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Label-Free, Sensitive, and Versatile Colorimetric Method for Molecule Detection via the G-Quadruplex-Based Signal Quenching Strategy

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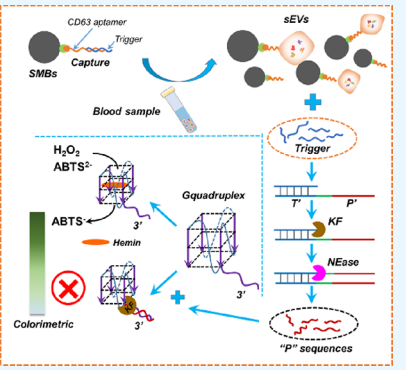
ABSTRACT: Signal amplification strategies have emerged as a prominent tool in the field of improving the detection sensitivity of small extracellular vesicles (sEVs). It is important to highlight that the utilization of signal quenching strategies is not commonly implemented. A detection technique for sEVs was established based on the unwinding of G-quadruplex using Klenow fragment polymerase (KF), which served as an inspiration for this study. This system is characterized by its simplicity and lack of labeling, making it an efficient approach for signal quenching. In the presence of sEVs, the CD63 aptamer in the capture@sMBs complex binds with the CD63 protein on the surface of sEVs to release trigger sequences, which were employed as a primer to mediate the DNA polymerase/endonuclease-assisted signal recycling. The signal recycling process produces numerous single-stranded DNA sequences that can bind to the toehold section of the G-quadruplex. This leads to the rupture of the G-quadruplex structure and the subsequent deactivation of a DNAzyme generated by the G-quadruplex structure and hemin, thereby inhibiting its biological catalytic function. Consequently, the G-quadruplex structure would undergo a transformation to a duplex structure, leading to the emergence of a discernible differential signal that can be noticed in a majority of instances, even without the aid of magnification devices. The decrease in the prominent signal allows for the efficient analysis of target sEVs, which exhibit a notably low detection limit. In addition to the detection of sEVs, the approach has also been utilized for the investigation of miRNA-21. The approach demonstrates a high level of selectivity and robustness in its capacity to differentiate between target miRNA and base-mismatched miRNA as well as other miRNA families. This statement suggests that the assay holds significant promise for use in biochemical research and clinical diagnosis.



1. INTRODUCTION

Small extracellular vesicles (sEVs) are nanosized vesicles that are generated by cells and consist of a lipid membrane.[1−3] Cargo molecules in sEVs, such as nucleic acids, proteins, lipids, and other bioactive compounds, play a significant role in a wide range of physiological and pathological processes by facilitating intercellular communication. In recent years, there has been significantly increasing attention on the sEVs as a potentially valuable biomarker for the early detection and treatment of various diseases, with a particular focus on the field of cancer.[4] There is a growing body of evidence indicating a strong correlation between the expression level of sEVs and the progression of cancer.[5,6] Nevertheless, the study of sEVs in a sensitive and direct manner presents significant challenges. Therefore, there is a pressing need to create simple, yet highly sensitive, techniques for the detection of sEVs.

Numerous platforms have been constructed for sEV analysis. One of the typical physical methods used for the identification of size distribution of sEVs is nanoparticle-tracking analysis (NTA).[7] Nevertheless, the lack of specificity in the NTA approach has restricted its applicability in further studies. Enzyme-linked immunosorbent assays (ELISAs) and western blots are reliable techniques for the precise identification of target sEVs originating from distinct cells by recognizing their surface membrane proteins.[8,9] Nevertheless, the limitations associated with these methods, including the need for extensive labor and huge sample volumes, the high costs associated with antibody usage, and limited sensitivity, have rendered these approaches inadequate for the detection of sEVs.

