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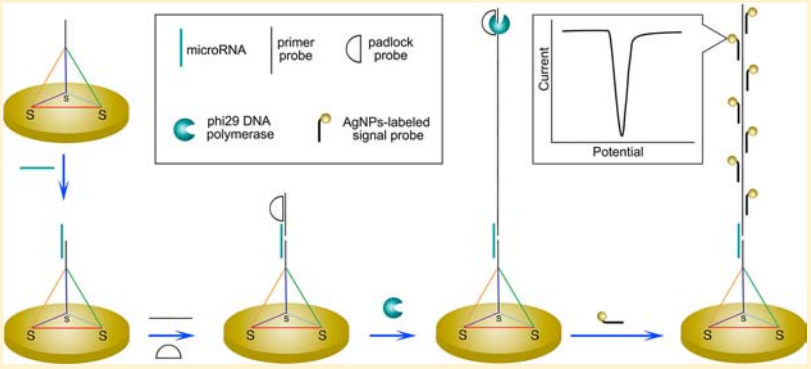
Ultrasensitive Detection of MicroRNA through Rolling Circle Amplification on a DNA Tetrahedron Decorated Electrode

Peng Miao,*[,†,§] Bidou Wang,[†] Fanyu Meng,[†,‡,§] Jian Yin,[†] and Yuguo Tang*[,†,§]

[†]CAS Key Lab of Bio-Medical Diagnostics, Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences, Suzhou 215163, China

[‡]Laboratory of Biosensing Technology, School of Life Sciences, Shanghai University, Shanghai, 200444, China

[§]University of Chinese Academy of Sciences, Beijing 100049, China



ABSTRACT: MicroRNAs are a class of evolutionally conserved, small noncoding RNAs involved in the regulation of gene expression and affect a variety of biological processes including cellular differentiation, immunological response, tumor development, and so on. Recently, microRNAs have been identified as promising disease biomarkers. In this work, we have fabricated a novel electrochemical method for ultrasensitive detection of microRNA. Generally, a DNA tetrahedron decorated gold electrode is employed as the recognition interface. Then, hybridizations between DNA tetrahedron, microRNA, and primer probe initiate rolling circle amplification (RCA) on the electrode surface. Silver nanoparticles attached to the RCA products provide significant electrochemical signals and a limit of detection as low as 50 aM is achieved. Moreover, homology microRNA family members with only one or two mismatches can be successfully distinguished. Therefore, this proposed method reveals great advancements toward improved disease diagnosis and prognosis.

INTRODUCTION

MicroRNAs are evolutionally conserved, single-stranded, small noncoding RNAs involved in the regulation of gene expression through the RNA interference process.[1−3] Increasing evidence has indicated that microRNAs are of great importance in a vast range of biological processes including cellular differentiation, immunological response, tumor development, and so on.[4−6] Aberrant levels of specific microRNAs are found to be associated with initiation or development of certain diseases.[7] For example, the miR-29a level correlates with cardiac hypertrophy;[8] miR-124-5p functions as a tumor suppressor and serves as a molecular marker for glioma diagnosis;[9] miR-138, miR-147a, miR-147b, and miR-511 levels in hepatitis C virus (HCV)-associated diffuse large B-cell lymphoma (DLBCL) can be used to predict overall survival;[10] let-7a is down-regulated in severe asthma patients, which can be used not only as a biomarker, but also a therapy target.[11]

Currently, the number of identified microRNAs is growing rapidly.[12] Despite the enthusiastic investigation of microRNA functions, the development of sensitive detection methods is lagged behind. The main obstacles include small size, low abundance in total RNA, and sequence similarity among family members.[13] To meet the urgent need for microRNA expression analysis, traditional techniques like Northern blotting and molecular cloning are now replaced by more powerful techniques including quantitative reverse transcription polymerase chain reaction (qRT-PCR)[14] and microarrays.[15] High sensitivity is one of the most important concerns for analytical methods.[16,17] However, limit of detection (LOD) of mainstream techniques may not meet the requirement of microRNA assay. Therefore, amplification strategies should be developed to assist effective measurements.[18,19] For example, qRT-PCR employs the amplification by polymerase during cycles of programmed temperature variation.[20] Recently, tremendous progress has been made in isothermal amplification, which is more convenient and cost-effective than PCR.[21,22] Among them, rolling circle amplification (RCA) is one of the most widely used isothermal amplification techniques and receives

