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Circle Padlock-Mediated Catalytic Hairpin Assembly Cooperating Primer Exchange Reaction for Sensitive and Label-Free MicroRNA Detection

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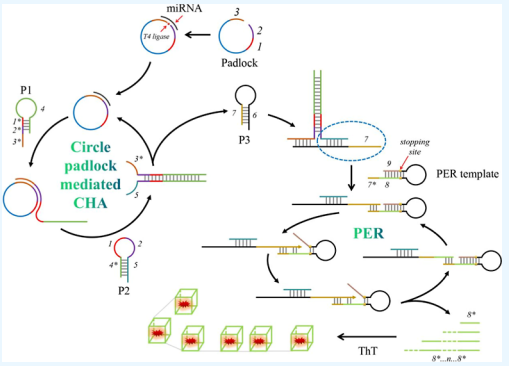
ABSTRACT: MicroRNAs (miRNAs), which play critical roles in regulating gene expression and cell functions, are recognized as potential biomarkers for various human diseases, including gastric ulcers. The reliable, specific, and sensitive detection of miRNA is highly recommended for the clinical diagnosis and therapy of different diseases. Herein, we depict a label-free and low-background fluorescent assay for the highly sensitive detection of miRNAs by coupling target miRNA-triggered cyclization of a padlock, circular padlock-mediated catalytic hairpin assembly (CHA), and primer exchange reaction (PER)-assisted signal generation. The padlock probe recognizes the target miRNA, forming a circular padlock that subsequently facilitates the CHA. The subsequent PER process generates substantial quantities of G-quadruplex sequences that rapidly combine with thioflavin T to create substantial fluorescence, thereby enabling the highly sensitive detection of the target miRNA. This method demonstrated significant potential for the early diagnosis of diseases such as gastric ulcers, as it could conclude the detection process in human serum samples within hours.



1. INTRODUCTION

MicroRNAs (miRNAs) are endogenous noncoding single-stranded RNAs that can control gene expression and regulate various biological processes, such as cell proliferation, differentiation, and apoptosis.[1−3] Hence, the expression level of miRNAs in cells, tissue, or body fluid strongly correlates with the pathological development of human diseases, such as gastric ulcers[3,4] and gastrointestinal cancer.[5] Extensive research studies suggest that the expression level of various miRNAs (e.g., miRNA-21 and miRNA-155) is significantly up-regulated in blood or tissue samples from patients with gastrointestinal cancers or gastric ulcer, which can serve as biomarkers for the early diagnosis of disease and holds great potential in gene therapy or pharmacological therapy of cancers.[6,7] Hence, it is imperative to devise techniques for sensitive and reliable miRNA detection in clinical samples such as serum and plasma.

The sensitive and accurate miRNA quantification is problematic due to its small size, low concentration, and great sequence similarity among family members.[8] In the 2000s, Northern blotting emerged as the preferred approach for analyzing miRNA. Nevertheless, it has low sensitivity and is time-consuming, making it unsuitable for rapid miRNA identification. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) has been developed to accurately measure the amount of miRNA due to its easy-to-operate character and high sensitivity.[9,10] However, the limited size of miRNAs poses a challenge in designing primers for effectively differentiating miRNAs from closely related sequences. To overcome the limitations of conventional approaches, various isothermal signal amplification strategies, such as rolling circle amplification,[11−13] catalytic hairpin assembly (CHA),[14] and primer exchange reaction (PER),[15−17] have been devised as alternative methods. These isothermal signal amplification strategies integrating with various signal modes (e.g., fluorescence, color, electrochemistry, and electrochemiluminescence) have been demonstrated to make the significant progression to the traditional PCR-based methods, such as the simplified experimental procedures and high signal amplification efficiency.[18,19] Among them, fluorescence-based amplification strategies have attracted abundant attention due to the programmable and easy-to-operate character.[20−22] However, fluorescence assays commonly require labeling the fluorescence moiety for signal generation, which is susceptible to experimental conditions (e.g., metal ions and temperature) and can induce high background signals and low stability. In addition, the fluorescence methods coupling with a single

