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Article

Single-Step Detection of microRNA via the Programmable DNAzyme Probe-Mediated Multiple Signal Recycling

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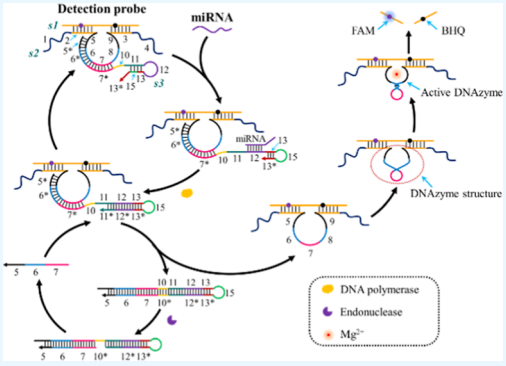
ABSTRACT: The significant role of microRNAs (miRNAs) in the regulation of pathological ocular neovascularization makes them both biomarkers and therapeutic targets in vascular eye diseases. However, their acute and sensitive detection and regulation continue to be a challenge. An integrative DNA probe (detection probe) was developed to enable the single-step detection of miRNAs with high sensitivity and specificity. This probe combines the functions of specific target recognition, the DNAzyme unit-based signal reaction, and the DNA polymerase/endonuclease-assisted signal cycle. In particular, a single-stranded sequence that engages with the DNAzyme unit of the s2 sequence can identify the target miRNA and liberate the DNAzyme unit to facilitate the signal reaction with the help of metal ions. In addition, the target recycling process and signal cycle were initiated by the DNA polymerase/endonuclease-assisted chain extension and displacement process, resulting in a low limit of detection of 4.56 fM. In addition, the method demonstrated a high level of septicity for the detection of target miRNAs and was capable of distinguishing interfering miRNAs with a one-base mismatch. Our research has shown that the integrative DNAzyme nanomachine is a promising biosensor for the sensitive detection and regulation of miRNA in a single step. It is anticipated that this technology will be used in the early diagnosis and therapy of vascular eye diseases.



1. INTRODUCTION

The research on miRNA biology has attracted abundant attention and has been extended to many biomedical research fields, including eye research, since the discovery of microRNAs (miRNAs). miRNAs are essential in regulating cell proliferation, development, and maturation at the posttranscriptional level, making them promising targets for novel therapeutic techniques and diagnostic biomarkers.[1−4] Ocular neovascularization (NV) is a major contributor to vision loss under various vascular-related eye conditions. Existing treatments targeting angiogenesis, including anti-VEGF therapies, exhibit certain drawbacks, highlighting the necessity of exploring alternative therapeutic approaches. Emerging research has identified multiple miRNAs as key regulators in the pathological development of ocular NV, positioning them as potential diagnostic markers and intervention points for vascular-related ocular disorders. Consequently, miRNAs are regarded as significant biomarkers for the early identification of eye diseases, prognosis, and the formulation of therapeutic strategies.[5] The precise identification and quantitative assessment of miRNAs are crucial for disease diagnosis and therapy, elucidating their biological mechanisms, and advancing the development of associated gene therapies. However, the diminutive size, sequence homology, and low abundance of miRNAs present significant hurdles to their accurate identification.

Various signaling approaches have been developed to assess the expression levels of miRNAs. Northern blotting is the traditional technique for miRNA detection, relying on hybridization and blotting with complementary radioactive or fluorescent probes.[6,7] This process is resource-intensive and laborious and lacks sensitivity, potentially leading to environmental contamination. The microarray analysis employs a high-throughput analytical platform that may identify disease-specific expression of many miRNAs across multiple samples.[8,9] This approach is hindered by inadequate sensitivity, prolonged hybridization duration, and the necessity for highly precise instrumentation, limiting its broader use. Consequently, effective amplification procedures are frequently necessary to provide extremely sensitive assay systems for miRNA detection. The rolling circle amplification (RCA) technology was investigated for the amplification detection of mRNAs because of its ease of use, high sensitivity, and high specificity.[10−13] Nonetheless, trade-offs, such as the protracted amplification process, challenges in template synthesis, and