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particular interest.[23−25] RCA involves the amplification of a circular DNA template with linear kinetics by phi29 DNA polymerase. A long single-stranded DNA (ssDNA) is synthesized under isothermal conditions, which provides possibilities for many advanced DNA machines and signal outputs.[26,27]

In this work, a DNA tetrahedron decorated electrode has been prepared and used for ultrasensitive detection of microRNA based on RCA. In addition, solid-state Ag/AgCl reaction from silver nanoparticles (AgNPs) provides the electrochemical signals. This proposed method yields high diagnostic accuracy toward the detection of microRNA. LOD as low as 50 aM is achieved and homology microRNA family members with only one or two mismatches can be successfully distinguished. This method also possesses other advantages such as simplicity, fast response, low cost, and convenient operation, which can provide a promising sensing platform for bioanalysis and clinical molecular diagnostics.

RESULTS AND DISCUSSION

Sensing Principle. DNA tetrahedron is formed by four ssDNAs named as tetrahedron A, B, C, and D.[28,29] Synthetic human microRNA, hsa-let-7a, is tested as the model microRNA in this electrochemical detection system. As shown in Figure 1,

Figure 1. Hybridization details of the four ssDNAs to form the DNA tetrahedron.

the hybrids of the four ssDNAs constitute the six edges of the DNA tetrahedron and the remaining pendant linear sequence of tetrahedron A is designed to capture microRNA. The principle of this electrochemical biosensor for RCA-based microRNA assay is illustrated in Figure 2. DNA tetrahedron is modified on the gold electrode surface via thiols on the three vertexes of tetrahedron. This scaffold contributes to the enhancement of molecular recognition efficiency. Moreover, common "backfilling" process like the employment of 6-mercapto-1-hexanol to modulate the electrode surface can be avoided. In the presence of microRNA, a sandwich structure forms on the electrode surface (tetrahedron A/microRNA/primer probe), which can initiate the subsequent RCA with the help of circular DNA template and phi29 DNA polymerase. Finally, a large number of AgNPs-labeled signal probes attach to the long ssDNA product through hybridization and provide significant electrochemical signals by the highly characteristic solid-state Ag/AgCl reaction,[30] which reflect the original microRNA concentration.

Characterization of Electrode Modification. Electrode modification can be characterized by electrochemical impedance spectra (EIS). As displayed in Figure 3, semicircle

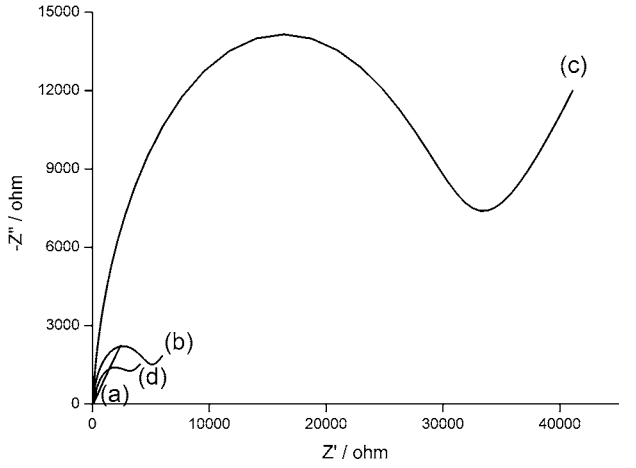

Figure 3. Nyquist diagrams of impedance spectra for (a) bare electrode, (b) DNA tetrahedron decorated electrode, (c) after microRNA-mediated RCA, and (d) after further incubation with AgNP-labeled signal probe.

domains of the Nyquist plots reveal the limited interfacial charge transfer resistance. EIS of bare electrode includes no semicircle. The diameter of the semicircle domain of the DNA tetrahedron decorated electrode is relatively small. A large increase is observed after microRNA-mediated RCA on electrode due to the generated long ssDNA which repels the negatively charged $Fe(CN)_6^{3-/4-}$. However, after hybridization

