Carbon nanotube enhanced label-free detection of microRNAs based on hairpin probe triggered solid-phase rolling-circle amplification†

Qianqian Tian,†a Ying Wang,†a,b Ruijie Deng,a Lei Lin,a Yang Liu,a and Jinghong Li*a

The detection of microRNAs (miRNAs) is imperative for gaining a better understanding of the functions of these biomarkers and has great potential for the early diagnosis of human disease. High sensitivity and selectivity for miRNA detection brings new challenges. Herein, an ultrasensitive protocol for electrochemical detection of miRNA is designed through carbon nanotube (CNT) enhanced label-free detection based on hairpin probe triggered solid-phase rolling-circle amplification (RCA). Traditionally, RCA, widely applied for signal enhancement in the construction of a variety of biosensors, has an intrinsic limitation of ultrasensitive detection, as it is difficult to separate the enzymes, templates, and padlock DNAs from the RCA products in the homogeneous solution. We purposely designed a solid-phase RCA strategy, using CNTs as the solid substrate, integrated with a hairpin structured probe to recognize target miRNA. In the presence of miRNA the stem-loop structure will be unfolded, triggering the CNT based RCA process. Due to the efficient blocking effect originating from the polymeric RCA products, the label-free assay of miRNA exhibits an ultrasensitive detection limit of 1.2 fM. Furthermore, the protocol possesses excellent specificity for resolving lung cancer-related let-7 family members which have only one-nucleotide variations. The high sensitivity and selectivity give the method great potential for applications in online diagnostics and in situ detection in long-term development.

Introduction

High sensitivity and selectivity for detection of miRNAs brings new challenges. MiRNAs are burgeoning as novel biomarkers for cancer diagnostics and therapies due to their crucial functions in gene expression.1–3 To better understand the role of miRNAs in biochemical processes, high-level detection techniques are extremely useful for all kinds of investigations, from basic scientific research to clinical applications.1–3 Hence, the development of a novel detection method for miRNAs with high precision and good sensitivity has been becoming an intractable issue at present.4–8 Even though great processes (e.g. northern blotting,9 microarrays10,11 and real time polymerase chain reaction,12,13 etc.) have been developed in the past several decades for improving analysis ability, huge obstacles are still challenging the existing methods because of the sequence homology, low abundance and short lengths of the miRNAs.14–18 We describe herein a special approach for achieving ultrasensitive and highly selective detection of miRNAs using carbon nanotube enhanced label-free electrochemical detection with hairpin DNA triggered solid-phase rolling-circle amplification.

To date, the specificity of a miRNA assay for discriminating one-base differences between the miRNAs is in great demand, in order to better understand the biological functions of individual miRNAs.19–22 For a proof-of-concept experiment, we selected members of the let-7 miRNA family (let-7a, let-7f, and let-7g) as ideal models because they have the same length and their sequences differ by only one or two bases. In addition, their expression levels are closely associated with cell development and human lung cancer.23,24 As shown in Scheme 1A, amino modified hairpin probes were immobilized on the carbonyl functionalized CNTs through chemical bonding. The hairpin structure is beneficial for binding target miRNAs efficiently and discriminating a mismatched sequence, since the presence of the stem makes the structure thermodynamically unfavorable for binding a mismatched sequence to the loop. Because the sequence of the hairpin probe (shown in green) is the complimentary sequence corresponding to the
target miRNA sequence, the target miRNA could unfold the hairpin probe anchored on the CNT’s surface, triggering a follow-on reaction with the circulate probe and phi29 enzyme under suitable conditions.

As an advanced DNA amplification technique, rolling-circle amplification (RCA) can realize great signal amplification via the production of thousands of repeated sequences. Generally RCA techniques are performed in solution-phase to achieve the fantastic amplification effects. However, the separation of the enzyme, template and padlock DNAs from the RCA products hinders its application to miRNA detection. The present method proposes a solid-phase RCA method, which is compellingly advantageous compared to the conventional solution-based cases.

We purposely designed the enhanced electrochemical sensing platform by using CNTs as the signal transducer. Due to their easy chemical modification and high electron transfer ability, CNTs are promising as an advanced electrode material. By using CNTs as the solid primer for hairpin probe immobilization, a sensitive electrochemical interface was thus accomplished using differential pulse voltammetry analysis. This protocol could detect target miRNA down to the femtomolar level (1.2 fM, s/n = 3σ), with dramatic selectivity for resolving lung cancer-related let-7 family members which have only a one-nucleotide variation.