In recent years, several methods utilizing aptamers have been developed, which have shown advancements compared to conventional procedures for the detection of sEVs.[10−13] We once proposed an aptamer-based method, wherein it presented a very sensitive approach for fluorescently detecting sEVs.[14]

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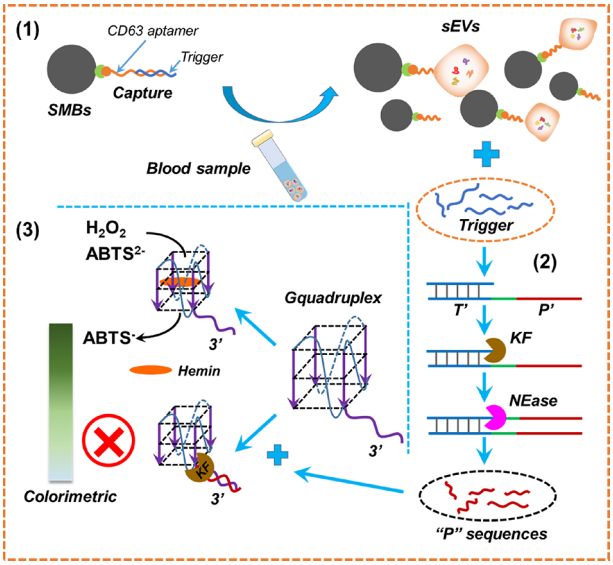
This method involved a capture probe that facilitated the conversion of signals from the CD63 protein, located on the surface of the sEV membrane, into nucleic acid signals. By using the CRISPR-Cas12a system, the approach ultimately demonstrated a detection range spanning from 10^3 to 10^6 particles/μL. In addition, Sun et al. developed a self-calibration sensor for the purpose of detecting exosomes.[15] This was achieved by integrating a methylene-blue-labeled aptamer onto a functional hybrid thin-film-based indium tin oxide substrate. The sensor demonstrated a low limit of detection of 100 particles/μL. The colorimetric method offers significant advantages over fluorescent and electrochemical approaches for the identification of sEVs due to its ability to be recognized by the naked eye. Gold nanoparticles (AuNPs) have garnered significant interest in colorimetric-based assays and have been widely utilized for the detection of biomolecules.[16,17] This is primarily attributed to their distinct optical properties, which are dependent on the distance between particles and can result in a color shift upon aggregation. Nevertheless, the intricate process of ligating oligonucleotide modifications onto the surface of AuNPs has hindered their potential for broader utilization. In comparison to AuNPs, the utilization of the G-quadruplex/hemin peroxidase mimicking DNAzyme in colorimetric-based assays is feasible due to its enhanced catalytic activities toward oxidation substrates related to hydrogen peroxide (H_2O_2), such as *o*-phenylenediamine (OPD), $ABTS^{2-}$, and TMB.[18,19] This results in the production of colorimetric signals based on the G-quadruplex/hemin DNAzyme.[20,21] However, only a limited number of studies have documented the utilization of signal quenching-based colorimetric techniques for the identification of sEVs. In addition, the quenching methods lack signal amplification processes and thus necessitate improved sensitivity.

Herein, we present a simple, highly responsive, and label-free colorimetric system that combines strand displacement amplification (SDA) with a signal quenching approach based on G-quadruplex DNAzyme catalysis. The detection result of the established approach can be easily observed through visual inspection or by employing UV-visible spectroscopy analysis. The newly developed method utilizes SDA-based signal amplification, which leads to a substantial reduction in the detected signal. This approach allows for the detection of target sEVs with a considerably lower detection limit while also being simple and cost-effective. Due to the exceptional performance exhibited by this sensing system, it possesses significant potential for use in biochemical research and clinical diagnosis.

2. RESULTS AND DISCUSSION

2.1. The Working Principle of the Colorimetric Method for sEVs detection. The working mechanism of the proposed colorimetric method for sensitive sEVs detection is illustrated in Scheme 1. The method is composed of three steps, including identification of sEVs by capture@sMBs , NEase-assisted production of "p" sequences, and G-quadruplex-based signal quenching. The capture probe, which is loaded on the surface of sMBs, is formed by a CD63 aptamer sequence and a trigger sequence (partly complementary with the CD63 aptamer sequence). The CD63 aptamer can specifically bind with CD63 protein on the surface of sEVs, as reported by former researches.[10,14] After the assembly of the CD63 aptamer and trigger sequence through a thermal cycle, a capture probe is constructed. When sEVs exist, the capture@sMBs complex identifies CD63 protein on the surface of sEVs via the interaction between CD63 aptamer and CD63 protein. The conformation of the CD63 aptamer after identifying CD63 protein releases trigger sequence from capture probe. A template is designed to perform the subsequent amplification process, which possesses a central NEase recognition sequence and a flanking region (P′). Based on this, the released trigger hybridizes with T′ at the 3′-end region of the template to form a complex, and serves as a primer for Klenow Fragment (KF) to synthesize a duplex with a complete NEase recognition section. Then, a nicking site is generated by NEase on the complementary strand in produced dsDNA duplex, and DNA polymerase can sequentially extend the cleaved complementary sequence while displacing the "P" sequences. With the cooperation of KP polymerase and NEase, a large amount of "P" sequences can eventually be synthesized. The coloremetric procedure is based on unwinding of G4 by KF using a "P" sequences—complementary toehold connected onto a 3′-Myc2345 G-rich sequence. Once hybridized with generated "P" sequences, KF mediates chain extension, which disrupts the G4 structure and guides the probe amplified into a duplex. Thus, the green color generation process with addition of hemin, $ABTS^{2-}$, and H_2O_2 will be abolished.