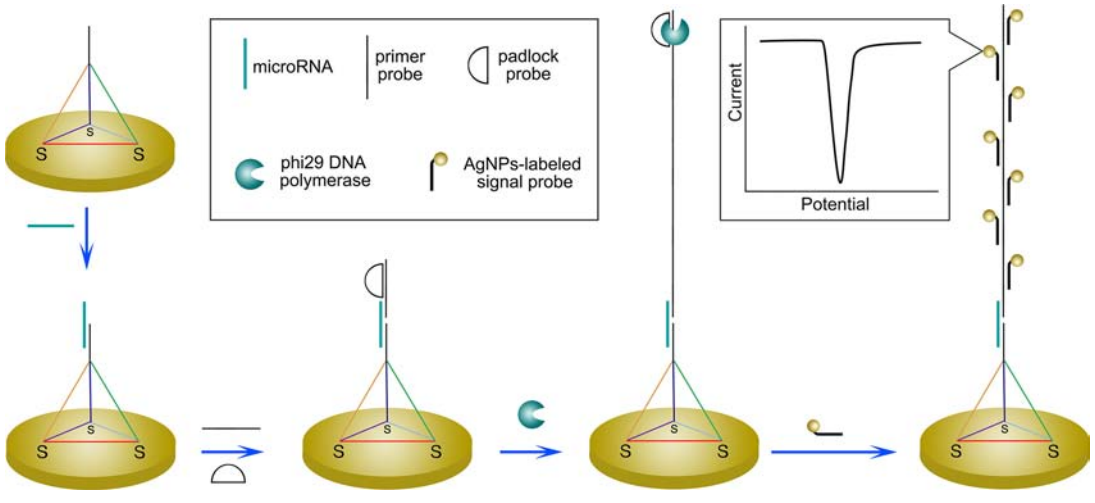

Figure 2. Scheme of the RCA-based microRNA assay.

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with AgNPs-labeled signal probe, the diameter declines, due to the oxidization of AgNPs to Ag^+.

Linear sweep voltammetry (LSV) is also performed to reveal electrochemical behaviors during the modification steps. As shown in Figure 4, no silver stripping peak exists on DNA

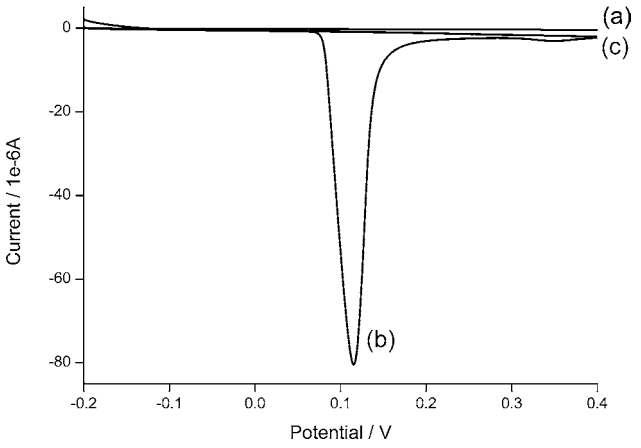

Figure 4. Linear sweep voltammograms of (a) DNA tetrahedron decorated electrode, after RCA and signal probe hybridization in the (b) presence and (c) absence of 1 nM microRNA.

tetrahedron decorated electrode. By the microRNA-mediated RCA and subsequent signal probe hybridization, a significant peak is obtained (curve b). However, in the absence of microRNA, primer probe cannot be localized on the electrode surface and the following RCA and hybridization cannot occur on the electrode, which results in a flat curve (curve c).

Quantification of Target MicroRNA. LSV can also offer quantitative information on microRNA. As shown in Figure 5,

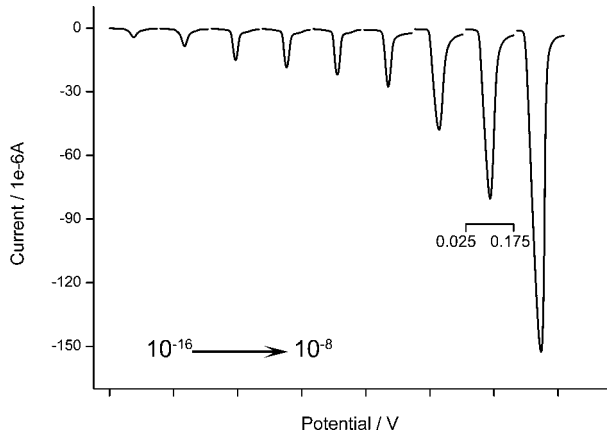

Figure 5. Linear sweep voltammograms of DNA tetrahedron decorated electrode for the detection of microRNA with the concentration of 10^{-16}, 10^{-15}, 10^{-14}, 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, and 10^{-8} M (from left to right).