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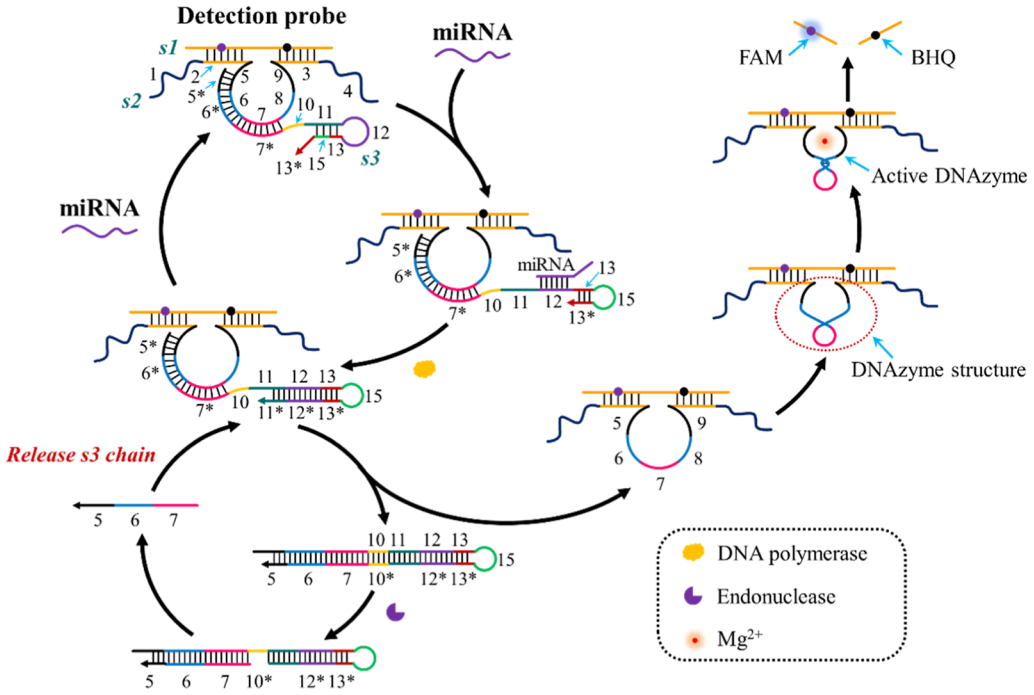

Figure 1. Working mechanism of the one-step miRNA detection method.

costly enzymes, presently hinder its broader use. Despite the quantitative reverse transcription polymerase chain reaction (qRT-PCR) being regarded as a gold standard for its high sensitivity and broad applicability in detecting various miRNAs, its extensive implementation continues to pose challenges due to the intricate primer design, requirement for costly equipment, and occurrence of false positives in miRNA detection.[14−16] Furthermore, other issues, including numerous experimental procedures and the participation of two or more detection probes, render these traditional methods inadequate for simple and sensitive miRNA analyses in complex experimental environments.

DNAzymes are a category of DNA-derived biocatalysts capable of facilitating various biological and chemical transformations in the presence of certain metal ion cofactors.[17,18] DNAzymes were employed to create DNAzymatic sensors for various metal ions in aqueous solutions by modifying these molecules with several functional agents, including fluorophores and thiol groups.[19] The identification of the target species can result in the amplified fluorescence intensity by over 10-fold, exhibiting greatly improved sensitivity with a reaction time of several minutes. However, these DNAzyme-based biosensors typically necessitate numerous probes to facilitate the signal amplification process, potentially resulting in false-positive outcomes.[20] Consequently, it is essential to create activatable DNAzyme-based probes by constructing a multifunctional DNA nanomachine that entirely excludes non-nucleic acid components.