Scheme 1. Working Mechanism of the Capture@sMBs-Based sEV Isolation and MG41 Probe-Based Color Development[a]



[a](1) SMB capture probe-based sEV identification and isolation; (2) released trigger sequence-based strand displacement amplification; (3) MG41 probe-based signal quenching reaction.

2.2. Feasibility of the Proposed Method. Several previous studies have demonstrated the validation of the capture probe assembly using a fluorescence test. In this experiment, the CD63 aptamer is labeled with FAM at its terminal end, while the trigger is labeled with BHQ at its terminal end. An equimolar ratio of the CD63 aptamer and trigger molecules was combined and subjected to incubation at 90 °C for 10 min. Following a gradual cooling process to reach room temperature, the fluorescence emitted by FAM was measured using a fluorospectro photometer. As depicted in Figure 1a, the fluorescence signal of the capture probe was noticeably diminished upon assembly, suggesting the effective hybridization of the trigger sequence with the CD63 aptamer.

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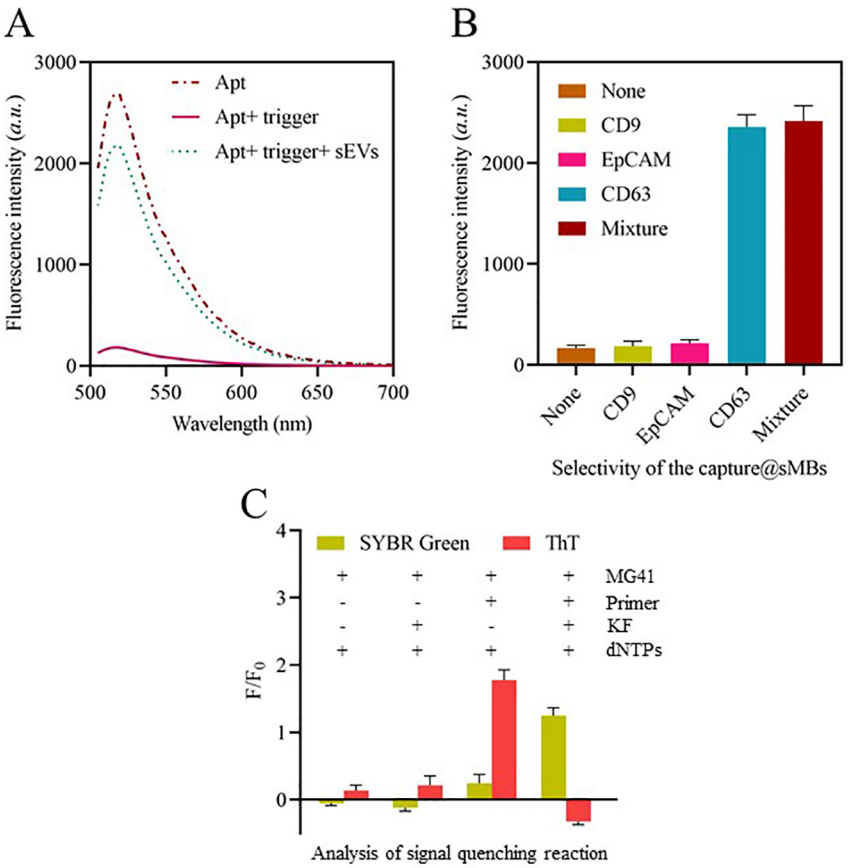

Figure 1. Feasibility of the proposed approach. (A) Fluorescence spectrum of the FAM-labeled CD63 aptamer during the construction of the capture probe. (B) Fluorescence intensities of the capture probe when detecting different molecules. (C) F/F_0 of the MG41 in the chain extension process.