**Experimental section**

**Reagents and materials**

DNA sequences and commercial RNA extraction kits (UNIQ-10 column Trizol total RNA purification kit) were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). miRNA sequences were purchased from TaKaRa Biotech Company (Dalian, China) and HPLC purified. All of the DNA and RNA sequences are listed in Table S1.

T4 DNA ligase, Exonuclease I, Exonuclease III, phi29 DNA polymerase, 10× T4 DNA ligase reaction buffer, 10× phi29 DNA polymerase reaction buffer and BSA were bought from New England Biolabs (Beijing, China). Diethyl oxalylcarbodiimide (DEPC), the deoxyribonucleotides mixture (dNTPs), and agarose were obtained from Beijing DingGuo Biotech. Co., Ltd. Hexaammineruthenium chloride, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and 2-(N-morpholino)ethanesulfonic acid (MES) were obtained from Alfa Aesar. Other regents of analytical grade were obtained from Beijing Chemical Co. (Beijing, China) and used without further purification. All DNA and miRNA reagents were diluted with 10 mM PBS (pH 7.4) containing 0.1 M NaCl. The washing buffer in the experiments was 10 mM PBS (pH 7.4). All of the solutions and deionized water used were treated with DEPC and autoclaved to protect from RNase degradation.

**Instruments**

Scanning electron microscopy (SEM) images were obtained with a field emission SEM system (JSM 7401F, Japan). Fourier transform infrared (FTIR) spectra were recorded on a Spectrum GX FTIR system. The cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were conducted on a CHI 1030b Instrument (CH Instrument Co.). Electrochemical impedance spectroscopy (EIS) was carried out on a PARSTAT 2273 potentiostat/galvanostat (Advanced Measurement Technology Inc., USA). The gel electrophoresis was visualized via ChampGel 5000 (Beijing Sage Creation Science Co., Ltd, China). The contact angle was measured using a Dataphysics OCA 15pro CA measuring instrument (DataPhysics Instruments GHPH, Filderstadt). The concentration of total RNA was measured using the absorbance at 260 nm with a spectrophotometer (NanoDrop 2000C, U. S.).

**Preparation of the CNTs-hairpin probe modified electrode (HP/CNTs/GCE)**

A glassy carbon electrode (GCE, diameter of 3 mm) was polished with a 0.05 mm Al_{2}O_{3} slurry on an abrasive cloth and successively cleaned with ethanol and distilled water under ultra-sonication, followed by drying in high-purity nitrogen gas. 6 μL of 1 mg mL⁻¹ carboxylic group functionalized carbon nanotube solution was dropped onto the pretreated GCE and then dried overnight at 25 °C to obtain the CNT-modified GCE (CNTs/GCE). Then, the electrode was immersed in 50 μL of a MES (25 mM, pH 6.0) solution containing 20 mM EDC and 20 mM NHS at 25 °C for 1 h. Then the immobilization of the hairpin probe was accomplished with the CNTs/GCE electrode by incubating in 50 μL of a 1 μM 5′-NH₂ modified hairpin probe (HP) solution at 25 °C for 2 h.
modified electrode (HP/CNTs/GCE) was obtained following a thorough rinse with PBS.

**Solid-phase rolling-circle amplification**

The hairpin probe-modified electrode (HP/CNTs/GCE) was soaked in 40 μL of target miRNA at a certain concentration and incubated at 45 °C for 2 h to produce miRNA/HP/CNTs/GCE. The obtained miRNA/HP/CNTs/GCE was carefully washed with 10 mM PBS to remove the noncaptured miRNA. Whereafter, the miRNA/HP/CNTs/GCE was immersed in 40 μL of 1 M NaCl and 1 μL of a circular probe (CP) solution for 2 h at 45 °C. The resulting CP/miRNA/HP/CNTs/GCE electrode was carefully washed with 10 mM PBS to remove the adsorbed DNA. Subsequently, the RCA reaction was conducted in a solution volume of 40 μL, which contained 4 μL of 10× phi29 DNA polymerase reaction buffer (500 mM Tris-HCl, pH 7.5 at 25 °C, 100 mM MgCl2, 100 mM (NH4)2SO4, 40 mM dithiothreitol), 0.8 μL of BSA (10 mg mL−1), 0.8 μL of dNTPs (10 mM for each of dATP, dGTP, dCTP, dTTP), 33.4 μL of 40 mM dithiothreitol, 0.8 μL of BSA (10 mg mL−1), 0.8 μL of dNTPs (10 mM for each of dATP, dGTP, dCTP, dTTP). After incubating at 30 °C for 2 h, the RCA/CP/miRNA/HP/CNTs/GCE was washed with 10 mM PBS to stop the reaction and then used for the following electrochemical measurements and contact angle measurements.