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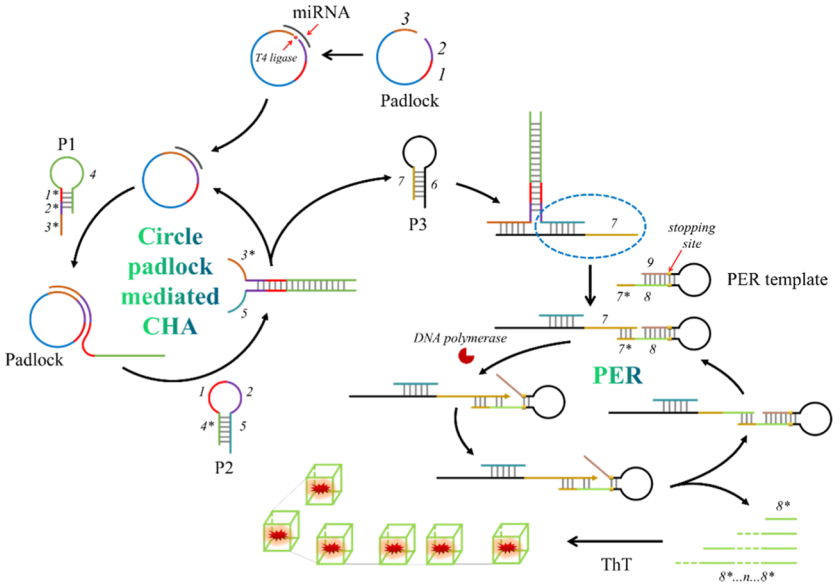
Scheme 1. Schematic Illustration of the Triple Amplification Strategy for Ultrasensitive Detection of Gastric Ulcer-Related miRNA[a]



[a]The strategy mainly contains three steps, including the miRNA-assisted cyclization of the padlock, circle padlock-mediated CHA, and the PER.

isothermal signal amplification strategy failed to provide adequate sensitivity.

Herein, we depict a novel label-free approach by integrating the target-assisted cyclization of padlock, circular padlock-mediated CHA, and PER-based signal generation for sensitive and reliable miRNA detection. In this method, target miRNA hybridizes with the two terminals of the padlock sequence to form the circular padlock with the assistance of the T4 DNA ligase. The circular padlock triggers the formation of numerous double-stranded structures of P1−P2 by activating CHA. The PER approach is a reliable isothermal amplification method used as a supplementary amplification step to improve signal amplification efficiency and generate multiple G-quadruplex sequences. Many G-quadruplex sequences form complexes with thioflavin T (ThT), resulting in substantial fluorescence and enabling the highly sensitive detection of target miRNA. By investigating the practical application potential of this approach in intricate biological systems and clinical samples, we expect it to offer a new and valuable detection tool for both biological research and clinical applications of miRNA.

2. EXPERIMENTAL SECTION

2.1. Reagents and Instruments. The DNA sequences (as illustrated in Table S1) were acquired from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The essential enzymes, including the Bst-DNA polymerase and T4 DNA ligase, were acquired from Thermo Fisher Scientific (Waltham, U.S.A.). Beyotime Biotechnology Co., Ltd. (Shanghai, China) provided the deoxyribonucleotide mixture (dNTP), diethylpyrocarbonate (DEPC) water, and commercial human serum sample. To safeguard against RNase degradation, all of the solution and deionized water utilized were treated with DEPC and autoclaved.

2.2. Phosphorylation of the Padlock. The linear padlock probe was phosphorylated by adding 2 μL of a 10 μM padlock probe, 2 μL of T4 polynucleotide kinase reaction buffer, 2 μL of a 10 mM ATP solution, 23 μL of DEPC-treated water, and 1 μL of T4 polynucleotide kinase (10 U/μL). The reaction was performed at a temperature of 37 °C for 1 h and was then stopped by incubating at 70 °C for 10 min.