We present a one-step approach for the sensitive and reliable detection of miRNA with a single detection probe, which is formed through the hybridization of three sequences (s1, s2, and s3) to incorporate the DNAzyme unit, target recognition area, and cleavage substrate. This approach involves target recognition by the detection probe, which triggers the self-priming process of s3, ultimately leading to DNA polymerase- or endonuclease-assisted chain extension. The chain extension process activates the recycling of target miRNA and the subsequent signaling cycle to perpetually release the DNAzyme unit in the detection DNA probe. The DNAzyme unit is activated by metal ions to cleave the substrate sequence and generate a fluorescence signal. This design demonstrates great sensitivity and stability, indicating a promising potential in vascular eye disease diagnosis, therapeutic strategy guidance, and prognosis evaluation.

2. EXPERIMENTAL SECTION

2.1. Materials and Reagents. High-performance liquid chromatography (HPLC) was employed to purify the oligonucleotides. TaKaRa Biotechnology (China) and Sangon Biotech Co., Ltd. (China) were responsible for the synthesis and purification of all oligonucleotides (Table S1). These sequences are designed according to former research studies.[21,22] The QuantiNova SYBR Green PCR Kit and QuantiNova Reverse Transcription Kit were acquired from QIAGEN Bioinformatics (Germany). Biological Industries (USA) supplied DMEM and BSA. The Nt.AlwI nicking endonuclease and Klenow fragment exo-DNA polymerase were procured from New England Biolabs Co., Ltd. SBS Genetech Co., Ltd. (Beijing, China) supplied the deoxyribonucleoside triphosphate (dNTP) compound. The CFX96 Real-Time System (Bio-Rad, USA) was employed to conduct the RTqPCR. An F-7000 fluorescence spectrophotometer (Hitachi, Japan) was employed to record the fluorescence spectra.

2.2. DNA Probe Preparation. The DNA assay was composed of three oligonucleotide chains (s1, s2, and s3). Table S1 contains the list of the sequences used in this research. A buffer (10 mM HEPES, 100 mM NaCl, and 50 mM $MgCl_2$, pH 6.0) was used to dissolve all of the sequences. Initially, 50 μL of DNA mixtures (s3 + s2, 1 μM, respectively) was denatured at 95 °C for 10 min, subsequently chilled to 20 °C, and stored at 4 °C to construct the s3/s2 complex. Following this, the detection probe was constructed by adding the 1 μM s1 sequence to the mixture.

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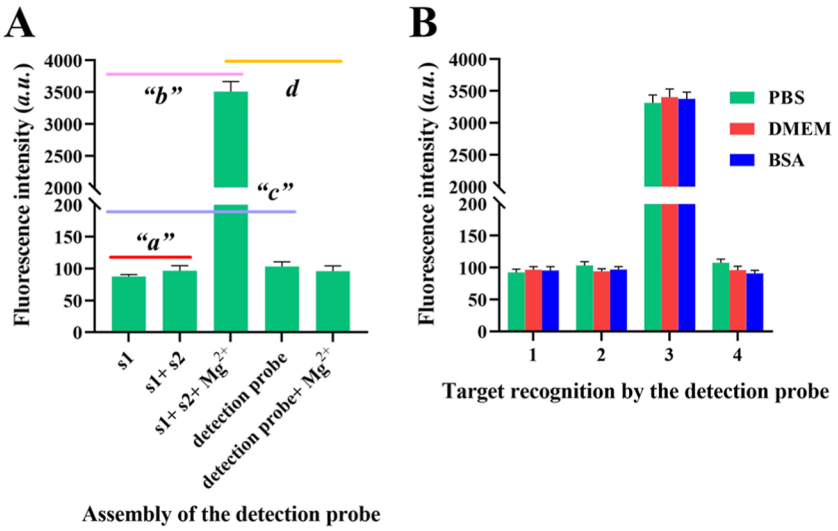

Figure 2. Construction of the detection probe and its feasibility in recognizing target miRNA. (A) Fluorescence intensity of the s1 sequence during the assembly of the detection probe when metal ions existed or did not exist. (B) Fluorescence intensity of the detection probe when target miRNA and DNA polymerase existed or did not exist. Column 1, "detection probe"; column 2, "detection probe + DNA polymerase"; column 3, "detection probe + DNA polymerase + miR-21"; column 4, "detection probe + DNA polymerase + miR-149".