LSV curves are recorded for the detection of microRNA with a series of concentrations from 10^{-16} M to 10^{-8} M. A larger amount of microRNA corresponds to the curve with larger current peak. The numerical calibration is plotted in Figure 6 and the linear relationship is established with the fitting equation of $y = 4.618x + 78.509$ ($n = 3$, $R^2 = 0.990$), where y is peak current and x is the logarithm of microRNA concentration

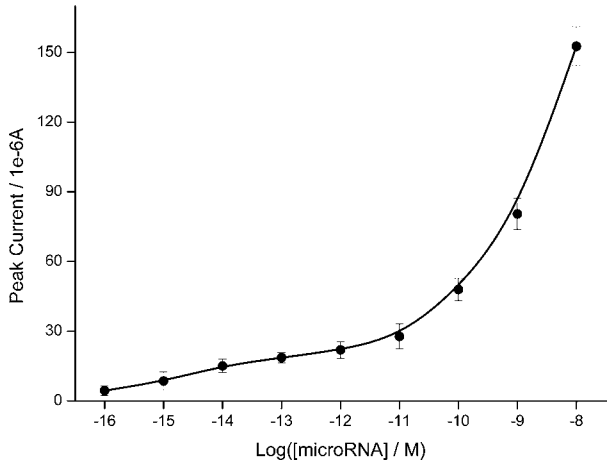

Figure 6. Calibration plot of the peak current versus the logarithmic microRNA concentration.

(Figure 7). LOD is calculated to be 50 aM (S/N = 3). The linear range is from 1 fM to 10 pM, which is quite wide.

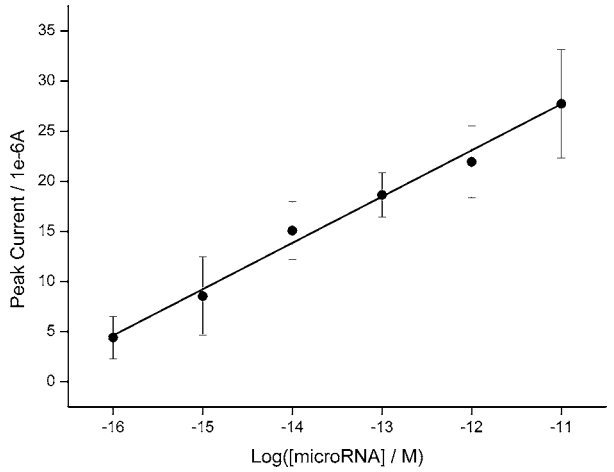

Figure 7. Linear relationship between peak current and logarithmic microRNA concentration.

To verify the high selectivity of this electrochemical biosensor, microRNAs of let-7 family are checked, which have similar sequences with only one or two different bases. From Figure 8, it is concluded that let-7a can be successfully distinguished from other negative controls. The biosensor does not respond to mismatched microRNAs and only gives negligible electrochemical signals.

Sample Analysis. Attempts are then made in utilizing the method for detection of microRNA levels in cells and serum samples. Total RNAs are first extracted from cells and serum samples and are then tested by the proposed method. The results are listed in Table 1. The detected microRNA levels are in good agreement with those obtained by qRT-PCR. The relative errors are also small, demonstrating the practical value of this method. Compared with qRT-PCR based strategy, isothermal amplification and DNA tetrahedron scaffold used in this method promise high sensitivity and excellent selectivity. In addition, the simple operation, low cost, and fine practical utility of this method make it a more advantageous tool for microRNA assay.