2.3. Cyclization of the Padlock. The ligation reaction was conducted by combining 4 μL of the phosphorylated padlock probe (100 nM), 4 μL of 10 × T4 ligase reaction buffer, 1 μL of the RiboLock RNase Inhibitor (40 U/μL), 23 μL of DEPC-treated water, 4 μL of miRNA at various concentrations, and 4 μL of the T4 DNA ligase (5 U/μL) in a total volume of 40 μL. The reaction was carried out at room temperature for 1 h.

2.4. Signal Amplification and Generation Procedure. For the CHA process, the volume of the reaction system for CHA was 40 μL. We introduced 5 μL of P1 (100 nM), 5 μL of P2 (100 nM), 5 μL of P3 (500 nM), 5 μL of a circular padlock, and varying concentrations of target miRNA into the mixture. The total volume was adjusted to 40 μL using DEPC water. The reaction was then carried out at a temperature of 25 °C for 0.5 h. Subsequently, we conducted the PER and G-quadruplex/ThT-facilitated signal reaction. Upon the initiation of PER formation, the G-quadruplex structures can interact with ThT, generating fluorescence signals. The reaction system has a volume of 40 μL and contains the following components: 5 μL of 10 × ThermoPol reaction buffer, 5 μL of 8 U/L Bst large-fragment polymerase, 6 μL of $MgSO_4$ (100 mM), 3 μL of dNTP solution (100 μM), and 6 μL of the CHA product. Following a 1 h reaction, the mixture was further incubated with a 10 mM ThT dye for an additional 30 min. The resulting solution was then diluted in Tris-HCl buffer to a final volume of 200 mL to record the fluorescence. In the recovery experiments, the serum was diluted by a factor of 10 using PBS buffer, and synthesized target miRNA was subsequently added and analyzed.

3. RESULTS AND DISCUSSION

3.1. Working Mechanism of the Proposed Method for Sensitive miRNA Detection. The detection procedure of the miRNA assay is depicted in Scheme 1. The linear padlock

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probe is designed with three distinct functional portions. The linear padlock sequence is cyclized in the presence of target miRNA, and a circular padlock is formed with the help of the T4 DNA ligase. During the signal amplification process, a hairpin1 probe (P1) that can be unfolded upon binding to the circular padlock is designed. When P1 is unfolded by a circular padlock, P1 can create a double-stranded structure with the complementary section of hairpin 2 (P2), and it can also substitute target miRNAs and a circular padlock when P2 binds to P1. Once released, the circular padlock subsequently opens an additional P1, leading to several P1−P2 double-stranded structures. The two toeholds ((3*) and (5)) in the P1−P2 duplex serve as a complete primer to unravel the P3 probe, revealing the "7" segment. The "7" sequence binds with the "7*" portion of the PER template probe. This interaction serves as a primer to facilitate the PER process with the help of Bst-DNA polymerase and dNTP (A, T, and G). More precisely, sequence "7" is duplicated and elongated until it reaches the C−G termination site (stopping site) on the PER template probe. Subsequently, segment 9 competes with the elongated sequence to form a union with the hairpin stem, thus causing the spontaneous release of the elongated sequence from the PER template probe. The newly released and extended "8*" identifies the primer binding site in the PER template again and begins a fresh round of extension toward the C−G termination site. This process generates many repetitive "8*" sequences with varying lengths. The PER template probe is designed so that the generated sequences contain several G-quadruplexes. These G-quadruplexes can bind with ThT to provide a significantly amplified fluorescent signal, enabling the sensitive detection of miRNA.

3.2. Feasibility Analysis of the Proposed Method for Label-Free miRNA Detection. Fluorescence analysis was conducted to demonstrate and testify to the target miRNA-based cyclization of the padlock sequence. Specifically, the two ends of the linear padlock sequence are labeled with Cy3 and its corresponding quenching moiety (BHQ). As shown in Figure 1A, the peak fluorescence intensity at 580 nm greatly decreased when the linear padlock sequence was mixed with the target miRNA (padlock + miR). This phenomenon could be explained by the fact that Cy3 was quenched by the BHQ, which indicates the proximity of the two terminals of the padlock. The padlock-miRNA complex was then heated to 90 °C and the fluorescence signal reappeared, indicating that the padlock sequence was disassociated with the target miRNA (padlock + miR +90 °C). Only when the T4 DNA ligase was mixed with the padlock-miRNA complex could no significant fluorescence signal increase be observed, indicating the formation of a circular padlock (padlock + miR + T4 + 90 °C).