2.3. Methods of Analysis. The sensing system was composed of 10 μL of the detection probe (500 nM), 10 μL of target miRNA, 2 μL of DNA polymerase (1.5 U/mL), and 2 μL of endonuclease (1 U/mL). The F-7000 fluorescence spectrophotometer (Hitachi, Japan) was used to record the fluorescence spectrum after the mixture was incubated at 25 °C for 90 min.

3. RESULTS AND DISCUSSION

3.1. Working Mechanism of the Method for Sensitive and Reliable miRNA Detection. The details of the working mechanism of the one-step miRNA detection approach are shown in Figure 1. In this method, a single detection probe is designed with three sequences to integrate the DNAzyme unit, target recognition module, and cleavage substrate. When target miRNA exists in the sensing system, the miRNA can bind with the "12" section in s3, thus exposing the "13" and "13*" sections. The "13*" section hybridizes with the "13" section and works as a primer to mediate the chain extension. Under the assistance of DNA polymerase, a sequence containing the transcribed "12*" section is attached to the "13*" and thus the miRNA is released from s3. The released s3 can bind with the next detection probe to mediate target recycling. Moreover, the "5"-"6"-"7"-"10*" sections are transcribed. The endonuclease recognizes the transcribed "10*" and generates a nicking site. Under the cooperation of the DNA polymerase and endonuclease, large amounts of "5"-"6"-"7" sequences are produced to hybridize with its complementary section in s3. Therefore, the s3 sequences are released from the detection probe, exposing the DNAzyme unit. The released DNAzyme unit can be activated by combining with the metal ions, and thus, the substrate sequences whose two ends were labeled with FAM and BHQ are cleaved. Consequently, the FAM signal, which was originally quenched by the BHQ, reappeared. The changes in the FAM signal are correlated with the concentrations of miRNA in the sensing system.

3.2. Assembly of the Detection Probe and Its Feasibility in Identifying Target miRNA. The detection probe is composed of three sequences, including s1, s2, and s3. The successful assembly of the detection probe is crucial for specific target recognition and the induction of subsequent signal amplification. Therefore, we first tested the assembly of the detection through a fluorescent assay. As shown in Figure 2A, the obtained fluorescence intensity of the s1/s2 duplex showed no significant difference with that of the s1 sequence alone, indicating the DNAzyme unit in s2 is in an inactive state and it could not cut the substrate sequence (s1) (statistic line "a"). When metal ions (Mg^{2+}) are added to the sensing system containing the s1/s2 duplex, the fluorescence intensity of the s1 sequence reappears because the DNAzyme unit in the s2 sequence is activated to cleave the s1 chain. The changes of the fluorescence signals demonstrated the active DNAzyme can cut the s1 chain to mediate the signal generation process. The assembly of the detection probe followed the heating of the mixture of s1, s2, and s3 sequences and cooling the mixture to room temperature. Compared with the fluorescence signals of the s1 chain alone and s1/s2 duplex, the fluorescence intensity of the detection probe showed no significant difference. When metal ions were mixed with the detection probe, the obtained fluorescence intensity remained at a low level and was comparable to that of the s1/s2 duplex because the s3 sequence blocked the DNAzyme unit of the s2 sequence, demonstrating the successful construction of the detection probe.