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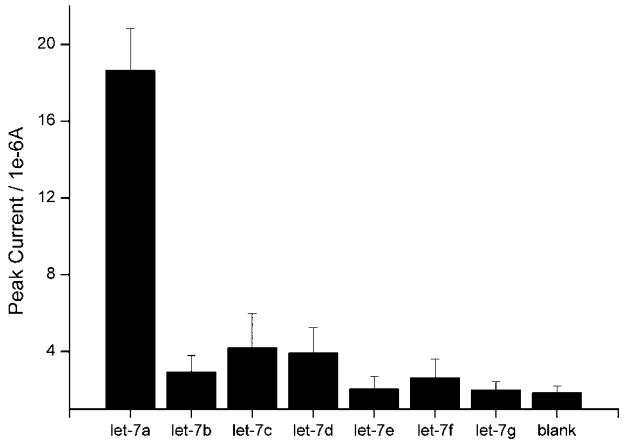

Figure 8. Selectivity assessment among homology microRNA family members. The concentrations are 100 fM.

Table 1. Let-7a Assay in Total RNAs Extracted from A549 Cells and Serum Samples

sample	detected (10^6 copy/μg)	qRT-PCR results (10^6 copy/μg)	relative error
A549 cells	4.5	4.7	4.3%
	6.6	6.7	1.5%
Serum	2.5	2.4	4.2%
	2.9	2.6	11.5%

CONCLUSIONS

In conclusion, we have introduced a novel electrochemical strategy for quantitative detection of microRNA. DNA tetrahedron decorated electrode is employed which reinforces molecular recognition efficiency and avoids spacer molecules for electrode surface modulation. By combining rolling circle amplification and solid-state Ag/AgCl reaction, enhanced electrochemical signals are obtained which can indicate the microRNA concentration sensitively and selectively. The proposed method not only exhibits excellent analytical characteristics like wide linear range and low LOD, but also displays fine reproducibility and practical utility in real samples. Therefore, this method is expected to be a powerful tool for bioanalysis and clinical molecular diagnostics.

MATERIALS AND METHODS

Materials and Chemicals. Silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), trisodium citrate tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) and diethypyrocarbonate (DEPC) were from Sigma-Aldrich (USA). Phi29 DNA polymerase and T4 DNA ligase were purchased from New England Biolabs Ltd. (Beijing, China). A549 cells were provided by the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Gaithersburg, USA). Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Material Co., Ltd. (Hangzhou, China). Human serum samples were supplied by local hospital (Suzhou, China). Other reagents were of analytical grade and were used as received. Water used to prepare all solutions was purified (18 MΩ·cm resistivity) from a Millipore system and treated with DEPC. Oligonucleotides were synthesized and purified by Takara Biotechnology Co., Ltd. (Dalian, China) and the corresponding sequences are listed in Table 2. Tetrahedron B, C, and D were thiolated with a -$(CH_2)_6$-spacer at the 5′ end. The padlock probe contained a 5′-phosphoryl end. The AgNPs-labeled signal probe was formed by the silver-amino interaction.[31] Compared with target let-7a, the mismatch sites of other let-7 family members were labeled with underlines.

Fabrication of DNA Tetrahedron. DNA tetrahedron was formed by the hybridization between the four ssDNAs.[32] Tetrahedron A, B, C, and D solutions were prepared in 10 mM Tris-HCl buffer containing 10 mM TCEP, 50 mM $MgCl_2$ (pH 8.0). The final concentration of each strand was 4 μM. Afterward, equivalent volumes of the four strands were blended together. The mixture was heated to 95 °C for 2 min and then cooled to 4 °C.

Electrode Cleaning and Modification. The substrate gold electrode (2 mm) was pretreated before surface modification following the reported protocol.[33] Piranha solution (98% H_2SO_4: 30% H_2O_2 = 3:1) was prepared and used to treat the gold electrode for 5 min (*Caution: Piranha solution reacts violently with organic solvents and should be handled with great care!*) Subsequently, the electrode was carefully polished to a mirror-like surface with P5000 sand paper and 1 μm, 0.3 μm, 0.05 μm alumina slurry, respectively. After that, it was cleaned by ultrasonication for 5 min in ethanol and then in double-distilled water. The electrode was then