In the presence of sEVs, the CD63 protein located on the surface of the sEV membrane interacted with the CD63 aptamer. This interaction resulted in the unwinding of the capture probe, leading to the reappearance of fluorescence from the FAM dye and the liberation of trigger sequences. The experiment further confirmed the specificity of the developed capture probe, as evidenced by the absence of any notable increase in fluorescence when the capture probe was treated with CD9 protein (Figure 1b).

In the colorimetric technique, the previously described G4 probes were chosen based on the linker sequence between G-rich and recognition areas. ThT and SYBR Green I were chosen as the fluorescent probes for the G4 structure and dsDNA, respectively, because they are commercially available. The results of the MG41-based colorimetric analysis showed that primer hybridization with the toehold section initiated hybrid duplex amplification, leading to the unwinding of the G4 structure in the presence of KF and dNTPs (Figure 1c). The more exposed G4 structure in the presence of "P" sequences may explain why the absence of KF resulted in only a mild fluorescence signal increase in SYBR Green I and a high ThT fluorescence incensement. Based on these findings, it appears that the G4 structure was dissociated with the help of KF in the presence of a primer and subsequently polymerized as a double chain.

2.3. Optimizing Experimental Conditions. For an improved sensing performance, various essential parameters of the approach were considered. These elements included the incubation duration of sEVs with capture@sMBs, the incubation time, the concentration of KF polymerase, the concentration of endonucleases, and the concentration of hemin. The measurement of absorbance was conducted in two conditions: in the absence of sEVs denoted as A and in the presence of sEVs denoted as A_{target}. As depicted in Figure 2a, the A-A_{target} had the most pronounced alteration prior to 30 min and subsequently attained a state of equilibrium beyond the 30 min threshold. Hence, a duration of 30 min was chosen for the capture of sEVs. The optimized reaction time for signal amplification and generation was determined at 80 min. The amounts of DNA polymerase and endonuclease play a crucial role in the process of signal amplification. The data presented in Figure 2b demonstrate that the migrations of KF polymerase from 0 to 0.2 U/mL led to an increase in absorbance. As the DNA polymerase concentration rose, however, so did the background absorbance. The addition of extra KF polymerase resulted in a decrease in the A/A0 ratio as the concentration of KF polymerase increased from 0.2 to 0.3 U/mL. The best condition for the KF polymerase concentration was determined to be 0.2 U/mL. A hemin concentration of 2 mM was employed to achieve the optimal signal-to-noise ratio, as depicted in Figure 2c.

2.4. Analytical Performance of the Established Colorimetric sEV Detection Method. Subsequently, the detection sensitivity of this proposed approach was evaluated. Figure 3a demonstrates that the signal at 420 nm was attenuated as sEV concentrations increased from 10^2 to 10^6 particles/μL. The linear relationship between A-A_{target} and sEV concentration is shown in Figure 3b. Our suggested signal

