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RESEARCH ARTICLE

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Duplex-specific nuclease and Exo-III enzyme-assisted signal amplification cooperating DNA-templated silver nanoclusters for label-free and sensitive miRNA detection

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Abstract

Development of novel miRNA detection strategies plays a crucial role in fundamental research and clinical diagnosis of various diseases, such as infantile pneumonia. We herein develop a rapid and sensitive DNA-templated AgNCs-based miRNA detection approach, pinning the hope on an improved detection sensitivity in an easy-to-operate way. In the method, a hairpin probe is designed to specifically bind with target miRNA, and to initiate the DSN enzyme and Exo-III-assisted dual signal recycles. The resultant guanine-rich DNA sequences after signal amplification turn on the fluorescence of the dark AgNCs by hybridizing with the DNA template of the dark AgNCs. The generated signals are correlated with the amounts of target miRNA in the sensing system. Through a series of experiments, the established approach exhibits a great dynamic range of more than seven orders of magnitude with a low limit of detection of 245 aM, holding great promises for miRNA-related researches and disease diagnosis.

Keywords: DSN enzyme, Exo-III enzyme, miRNA, AgNCs

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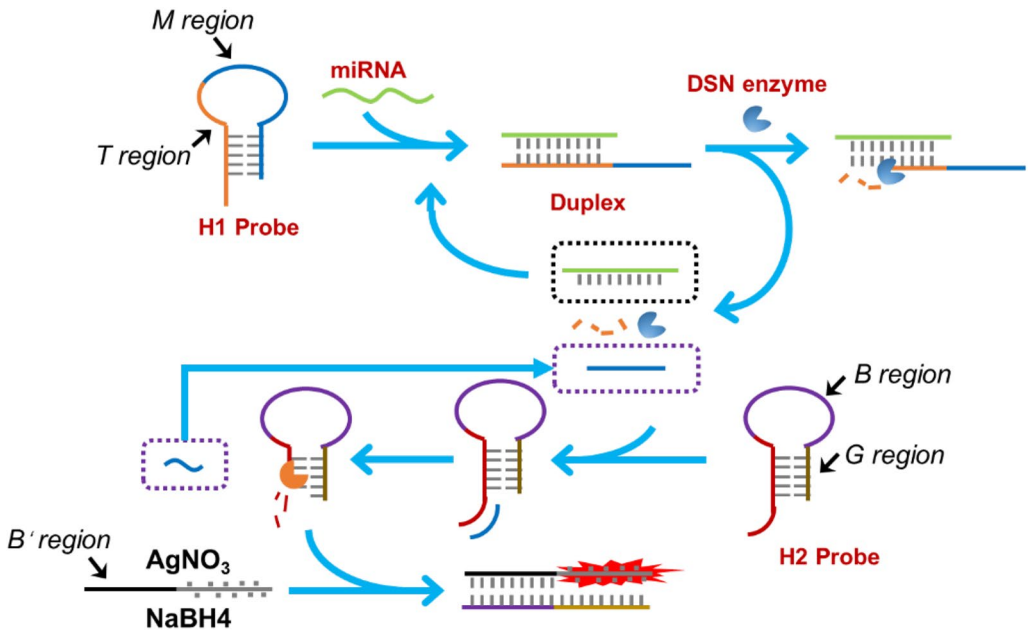
Graphical abstract



Introduction

MicroRNA (miRNA) is a kind of non-coding RNA that plays a crucial role in regulating the gene transcription and translation, and thus affecting biological development, reproduction, cell differentiation (Yin et al. 2021; Zhang et al. 2021). The recent researches have implied the miRNAs participant in regulating the inflammatory response in the development of infantile pneumonia (Lee et al. 2018), which is induced by *mycoplasma pneumoniae* and is common in infants, school-age children and adolescents (Cui et al. 2021; Li et al. 2019; Zhang et al. 2020). Despite that most of the cases have a good prognosis, the high recurrence and possibility to develop sever lung lesion cannot be ignored. For example, Xiao et al. reported that miRNA-21 can down-regulate enterovirus71-induced innate immune response and inflammation, and illustrated its potential role in protective mechanisms of infantile pneumonia (Wang et al. 2019). Therefore, developing novel miRNA detection methods can possibly contribute to the biological researches of infantile pneumonia.