Table 2. DNA and RNA Sequences Used in This Work

name	sequence (5′-3′)
Tetrahedron A	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTATTTAACTATACAA
Tetrahedron B	SH-C_6-TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGGGTCCAATAC
Tetrahedron C	SH-C_6-TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCGGCTCTTC
Tetrahedron D	SH-C_6-TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCTCGCAT
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU
hsa-let-7b	UGAGGUAGUAGGUUGUGU GGU
hsa-let-7c	UGAGGUAGUAGGUUGUAUGGUU
hsa-let-7d	AGAGGUAGUAGGUUGCAUAGUU
hsa-let-7e	UGAGGUAGGAGGUUGUAUAGUU
hsa-let-7f	UGAGGUAGUAGAUUGUAUAGUU
hsa-let-7g	UGAGGUAGUAGUUUGUACAGUU
primer probe	TACTACCTCAATCCCTATAAATACCCTAAC
padlock probe	phosphoryl-TTATAGGGTATCTCTATCTCTTAGGGTAT
AgNPs-labeled signal probe	AgNPs-NH_2-TATCTCTATCTC

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soaked in 50% HNO_3 for 30 min and electrochemically cleaned with 0.5 M H_2SO_4 to remove any remaining impurities. Afterward, it was dried with nitrogen and was ready for surface modification. Then, 10 μL of DNA tetrahedron was dipped on the gold electrode surface. After 8 h, the electrode was rinsed with double-distilled water.

Preparation of AgNPs-Labeled Signal Probe. AgNPs-labeled signal probe was formed by the conjugation of AgNPs and DNA probe labeled with amino group at the 5′ end. Bare AgNPs were synthesized by the borohydride reduction of $AgNO_3$.[34] Briefly, 100 mL of $AgNO_3$ (0.25 mM) and trisodium citrate (0.25 mM) solution was prepared, which was then mixed with 3 mL of $NaBH_4$ solution (10 mM) under violent stirring for 30 min. The solution was left to sit overnight in the dark. The prepared bright yellow AgNPs were purified by centrifugation at 12 000g for 30 min. Then, AgNPs were mixed with 10 μM DNA probe for 24 h to achieve the silver−amino binding.[35] A further centrifugation procedure was performed for purification purposes.

MicroRNA-Mediated Rolling Circle Amplification. MicroRNA and primer probe were dissolved in 10 mM phosphate buffered saline (PBS) containing 0.25 M NaCl (pH 7.4) separately. The DNA tetrahedron decorated gold electrode was incubated in microRNA solutions with different concentrations for 1 h. Then, the electrode was rinsed and incubated in 1 μM primer probe for another 1 h. Consequently, it was immersed in the 50 mM Tris-HCl buffer solution containing 1 μM padlock probe, 5 unit/mL T4 DNA ligase, 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP at 22 °C for 1 h. During this process, the linear padlock probe hybridized with primer probe and the created nick was linked by T4 DNA ligase.[36] The circle template was thus formed. Afterward, the electrode was treated with 50 unit/mL phi29 DNA polymerase at 37 °C for 1 h, which was prepared in 50 mM Tris-HCl buffer containing 10 mM $MgCl_2$, 10 mM $(NH_4)SO_4$, 4 mM DTT, 0.2 mg/mL BSA, and 0.5 mM dNTP. Long ssDNA was thus produced.

Electrochemical Measurements. Electrochemical experiments were carried out on a CHI 660D electrochemical workstation (CH instruments, Shanghai, China), employing a three electrode system. The pretreated gold electrode was immersed in signal probe for 1 h before measurement, and was used as the working electrode. Platinum wire was used as the auxiliary electrode and Ag/AgCl electrode acted as the reference electrode. EIS experiments were performed in 5 mM $Fe(CN)_6^{3-/4-}$ with 1 M KNO_3. The 0.204 V bias potential and 5 mV amplitude were set and the frequency range was from 1 to 100 000 Hz. LSV experiments were performed in 0.1 M KCl with the scan rate of 0.1 V/s.

Cell and Serum Assays. Human lung cells (A549) were cultured in DMEM medium with 10% (v/v) fetal bovine serum at 37 °C in 5% CO_2 atmosphere. After reaching a confluence of 80%, the cells were washed with PBS, detached, and collected. Then, total RNAs were extracted from the cells and human serum samples, separately, using a RNA extraction kit from Qigen according to the manufacturer's procedures. Let-7a levels were then analyzed in the extracted total RNA as described above. qRT-PCR experiments were performed to obtain the reference values.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: miaopeng@sibet.ac.cn. Tel.: +86 512 69588279. Fax: +86 512 69588283.

*E-mail: tangyg@sibet.ac.cn.

Notes

The authors declare no competing financial interest.

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