The results in Figure 2B showed that the constructed detection probe maintained high stability even in complicated experimental conditions (PBS, DMEM, and BSA), implying its potential in clinical applications. Meanwhile, the obtained fluorescence signal of the detection probe in various experimental conditions greatly elevated when target miRNA and DNA polymerase existed in the sensing system. On the contrary, the obtained fluorescence signal remained at a low level when interfering miRNAs existed. The above results demonstrated the construction of the detection probe and its feasibility in specifically recognizing target miRNA even in complicated conditions.

3.3. Feasibility of the Whole Sensing System. With the constructed detection probe, we then tested the feasibility of

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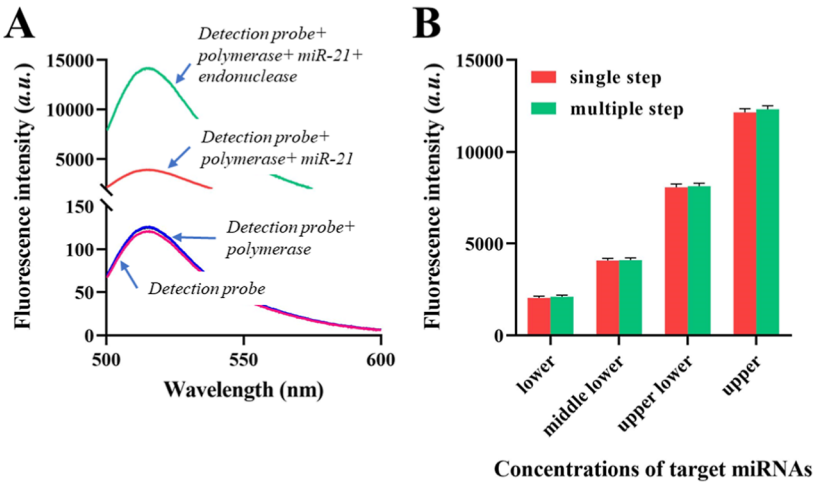

Figure 3. Feasibility analysis of the whole sensing system. (A) Fluorescence intensity of the method when essential experimental components existed or did not exist. (B) Fluorescence intensity of the method when detecting different concentrations of target miRNA using a single step and multiple steps.