**Cell culture and total RNA extraction**

The Hela cell line was provided by the Medicine School of Tsinghua University (Beijing, China). The Hela cells were cultured in DMEM medium supplemented with 10% fetal calf serum, 100 U mL−1 penicillin, and 100 μg mL−1 streptomycin at 37 °C in a humidified atmosphere of 5% CO2. The total RNA was extracted from the cultured cancer cells using a UNIQ-10 column Trizol total RNA purification kit from Sangon Biotech Co., Ltd (Shanghai, China), according to the manufacturer’s procedure.

**Results and discussion**

**Concepcion of the strategy**

The ultrasensitive detection of target miRNA was realized on the CNT modified glassy carbon electrode with hairpin probe triggered RCA through DPV analysis. As shown in Scheme 1B, the electrochemical sensing system was prepared by depositing the hairpin/CNTs on the electrode and employing Ru(NH3)63+ as the signal probe. In the absence of miRNA, the stable stem-loop “hairpin” structure was unable to recognize the circular template and the phi29 DNA polymerase, as illustrated in Scheme 1B. Without the RCA products, the CNTs were completely exposed to the signal probe (Ru(NH3)63+), resulting in a strong adsorption of positively charged Ru(NH3)63+ on the negatively charged surface of the carboxylic acid group modified CNTs. Accordingly, an evident DPV signal was observed due to the electrochemical activity of Ru(NH3)63+.

However, when miRNA was introduced into the sensing system, the hairpin structured DNA probe anchored on the CNTs was unfolded by its complementary target, shown in the picture of Scheme 1B. By adding a circulate probe and phi29 DNA polymerase, solid-phase RCA was performed at the interface of the CNTs, producing super-long DNA strands with hundreds of repeated sequences. The RCA products were intertwined with each other, forming a DNA cross-linked film, covering the CNT layer on the electrode. Compared to the CNTs, the RCA products formed an insulating block layer, hindering electron transfer between Ru(NH3)63+ and the electrode surface. As a result, an obvious DPV signal loss was observed during the electrochemical detection. Based on the principle of CNT enhanced label-free electrochemical detection with hairpin DNA triggered solid-phase RCA, ultrasensitive detection could be realized successfully for target miRNA with good selectivity.

**Morphology characterization of the solid-phase RCA**

To realize the solid-phase RCA, amino modified hairpin probes were immobilized onto the carboxyl functionalized CNTs through chemical bonding. Hence, an FTIR spectrum (Fig. S1A†) was obtained to confirm the presence of carboxylated acid groups on the CNTs. Before modification, the CNTs show a band at 1100 cm−1, which is assigned to C−O stretching vibrations, while a band at 1630 cm−1 is ascribed to the C=O stretching vibration. After acid treatment, a new absorption band appeared at 1730 cm−1, which corresponded to C=O stretching that was attributed to −COOH. The FTIR spectrum of the carboxyl functionalized CNTs shows a new broad absorption band with a characteristic −COOH peak at 3000 cm−1. Fig. S1B† shows that carboxyl functionalized CNTs disperse well in water, however, the unmodified CNTs are barely dispersed. It is impossible to apply the unmodified CNTs as the solid substrate for in situ RCA due to their obvious hydrophobicity.

To demonstrate the morphology of the CNT enriched electrode before and after the solid-phase RCA, SEM and contact angle measurements were employed to explore the microstructure changes on covering the CNT layer on the electrode with a DNA cross-linked film. Fig. 1 displays the SEM pictures (A, B and C) and contact angle photos (D, E and F) of the CNT-modified CNTs/GCE and RCA/miRNA/hairpin probe/ CNTs/GCE, respectively. The CNTs formed a 3D and porous fibrous network as shown in Fig. 1A. The 3D interconnected network and the nanopores could efficiently enlarge the interfacial area and provide sufficient pathways for the absorption of Ru(NH3)63+ and for electron transfer in the film. However, RCA produced super-long and repeated DNA strands which were binding to the CNTs, forming a DNA cross-linked film, which can be seen in Fig. 1C. Contact angles are extremely sensitive to the wetting properties of the surface material. Generally, if the water contact angle is smaller than 90°, the solid surface is considered more hydrophilic. And the smaller the contact angle is, the more hydrophilic the solid surface is. Hence, the contact angle was tested for CNTs−COOH (Fig. 1D), the CNTs-hairpin probe (Fig. 1E), and the CNTs-hairpin probe after the RCA process (Fig. 1F). Apparently, the contact angle
of CNTs-COOH was 58.4°, which was the most hydrophobic. Modification with hairpin DNA increased the wettability of the CNTs, making the contact angle 53.6°. After the RCA process, the long single-stranded RCA products enable prominent enhancement of the surface wettability, with a resulting contact angle of 43.0°. Contact angle experiments demonstrated that the solid-phase RCA on CNTs was accomplished successfully.