The feasibility of the circular padlock-mediated CHA process was verified by labeling Cy3 and BHQ on the two terminals of the P2 probe. From the result in Figure 1B, the Cy3 signal was high when the P2 probe was in a linear state. When the P2 probe was assembled into a hairpin structure, the Cy3 signal was greatly quenched. When the P2 probe was incubated with the P1 probe, no significant increase in fluorescence signal was recorded, indicating that the P2 probe was in hairpin strutted, and no hybridization occurred between the P1 probe and the P2 probe. A greatly increased fluorescence signal was observed only when the circular padlock was added to the mixture, indicating that the CHA process was initiated.

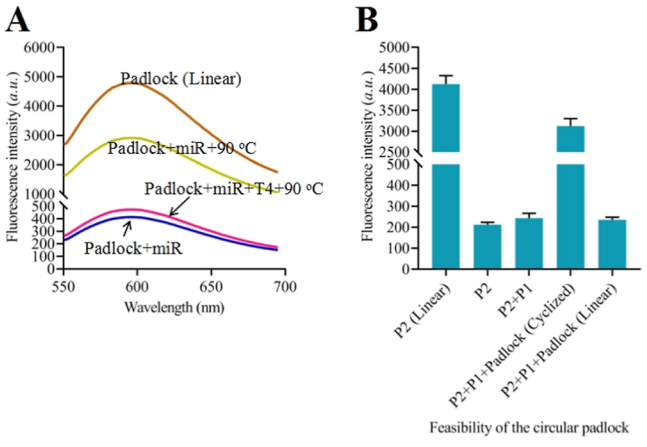

Figure 1. Feasibility of the proposed method. (A) Fluorescence spectrum of the Cy3-labeled padlock sequence when miRNA and T4 ligase existed or not. (B) Fluorescence intensity of the P2 probe during the circular padlock-mediated CHA process.

3.3. Optimizing Reaction Conditions on the Assay. The optimal amounts of T4 DNA ligase, probes (P1, P2, and P3), Bst-DNA polymerase, and reaction duration were determined to achieve the highest signal amplification efficiency and detection performance of the method. Figure 2A demonstrates a rapid increase in fluorescence signal until the concentration of the T4 DNA ligase reached 8 U/L, suggesting the successful ligation of the padlock. Figure 2B shows a direct correlation between the fluorescence intensity and the concentration of the P1 probe, with the maximum signal observed at 100 nM. Subsequently, a diminished signal was seen with more than 100 nM P1 probe, signifying the attainment of saturation in the CHA reaction. The optimized

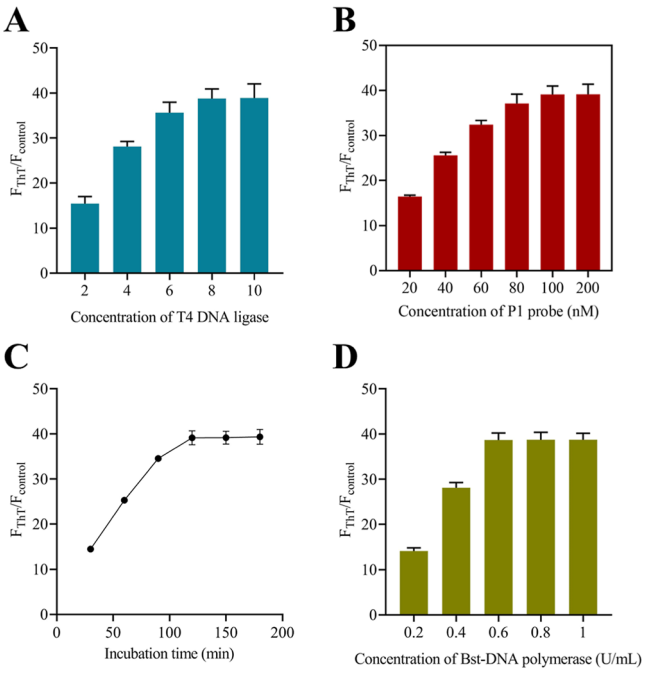

Figure 2. Optimization of experimental parameters. Fluorescence signal ($F_{ThT}/F_{control}$) of the method when detecting miRNA with different concentrations of the T4 DNA ligase (A), different P1 probe concentrations (B), different incubation time (C), and different concentrations of the Bst-DNA polymerase (D).