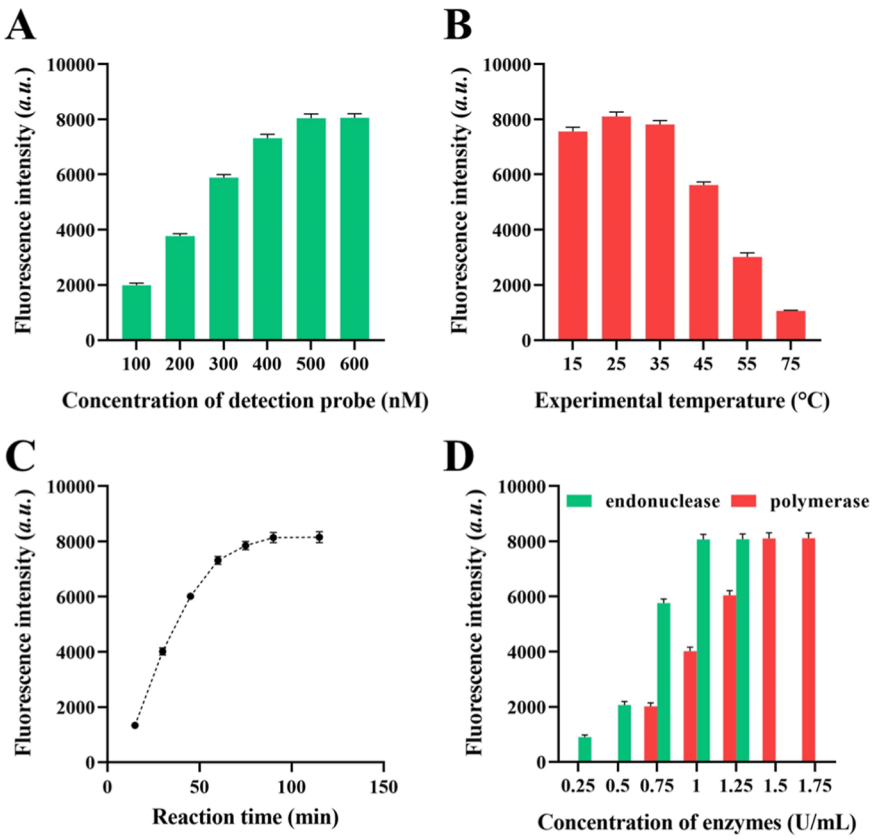

Figure 4. Optimization of experimental parameters. (A) Fluorescence intensity of the method detection target miRNA with different concentrations of the detection probe. (B) The detection performance of the method when performed under different experimental temperatures. (C) Fluorescence signals of the method when detecting target miRNA with various reaction times. (D) Fluorescence intensity of the method when detecting target miRNA with different concentrations of enzymes.

the whole sensing system and evaluated the signal amplification efficiency. The results in Figure 3A showed that when target miRNA, DNA polymerase, detection probe, and metal ions existed in the sensing system, high fluorescence intensity was recorded because the active DNAzyme unit cut the s1 sequence to release FAM signals. When the endonuclease was added to the sensing system, the recorded FAM intensity further elevated to 3.45 times that without endonuclease. The above results demonstrated that the sensing system could work only when all essential components existed, and the endonuclease-induced signal cycle led to a high amplification efficiency (3.45 vs 1).

We then validated whether the method could detect target miRNA in a single step. Specifically, the detection results of the method when the detection probe was premixed with enzymes (DNA polymerase and endonuclease) or mixed with the two

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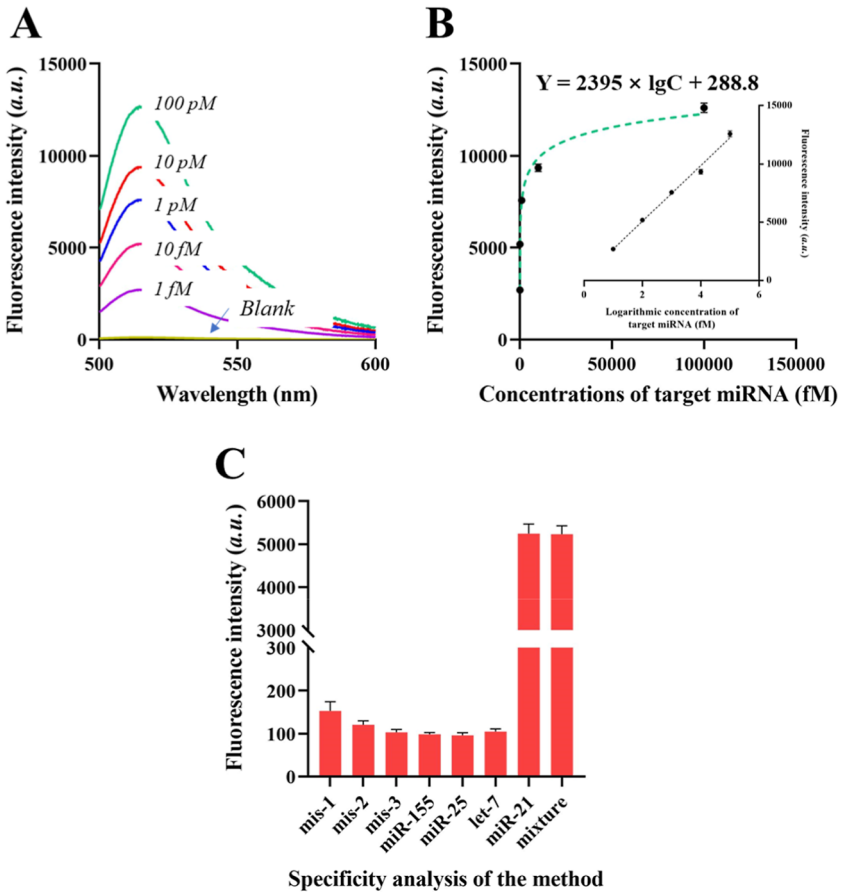