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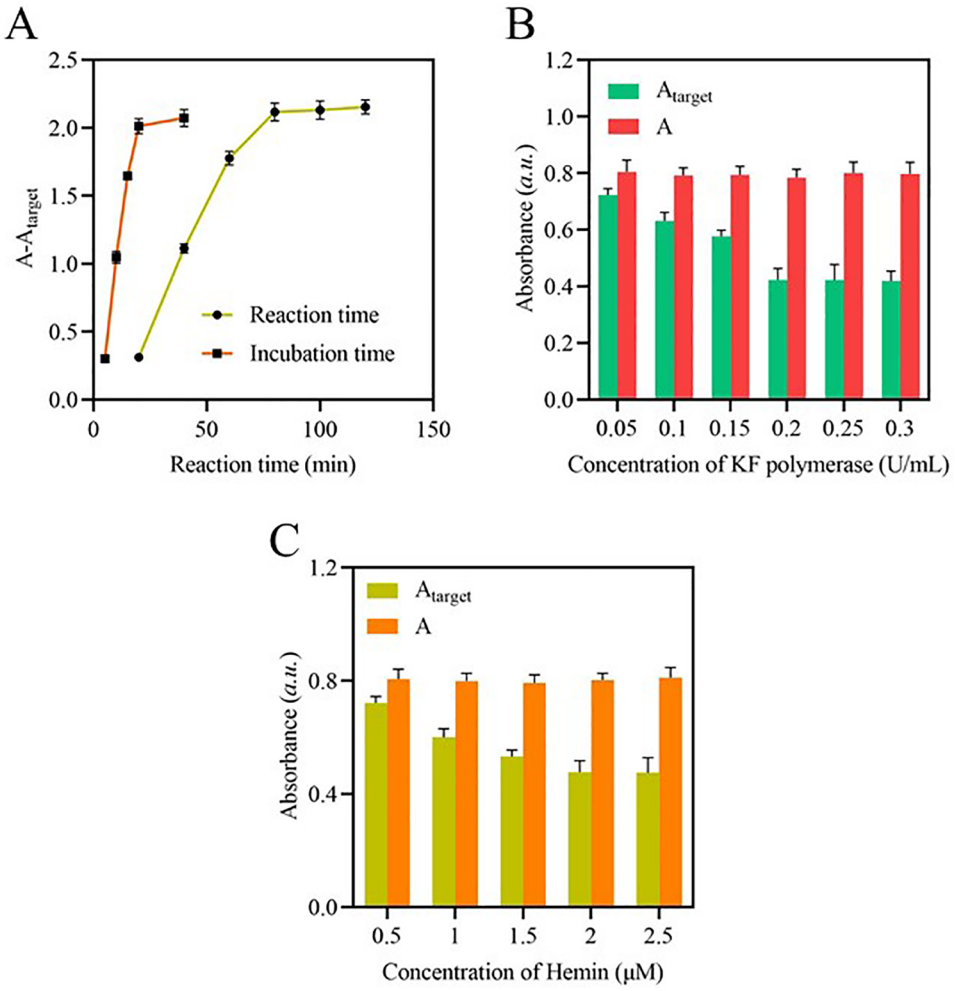

Figure 2. Optimization of experimental parameters. (A) Absorbance in the absence (A) and presence of sEVs (A_{target}) under different incubation times and reaction times. A-A_{target} of the approach with different KF polymerase concentrations (B) and hemin concentrations (C).

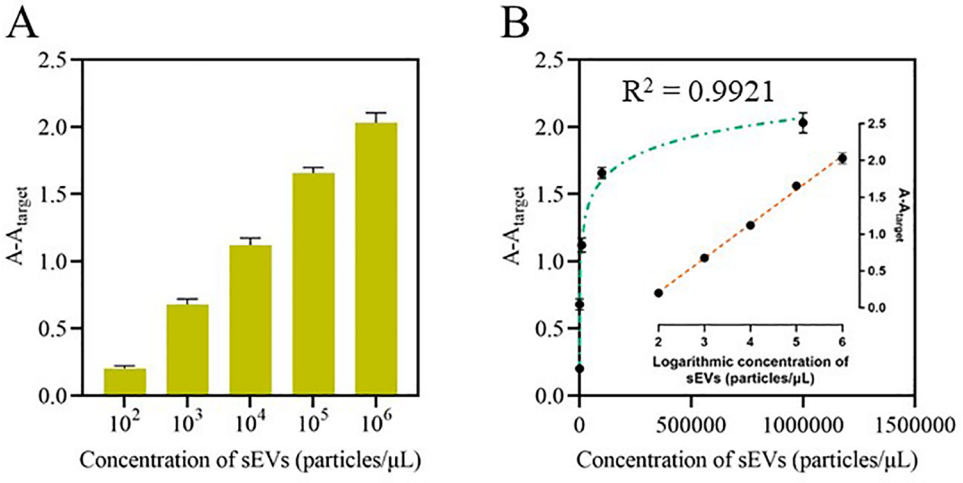

Figure 3. Analytical performance of the approach. (A) A-A_{target} of the approach when detecting different concentrations of sEVs. (B) Correlation between the A-A_{target} and the concentration of sEVs.

quenching strategy was able to attain the same level of sensitivity as previously described colorimetric methods based on signal amplification but with a much more straightforward means of outputting the results. The spike recovery in human serum was performed by adding several known amounts of sEVs, and recoveries ranging from 98.6 to 104.8% were obtained (Table 1), indicating the potential utility of this proposed technique for actual samples. The suggested approach's reproducibility is assessed by repeatedly identifying 10 sample duplicates; the resultant A-A_{target} shows a coefficient