**Electrochemical impedance characterization of the solid-phase RCA**

CV and EIS are powerful and facile electrochemical techniques for verifying the assembly processes of the modified electrodes step by step. CV curves of the modified GCE are shown in Fig. S2.† The bare GC electrode exhibited a couple of reversible redox peaks in curve a. After subsequent capture of miRNA, the peak currents in the CV curve were further decreased, and the gap between the anodic and cathodic peaks became wider (curve d). Analogical results were obtained following capture of the circular probe (curve e) and after RCA (curve f). The phenomenon resulting from the electrostatic repulsion between negatively charged DNA and Fe(CN)₆³⁻/⁴⁻ blocks electron transfer.

Nyquist plots are composed of a straight linear part at a lower frequency range and a semicircle part at a higher frequency range. The diameter of the semicircle indicates the electron transfer resistance \( R_{et} \) at the electrode interface. The EIS plots are illustrated in Fig. 2A, along with the equivalent circuit analyzed using a nonlinear least-squares program, where \( R_s, R_{mem}, Q, R_{ct}, \) and \( Z_{we} \) are the electrolyte resistance, the resistance of the CNTs or the CNTs-RCA membrane, the constant phase element, the electron-transfer resistance, and the Warburg impedance, respectively. Due to a good electron transfer ability, the CNT-modified GCE (curve b) exhibited a smaller \( R_{et} \) than the bare GC electrode (curve a). It is obvious that the diameter of the semicircles increased gradually with sequential assembly of the molecular beacon (curve c), hybridization with miRNA (curve d), capture of the circular probe (curve e), and RCA process (curve f), indicating that the modification was conducted successfully step by step. A visual schematic display of the EIS curves corresponding to each step of the sensing platform construction is shown in Fig. 2B. The EIS curve of the CNTs-hairpin probe after the RCA process (Fig. 2B, curve f) provided the biggest diameter of the semicircle, indicating that the biggest \( R_{et} \) of the sensing electrode occurred after RCA. The increased \( R_{et} \) is one of the key reasons for the decreased Ru(NH₃)₆³⁺ signal in the following DPV analysis.

**Time dependence of CNT based solid-phase RCA**

To realize the sensitive detection of miRNA with the best experimental parameters, optimization of the DPV assay was focused on the effects of the RCA reaction time and signal probe adsorption time. First of all, the RCA reaction time was evaluated from 0.5 h to 3.0 h. As shown in Fig. S3A,† the peak current of the biosensor decreased after 2 h. Therefore, 2 h was confirmed as the optimal RCA reaction time for the present miRNA biosensor. Next, the electro-active probe incubation time was examined from 10 min to 40 min. As Fig. S3B† shows, when the incubation time reached 35 min, the biosensor exhibited the highest peak current for Ru(NH₃)₆³⁺. In conclusion, 2 h and 35 min were selected as the optimal parameter timings for miRNA detection in the following experiments.

**Differential pulse voltammetry analysis of miRNA**

According to the design of this protocol, as shown in Scheme 1A and B, in the presence of target miRNA (1 pM), solid-phase RCA based on CNTs would be performed and would result in the blocking of electron transfer between Ru(NH₃)₆³⁺ and the GCE. DPV detection and gel electrophoresis (Fig. 3) confirmed the hypothesis of the protocol design. As indicated by gel electrophoresis, the RCA could produce super-large DNA products due to its high amplification efficiency. After the RCA process was completed, the products of the RCA covered the surface of the CNTs. Although Ru(NH₃)₆³⁺
molecules would be adsorbed onto the RCA products on the electrode surface, the electrostatic interaction between Ru(NH$_3$)$_6$$^{3+}$ and DNA is weaker than the interaction between Ru(NH$_3$)$_6$$^{3+}$ and the carboxylic functionalized CNTs. The product of the solid-phase RCA was then characterized by DPV measurements. After hybridization of the hairpin probe (curve b), the peak current of Ru(NH$_3$)$_6$$^{3+}$ decreased slightly. Moreover, the peak current decreased dramatically after the RCA process (curve c). Accordingly, a solution-based RCA was conducted and the results are shown as the right-hand part of Fig. 3, which indicated that the CNT-based sensing strategy could realize a sensitive response to the RCA products. Detailed information about the solution-based RCA is shown in the ESI (Fig. S4†).