Figure 5. Analytical performance of the method. (A) Fluorescence intensity of the method when detecting different concentrations of target miRNA. (B) Regression of the concentrations of target miRNA and the peak fluorescence intensities. (C) Fluorescence signals of the method when detecting target miRNA and interfering miRNAs.

enzymes, respectively, were recorded and compared. The results in Figure 3B show that the obtained fluorescence intensities showed no significant difference, indicating the single-step detection of miRNA by the method is feasible.

3.4. Optimizations. To attain optimal performance, various conditions likely to influence the nanomachine were refined; these conditions encompassed the reaction temperature, reaction duration, concentrations of the enzymes (DNA polymerase and endonuclease), and quantity of the detection probe utilized in this sensing system. Initially, we adjusted the doses of the detection probes from 0 to 600 nM to optimize detection sensitivity (Figure 4A). We determined that the 500 nM detection probe produced the most significant alteration in fluorescence signals.

Furthermore, the reaction temperature influenced the efficacy of the nanomachine (Figure 4B). The peak signal alterations occurred at a reaction temperature of 25 °C. As the temperature rises, the fluorescence intensity changes diminish progressively. The cleavage activity of DNAzyme is contingent upon the presence of a hairpin structure. As the reaction temperature rises, DNAzyme is inclined to adopt a single-stranded configuration, hence resulting in a reduction of cleavage activity. Consequently, 25 °C was selected as the ideal temperature for the following experiments.

The reaction time was also analyzed (Figure 4C). As the reaction time grew, the fluorescence intensity first rose sharply before gradually leveling off after 90 min. The ideal reaction time for detection, to attain maximum efficiency in a reduced time frame and at a lower expense, was established at 90 min. The optimal concentrations of DNA polymerase and endonuclease were established at 1.5 U/mL and 1 U/mL, respectively (Figure 4D). The optimized concentration of endonuclease was lower than the optimized concentration of DNA polymerase because the DNA polymerase played crucial roles in mediating the self-priming-mediated chain extension and displacement, while the endonuclease was used to generate a nicking site to induce the production of "5"-"6"-"7" sequences.

3.5. Detection Performance of the Sensing System. In order to quantitatively assess the sensitivity of the method, we observed the corresponding variations in fluorescence intensity as the concentration of miRNA targets increased from 10 fM to 100 pM under optimal conditions. The peak intensity at 523 nm gradually increased (Figure 5A) with the increment of the miRNA concentrations. The linear regression ($Y = 2395 \times \lg C + 288.8$) and the rule of 3σ/slope (Figure 5B) were employed to determine the detection limit, which was 4.56 fM. This method was superior or comparable to most DNAzyme-based detection methods. It was demonstrated that the design of the detection probe in the method was capable of effectively cleaving the substrates under the induction of the target miRNA, resulting in high fluorescence signals for highly sensitive detection.

Another critical factor is specificity, which demonstrates that only molecules of interest can be identified. In order to further assess the specificity of the method, three other homologous