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concentrations for P2 and P3 probes are determined at 100 and 500 nM, respectively (Figures S1 and S2). Figure 2C demonstrates that fluorescence signals exhibited rapid development before 2 h for both CHA and PER signal generation. Subsequently, the growth rates steadily decreased, indicating a nearing process saturation. Therefore, we selected a reaction time of 2 h as the most favorable duration for the method. The concentration of Bst-DNA polymerase, as indicated in Figure 2D, was determined to be 0.6 U/mL, representing the optimal level. The concentration of the dNTPs was determined to be 100 μM (Figure S3).

3.4. Sensitivity of the Proposed Method. Additional research was performed on the fluorescence response in response to varying concentrations of the target miRNA under the optimized conditions. The fluorescence signals exhibit a progressive increase in response to the increased concentration of target miRNA, as demonstrated in Figure 3A. A strong

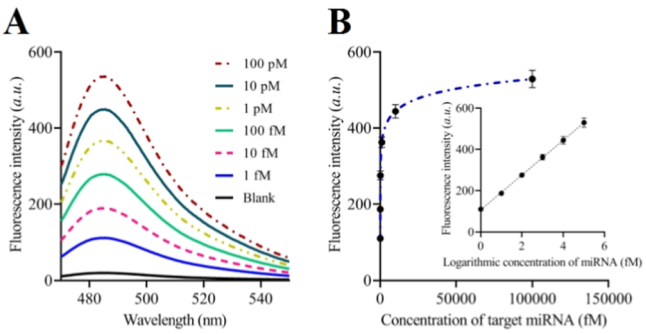

Figure 3. Analytical performance of the proposed method for sensitive miRNA detection. (A) Fluorescence spectrum of the method for different concentrations of miRNA detection. (B) Correlation between the recorded peak fluorescence value and the concentration of target miRNA. Inserted is the linear correlation between the peak fluorescence intensity and the logarithmic concentration of miRNA.

linear correlation between the logarithm of the target miRNA concentration and fluorescence intensity is demonstrated in Figure 3B, which ranged from 1 fM to 100 pM. The correlation equation is $F = 84.34 \times \lg C + 107.2$, with a correlation coefficient (R^2) = 0.9943, where F represents the fluorescence intensity in 485 nm and C represents the concentration of the target miRNA (fM). The limit of detection was estimated to be 712 aM using the 3δ rule. The sensitivity of the method is comparable or superior to most of the recently proposed fluorescence sensors while maintaining a label-free detection mechanism. The high sensitivity of the method was attributed to the high efficiency of the dual-amplification process, which combines circular padlock-mediated CHA and PER process-based low background signaling.

3.5. Selectivity and Reliability of the Assay. To assess the selectivity of the assay, the detection process was performed using various miRNAs, including miR-21, miR-141 (at a concentration of 1 nM), let-7a, and let-7c (at a concentration of 10 pM). Figure 4A shows that the fluorescence intensity was similar to that of the blank sample when all of these interfering miRNAs were added, indicating excellent selectivity of the method to interfering sequences. A negligible change was observed when miR-21 and miR-141 (at a concentration 100 times higher than the target miRNA) were mixed with the target miRNA instead of the sample containing only the target miRNA. The results demonstrate that the