Based on the detection strategy, target let-7a was the trigger for unfolding the hairpin probe loop and inducing the CNT based solid-phase RCA assay. Therefore, in the absence of miRNA, the biosensor shows the highest peak current in Fig. 4. The RCA process was active when miRNA was introduced to the biosensor, which resulted in the signal loss in the DPV analysis. Then, let-7a in different concentrations (0–1000 fM) was used to induce the CNT based solid-phase RCA and DPV analysis was carried out. With the increase of the let-7a concentration, the DPV peak current decreased sharply (Fig. 4). Correspondingly, a good linearity was obtained between the DPV current of the signal probe Ru(NH$_3$)$_6$$^{3+}$ and the concentration of the miRNA ($I = -3.5 \times \log C + 23.6, R^2 = 0.9880$), with a detection limit of 1.2 fM (s/n = 3), shown in the inset of Fig. 4. The miRNA detection method using CNT based solid-phase RCA proposed in the present work exhibited a promising ability for ultrasensitive analysis of miRNAs with a very low abundance. To estimate the ability of the CNT enhanced label-free detection strategy based on hairpin probe triggered solid-phase RCA, we illustrated a comparison of several biosensors based on electrochemical techniques with various substrates and strategies in Table 1. Compared to the selected strategies, CNT based solid-phase RCA shows a low detection limit and an obvious high ratio of signal to noise.

Specificity of the CNT based solid-phase RCA strategy for miRNAs

To date, requirements for the selectivity and specificity of the new methods for miRNA detection are growing rapidly with the development of miRNA science. Generally, the miRNA family shows a high similarity between its members, hindering the progress of the development of a detection platform. In the present design, we purposely engineered a hairpin structured probe as the element for target miRNA detection. Due to the strong specificity between the loop-structure and the target sequence, highly selective detection of homologous members of one miRNA family could be accomplished successfully. Three different members of the miRNA let-7 family, let-7a, let-7f, and let-7g, which are highly homologous were selected for carrying out the experiments. As shown in Fig. 5A, the peak currents of the non-target miRNAs (let-7f and let-7g) were both close to the background curve, and only the let-7a curve shows an obvious signal decrease compared to the background, indicating that only the target miRNA (let-7a) was recognized and detected.

Since the stability of the hairpin probes is greatly dependent on the stem length, the stem length is considered as a key factor which might have impressive effects on the specificity for miRNA detection. To explore the specificity of the designed hairpin structured probe towards the target miRNA, five hairpin probes with different stem lengths in the range of 6 nt to 10 nt were investigated. As shown in Fig. 5B, no matter the length of the hairpin probe (6, 7, 8, 9 or 10 nt), the sensing specificity for miRNA detection was not compromised.
The content of let-7a miRNA in the total RNA sample was calculated to be $3.7 \times 10^8$ copies $\mu$L$^{-1}$ from Hela cells was diluted to 45.6 ng $\mu$L$^{-1}$ with RNase free PBS buffer. Aliquots of the total RNA extracted from Hela cells (1 $\mu$L) were spiked with a standard solution containing synthesized let-7a at concentrations of 0, 10, 20, 30, 40 and 50 fm. The amount of let-7a miRNA in the Hela cells total RNA sample was calculated to be $3.7 \times 10^8$ copies $\mu$L$^{-1}$ (shown in Fig. S5†), which is consistent with reported results. These results demonstrate that the present method holds great promise for real sample analysis with great accuracy and reliability.

### Conclusion

In this work we have designed an electrochemical platform for ultrasensitive detection of miRNA (members of the let-7 family) with a detection limit as low as 1.2 fm. The versatile platform was combined with highly specific recognition between the hairpin probe and the complementary miRNA, and CNT based solid-phase RCA. First of all, the highly specific recognition between the hairpin probe and the miRNA guaranteed the selectivity. Under the optimized experimental conditions, three members of the let-7 family with only one base mismatches could be differentiated easily from each other. Secondly, solid-phase RCA is masterly employed as the signal amplification technique to enhance the sensitivity of this electrochemical method. Label-free detection is the third advantage of this work. No treatments were implemented on the target miRNAs during the detection.

### Acknowledgements

This work was financially supported by the National Basic Research Program of China (no. 2011CB935704), National...
Notes and references