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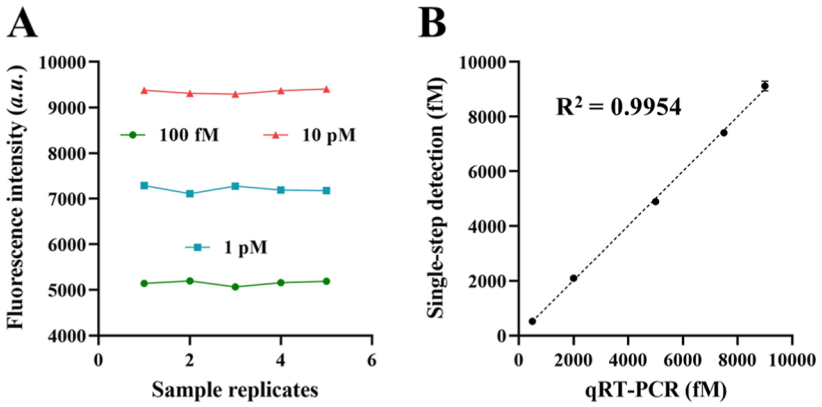

Figure 6. Stability and repeatability analysis. (A) Fluorescence intensity of the method when detecting different concentrations of target miRNA from sample duplicates. (B) Calculated miRNA concentration by the proposed single-step method and the qRT-PCR method.

miRNAs (miR-155, miR-25, and let-7), single-base, double-base, and triple-base discordant miRNA (mis-1, mis-2, and mis-3), and the mixture were employed as contrastive molecules (Figure 5C). As a result, the fluorescent signals generated by the base-mismatched miRNA-21 and the noncomplementary miRNAs were only marginally greater than the baseline signal. In contrast, the method demonstrated a substantial signal enhancement in target binding (miRNA-21 and mixture), suggesting that it was highly specific in distinguishing between target miRNAs and similar substances ($P < 0.05$) and was also capable of efficiently reducing false positives. Nevertheless, the mixture detection was not substantially different from the one detected for miR-21 ($P > 0.05$), indicating that the biosensing of miRNA-21 was not disrupted by the presence of other homologous miRNAs.

3.6. Stability and Repeatability Analysis of the Method. In order to assess the repeatability of the method, five replicated assay experiments were conducted with target miRNA concentrations of 100 fM, 1 pM, and 10 pM. The relative standard deviations (RSDs) of the three panels of experiments were determined to be 6.7%, 5.0%, and 5.1%, respectively, by documenting their signal responses using the constructed analytical procedure. The results indicated that this procedure has exceptional repeatability for miRNA-21 assays (Figure 6A).

The applicability reliability was ultimately confirmed by applying the method to the detection of miRNA from clinical samples. The miRNA concentrations calculated by the method exhibited a high degree of consistency with those calculated by the qRT-PCR method, as evidenced by the high correlation coefficient of 0.9954 in Figure 6B.

4. CONCLUSIONS

In our research, we created an integrative and intelligent detection probe capable of specifically responding to miRNA targets and releasing a DNAzyme unit to facilitate a signaling reaction. Due to its distinctive architecture, the probe facilitates dependable miRNA identification with exceptional specificity (including one-base mismatch discriminating) and sensitivity in a single step. In comparison to the recently reported DNAzyme-based biosensors and other methods for miRNA detection,[23−25] the proposed method offers several advantages: (i) all functions (e.g., target recognition, DNAzyme cleavage-based signal reaction, and signal cycles) are consolidated within a single probe, enhancing stability and resistance to interference by reducing the number of probe addition steps; (ii) only one step is necessary for miRNA detection, simplifying the process and facilitating promotion; (iii) target recycling and DNA polymerase/endonuclease-assisted signal cycles significantly amplify fluorescence signals, thereby enabling miRNA detection with a low limit of detection of 4.56 fM. In summary, this integrative nanomachine serves as a comprehensive instrument for the simultaneous detection and modulation of miRNA targets, with anticipated applications in the early diagnosis and personalized treatment of vascular eye diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c01877.

DNA and RNA sequences used in this work (PDF)

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Author Contributions

L.W.: writing—review and editing and project administration, writing—original draft, data curation, investigation, and resources. W.S.: methodology and formal analysis. L.D. and W.C.: data analysis. All authors adhered to the Committee on Publication Ethics guidelines.

Notes

The authors declare no competing financial interest.

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