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Table 1. Recovery Test of the Approach

samples	added (particles/μL)	recovery (particles/μL)	recovery rate (%)
1	500	524	104.8
2	1000	987	98.7
3	2500	2465	98.6
4	5000	5187	103.7

of variation (R^2) of 2.63% (Figure S1), which is sufficient for clinical application. Finally, the clinical practicality is evaluated by using the suggested method and NTA method to detect sEVs in serum samples obtained from patients with NSCLC. This method has great promise for use in the identification of clinical specimens as the results of sEVs detected using this method are highly consistent with NTA results (R^2 = 0.9456; Figure S2). These findings demonstrated the sensing strategy's potential for use in the detection of real samples with high sensitivity and selectivity.

2.5. Extensive Application for Biomolecule Detection. The application of the suggested approach for miRNA detection was extensively investigated. The investigation focused on evaluating the detection sensitivity of the proposed approach. According to the data presented in Figure 4a, the signal at a wavelength of 420 nm exhibited a progressive decrease when the concentration of target miRNA-21 increased from 100 fM to 1 nM. The results inserted an equation that demonstrated a strong positive connection between A-A_{target} and the concentration of miRNA-21. The equation representing the linear correlation is $Y = 0.4401 \times \lg C - 0.3260$. The detection limit, determined using the principle of 3 s/slope, was 45 fM. In contrast to colorimetric methods that rely on signal amplification, our proposed signal quenching strategy demonstrates comparable sensitivity and a broad linear range for miRNA-21. However, it is worth noting that bioluminescence and fluorescence strategies, which exploit the accumulation of the target signal, exhibit higher sensitivity. To assess the specificity of the test, we conducted an investigation into the signal response using a variety of single-base and three-base-mismatched sequences. According to the data presented in Figure 4b, the signal response generated by the target miRNA-21 was significantly higher compared to those with one or three base mismatches. This observation indicates that our technique exhibits a strong ability to differentiate between base mismatch sequences and the corresponding target sequence.

3. CONCLUSIONS

In summary, we have successfully constructed a signal quenching approach through the development of a sequence-specific and visually perceptible detection method for sEVs and miRNA detection. The approach employed in this study involved the targeted capture of sEVs and the subsequent disruption of G-quadruplex structures by KF polymerase following the release of a "P" sequence that forms a complementary pairing with a toehold section attached to the 3′-Myc2345 G-rich sequence. The sensing system that has been recently created exhibits a notable reduction in signal quenching, enabling it to identify target sEVs with a significantly low detection limit (Table S2). The strategy's remarkable anti-interference capability and practicality when applied in a serum environment would undoubtedly contribute to the advancement of trace sEV detection in biochemical research and clinical diagnostics.

4. EXPERIMENTAL SECTION

4.1. Materials and Reagents. The HPLC-purified DNA sequences and miRNAs were produced by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). The details of the sequences can be found in Table S1. A solution of deionized water treated with 0.1% diethylpyrocarbonate (DEPC) was obtained from Beijing Leagene Biotechnology Co., Ltd. (Beijing, China). The chemicals used in this study, including potassium chloride (KCl), sodium chloride (NaCl), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), and Triton X-100 (BioUltra), were procured from Sigma-Aldrich. A 30% concentration of hydrogen peroxide (H_2O_2) was acquired from Aladdin. The chemicals hemin and ABTS were procured from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). The Klenow fragment (3′-5′ exo-) (KF) and 10× NEB buffer 2 (composed of 100 mM Tris-HCl, 500 mM NaCl, 100 mM $MgCl_2$, and 10 mM DTT, with a pH of 7.9) were acquired from New England Biolabs Co., Ltd. (Beijing, China). The

