast cancerous cells can be a justification for potential application in early breast cancer diagnosis.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +86-20-39943044. Fax: +86-20-39943071. E-mail: chenzg@mail.sysu.edu.cn.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grant Nos. 21375152 and 21075139). We thank Dr. Qianqian Ji (State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University) for assistance on total RNA exaction and Dr. Peiqing Liu (New Drug Screening Center, Sun Yat-sen University) for the help of cell culture.

REFERENCES

(1) He, L.; Hannon, G. J. *Nat. Rev. Genet.* **2004**, *5*, 522–531.
(2) Calin, G. A.; Croce, C. M. *Nat. Rev. Cancer* **2006**, *6*, 857–866.
(3) Liu, N.; Olson, E. N. *Dev. Cell* **2010**, *18*, 510–525.
(4) Sullivan, C. S.; Ganem, D. *Mol. Cell* **2005**, *20*, 3–7.
(5) Arenz, C. *Angew. Chem., Int. Ed.* **2006**, *45*, 5048–5050.
(6) Yanaihara, N.; Caplen, N.; Bowman, E.; Seike, M.; Kumamoto, K.; Yi, M.; Stephens, R. M.; Okamoto, A.; Yokota, J.; Tanaka, T.; Calin, G. A.; Liu, C. G.; Croce, C. M.; Harris, C. C. *Cancer Cell* **2006**, *9*, 189–198.
(7) Kosaka, N.; Iguchi, H.; Ochiya, T. *Cancer Sci.* **2010**, *101*, 2087–2092.
(8) Valoczi, A.; Hornyik, C.; Varga, N.; Burgyan, J.; Kauppinen, S.; Havelda, Z. *Nucleic Acids Res.* **2004**, *32*, No. e175.
(9) Houbaviy, H. B.; Murray, M. F.; Sharp, P. A. *Dev. Cell* **2003**, *5*, 351–358.
(10) Lee, I.; Ajay, S. S.; Chen, H.; Maruyama, A.; Wang, N.; McInnis, M. G.; Athey, B. D. *Nucleic Acids Res.* **2008**, *36*, No. e27.
(11) Lee, J. M.; Cho, H.; Jung, Y. *Angew. Chem., Int. Ed.* **2010**, *49*, 8662–8665.
(12) Baker, M. *Nat. Methods* **2010**, *7*, 687–692.
(13) Allawi, H. T.; Dahlberg, J. E.; Olson, S.; Lund, E.; Olson, M.; Ma, W. P.; Takova, T.; Neri, B. P.; Lyamichev, V. I. *RNA* **2004**, *10*, 1153–1161.
(14) Hartig, J. S.; Grune, I.; Najafi-Shoushtari, S. H.; Famulok, M. *J. Am. Chem. Soc.* **2004**, *126*, 722–723.
(15) Kulcheski, F. R.; Marcelino-Guimaraes, F. C.; Nepomuceno, A. L.; Abdelnoor, R. V.; Margis, R. *Anal. Biochem.* **2010**, *406*, 185–192.
(16) Cheng, Y.; Zhang, X.; Li, Z.; Jiao, X.; Wang, Y.; Zhang, Y. *Angew. Chem., Int. Ed.* **2009**, *48*, 3268–3272.
(17) Wen, Y.; Xu, Y.; Mao, X.; Wei, Y.; Song, H.; Chen, N.; Huang, Q.; Fan, C.; Li, D. *Anal. Chem.* **2012**, *84*, 7664–7669.
(18) Fang, S.; Lee, H. J.; Wark, A. W.; Corn, R. M. *J. Am. Chem. Soc.* **2006**, *128*, 14044–14046.
(19) Alhasan, A. H.; Kim, D. Y.; Daniel, W. L.; Watson, E.; Meeks, J. J.; Thaxton, C. S.; Mirkin, C. A. *Anal. Chem.* **2012**, *84*, 4153–4160.