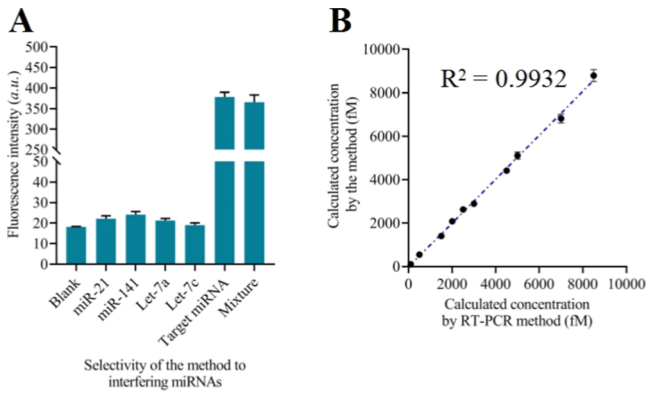

Figure 4. Specificity and clinical application potential of the method. (A) Fluorescence intensity of the technique when detecting different miRNAs. (B) Relations between the detection results by the method and by the RT-PCR method from constructed clinical samples.

proposed test exhibits exceptional selectivity toward target miRNAs. The method also showed a high selectivity to target miRNA in artificial actual samples, which was constructed by diluting the same concentrations of miRNA to the commercial serum samples, indicating the application potential in actual samples (Figure S4).

Circulating miRNAs have emerged as critical biomarkers for the diagnosis and prognosis of diseases such as gastric ulcers. Therefore, it is imperative to monitor the levels of miRNAs in clinical samples for the early diagnosis of diseases. Recovery experiments were conducted using commercial human serum samples to ascertain the reliability of the proposed assay for target miRNA detection. Various concentrations of target miRNA were introduced into 100-fold diluted serum samples, resulting in satisfactory recoveries ranging from 97.46% to 103.21% (Table 1). The validity of our approach was also

Table 1. Recovery Test of the Method

samples	added	detected	recovery rate (%)
1	10 fM	9.746 fM	97.46
2	100 fM	103.21 fM	103.21
3	5 pM	4.89 pM	97.8
4	10 pM	10.12 pM	101.2
5	50 pM	49.32 pM	98.64

evaluated by comparing the detection results to those of the most conventional RT-PCR method. The proposed method and RT-PCR exhibited a high degree of agreement with a high correlation coefficient of 0.9932, as illustrated in Figure 4B. These findings indicate that the assay is highly applicable for detecting miRNAs in clinical samples and biological fluids.

4. CONCLUSIONS

In summary, we have developed a novel fluorescent miRNA assay by coupling target miRNA-triggered cyclization of the padlock, circular padlock-mediated CHA, and PER-assisted signal generation. Compared with the former miRNA detection method based on CHA or PER, this method exhibits several advantages: (i) the padlock is cyclized only in the presence of target miRNA, and only the circular padlock unfolds the P1 probe to activate CHA, which allows for the accurate target identification and avoidance of false signals caused by homologous linear RNA; (ii) the low abundance of

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miRNAs in clinical serum samples is effectively addressed by the dual-amplification approach, which enables the sensitive detection of low-abundance miRNA; and (iii) the method is demonstrated with high specificity and outstanding anti-interference properties even for the detection of miRNA from complicated biological systems and environments. A brief comparison of the advantages and disadvantages of the method with the former is shown in Table S2.

However, the proposed method also possesses certain constraints such as a relatively lengthy detection time and unsuitability for cellular miRNA detection. In the future, we aim to create expedited assays to facilitate the in situ detection of miRNAs with a reduced detection period. In summary, the proposed method enables the identification of specific miRNAs, providing a novel method for investigating miRNA expression levels in patient blood samples and exploring their involvement in pathological mechanisms. Additionally, this method can be modified to identify a variety of other miRNAs, thereby offering a valuable alternative instrument for biomedical research and clinical applications related to miRNAs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c06859.

Sequences of the oligonucleotides used in present study; brief comparison of the approach with former ones; fluorescence signal ($F_{ThT}/F_{control}$) of the method when detecting miRNA with different concentrations of dNTPs; and selectivity of the method for miRNA detection from artificial actual samples (PDF)

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Author Contributions

The study was conceived by Z.C. M.C. conducted the lab work and wrote the manuscript. Q.K., A.Z., and S.L. assisted data analysis. All authors discussed and aided in interpreting the results.

Notes

The authors declare no competing financial interest.

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