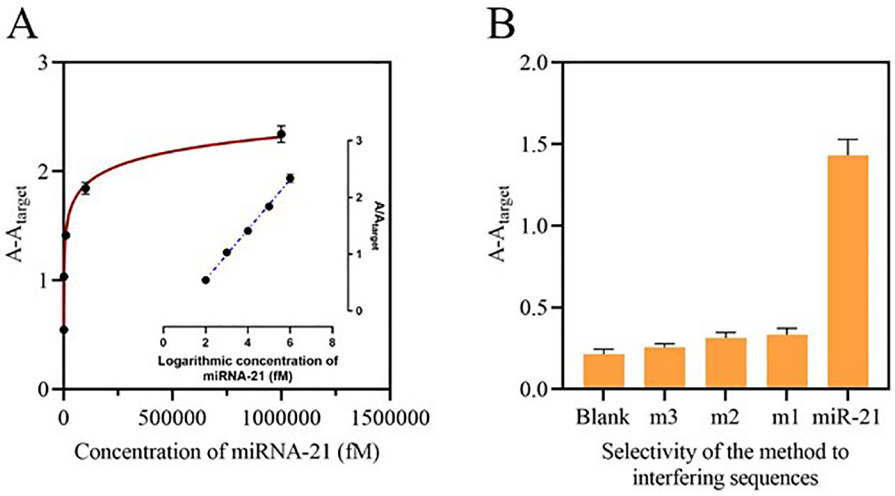

Figure 4. miRNA detection performance of the approach. (A) A-A_{target} of the approach with different concentrations of miRNA-21. (B) Selectivity of the approach.

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SYBR Green I (10,000×) and dNTPs Mix (10 mM) were procured from Beijng Solarbio Science & Technology Co., Ltd. (Beijing, China). The thioflavin T (ThT) compound was acquired from the Sigma-Aldrich company.

4.2. Cell Culture and sEV Purification. The HL-60 cell line was cultured in a Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin−streptomycin. The HEK293T cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% (v/v) penicillin−streptomycin. The culture incubator was kept at a temperature of 37 °C and a CO_2 atmosphere concentration of 5%. After being cultured for 48 h in a medium containing sEV-free FBS, the sEVs were obtained from the supernatant of the cell medium. The isolation procedures were conducted as described below. Initially, the intact cells were separated from the culture supernatant by a centrifugation process at a rate of 1000*g* for 5 min. Subsequently, the supernatant that was acquired was subjected to additional centrifugation steps at 2000*g* for 10 min and 10,000*g* for 30 min to eliminate cellular debris and big vesicles, respectively. In the present study, ultracentrifugation was employed to acquire sEVs by subjecting the sample to centrifugal forces of 120,000*g* for a duration of 120 min. The collected sEVs underwent an additional washing step using PBS buffer followed by resuspension in PBS buffer.

4.3. Detection of the Target. *4.3.1. Capture Process.* First, 5 μL sEVs with different concentrations were added into capture@sMBs for 30 min. After magnetic separation, the liquid supernatant was collected.

4.3.2. Detection Process. A total volume of 8 μL of MG41/45 at a concentration of 10 μM, 4 μL of the liquid supernatant at a specified concentration, 8 μL of 10× S5 NEB buffer 2, 4 μL of dNTPs at a concentration of 10 mM, and 48 μL of Tris-HCl buffer at a concentration of 10 mM was gently mixed and subjected to incubation at a temperature of 37 °C for a duration of 80 min. Subsequently, a volume of 8 μL of KF polymerase (2 U/μL) was introduced into the hybrid solution followed by an incubation period of 50 min at a temperature of 37 °C. Following that, the polymerization solution was halted at 80 °C for a duration of 10 min. After the sample reached ambient temperature, the polymerization solution was supplemented with 4 μL of hemin (200 μM, dissolved in DMSO), 160 μL of dilution buffer (containing 10 mM Tris-HCl, 25 mM KCl, 500 mM NaCl, and 0.1% Triton X-100), and 126 μL of Tris-HCl buffer (10 mM). This mixture was maintained at room temperature for a duration of 1 h. Subsequently, volumes of 10 μL of ABTS (40 mM) and 20 μL of freshly produced H_2O_2 (10 mM) were introduced into the resultant solution. The absorption spectra were collected after a period of 15 min.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09888.

Sequences of the oligonucleotides used in the present study (Table S1); brief comparison of the approach with the former ones (Table S2); the A-A_{target} of the approach when detecting 10 sample duplicates containing 10^4 particles/L sEVs (Figure S1); calculated sEV concentration by the proposed method and the NTA method (Figure S2) (PDF)

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Author Contributions

#Q.M. and B.G. contributed equally to the research. X.Z. and J.C.: visualization, writing-review, editing, supervision, and funding acquisition; Q.M. and B.G.: methodology, formal analysis, investigation, data curation, and writing-original draft; Y.J., Y.W., W.L., and H.C.: formal analysis and investigation.

Notes

The authors declare no competing financial interest.

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