(20) Chen, C.; Ridzon, D. A.; Broomer, A. J.; Zhou, Z.; Lee, D. H.; Nguyen, J. T.; Barbisin, M.; Xu, N. L.; Mahuvakar, V. R.; Andersen, M. R.; Lao, K. Q.; Livak, K. J.; Guegler, K. J. *Nucleic Acids Res.* **2005**, *33*, No. e179.
(21) Connolly, A. R.; Trau, M. *Angew. Chem., Int. Ed.* **2010**, *49*, 2720–2723.
(22) Jia, H.; Li, Z.; Liu, C.; Cheng, Y. *Angew. Chem., Int. Ed.* **2010**, *49*, 5498–5501.
(23) Wang, G. L.; Zhang, C. Y. *Anal. Chem.* **2012**, *84*, 7037–7042.
(24) Zhang, Y.; Hu, J.; Zhang, C. Y. *Anal. Chem.* **2012**, *84*, 9544–9549.
(25) Duan, R.; Zuo, X.; Wang, S.; Quan, X.; Chen, D.; Chen, Z.; Jiang, L.; Fan, C.; Xia, F. *J. Am. Chem. Soc.* **2013**, *135*, 4604–4607.
(26) Polsky, R.; Gill, R.; Kaganovsky, L.; Willner, I. *Anal. Chem.* **2006**, *78*, 2268–2271.
(27) Zhang, J.; Song, S.; Zhang, L.; Wang, L.; Wu, H.; Pan, D.; Fan, C. *J. Am. Chem. Soc.* **2006**, *128*, 8575–8580.
(28) Cheng, W.; Yan, F.; Ding, L.; Ju, H.; Yin, Y. *Anal. Chem.* **2010**, *82*, 3337–3342.
(29) Ren, Y.; Deng, H.; Shen, W.; Gao, Z. *Anal. Chem.* **2013**, *85*, 4784–4789.
(30) Ji, H.; Yan, F.; Lei, J.; Ju, H. *Anal. Chem.* **2012**, *84*, 7166–7171.
(31) Li, D.; Shlyahovsky, B.; Elbaz, J.; Willner, I. *J. Am. Chem. Soc.* **2007**, *129*, 5804–5805.
(32) Xiao, Y.; Rowe, A. A.; Plaxco, K. W. *J. Am. Chem. Soc.* **2007**, *129*, 262–263.
(33) Pelossof, G.; Tel-Vered, R.; Willner, I. *Anal. Chem.* **2012**, *84*, 3703–3709.
(34) Liu, S.; Wang, C.; Zhang, C.; Wang, Y.; Tang, B. *Anal. Chem.* **2013**, *85*, 2282–2288.
(35) Yang, N.; Cao, Y.; Han, P.; Zhu, X.; Sun, L.; Li, G. *Anal. Chem.* **2012**, *84*, 2492–2497.
(36) Zhu, X.; Zhang, W.; Xiao, H.; Huang, J.; Li, G. *Electrochim. Acta* **2008**, *53*, 4407–4413.
(37) Zhang, K.; Zhu, X.; Wang, J.; Xu, L.; Li, G. *Anal. Chem.* **2010**, *82*, 3207–3211.
(38) Huang, J.; Su, X.; Li, Z. *Anal. Chem.* **2012**, *84*, 5939–5943.
(39) Harcourt, E. M.; Kool, E. T. *Nucleic Acids Res.* **2012**, *40*, No. e65.
(40) Tavazoie, S. F.; Alarcon, C.; Oskarsson, T.; Padua, D.; Wang, Q.; Bos, P. D.; Gerald, W. L.; Massague, J. *Nature* **2008**, *451*, 147–152.
(41) Sempere, L. F.; Christensen, M.; Silahtaroglu, A.; Bak, M.; Heath, C. V.; Schwartz, G.; Wells, W.; Kauppinen, S.; Cole, C. N. *Cancer Res.* **2007**, *67*, 11612–11620.