QRT-PCR is currently one of the most-widely used strategies for sensitive and quantitative detection of miRNA, making remarkable contribution to the early diagnosis of diseases and basic medical researches (Chen et al. 2011; Deng et al. 2017). Despite that qRT-PCR method can provide robust amplification efficiency, the high labor intensity, complicated primer design and time-consuming characteristic greatly restrict its further application. Apart from qRT-PCR method, Northern blotting and DNA microarray are also conventional miRNA detection strategies, but are faced with the drawbacks of insufficient sensitivity and large sample consumption (Eulalio et al. 2012; Li et al. 2009; Pall and Hamilton 2008). To cope with the issues in conventional methods, a variety of fluorescent miRNA detection approaches have been developed with an improved sensitivity and selectivity in recent years through integrating with elegantly designed amplification strategies. For example, Ruixuan Wang et al. developed a sensitive miRNA detection method through integrating rolling circle amplification for signal amplification and CRISPR-Cas9 for cutting FAM and BHQ labeled report probes (Wang et al. 2020). With the excitation of FAM signal, the method exhibited a favorable detection performance with a limit of detection (LOD) in fM level. Despite that the organic dyes could provide enhanced fluorescence signal, they are also criticized by the limited brightness and photostability. Silver nanoclusters (AgNCs) have emerged in recent years due to superior capability in absorption and emission of light and have been applied in chemical/biomolecular detection (Guo et al. 2018, 2021; Lin et al. 2020). In addition, the AgNCs also possess smaller size and lower toxicity compared with quantum dots. AgNCs has

been utilized in establishment of fluorescence biosensors because its fluorescence wavelength of AgNCs depending on the DNA template as a scaffold (DNA-templated AgNCs) changes when the sequences of DNA template and microenvironment change (Guo et al. 2020; New et al. 2016). For example, Gui-min Ma proposed a label-free miRNA detection method through integrating DNA-templated AgNCs for signal output and duplex-specific nuclease (DSN)-assisted signal amplification (Ma et al. 2021). The method was brilliant and exhibited a favorable detection performance in laboratory experiments. However, the sensitivity needs to be improved with only DSN enzyme for signal amplification, which could not meet the high requirement to detect trace amount of miRNA.

Herein, we proposed a label-free miRNA detection approach through integrating DSN enzyme and Exo-III enzyme to establish dual signal recycles and using DNA-templated AgNCs for signal generation (Fig. 1). In this method, we designed a hairpin structure probe (H1 probe) which contains two functional sections, one is T section for target miRNA recognition and hybridization, and the other is M region to mediate the Exo-III enzyme-assisted signal. When target miRNA, with miRNA-21 as example, existed in the system, it can bind with H1 probe and gradually unfold it to form a miRNA-H1 probe duplex. In the presence of DSN enzyme, it can identify the miRNA-T region section in the duplex and digest T region to release target miRNA. The liberated miRNA can bind with the rest H1 probe to form a recycle. After incubating for 60 °C for 10 min to degrade DSN enzyme, H2 probe is added to the mixture. The M region binds with the toehold section in H2 probe forming a blunt 3' terminal in the probe, which can be recognized by Exo-III enzyme. Under the assistance of Exo-III enzyme-based digestion of H2 probe, the resultant sequence which is composed of B region and G region, hybridize with B' region in the dark AgNCs sequence, narrowing the distance between dark AgNCs and the G-rich sequence in the resultant sequence. Consequently, dark AgNCs were active to emit strong red fluorescence. Currently, the mechanism of enhanced red fluorescence generated by DNA-AgNCs remains unknown. The former researches by Werner hypothesis electron transfer from guanine to the nanoclusters lead to the generation of red fluorescence (Yeh et al. 2010). Through recording the generated fluorescence signals, the level of target miRNA can be calculated.

Experimental section

Materials and reagents

The Silver nitrate (AgNO3) was brought from Tianjin FengchuanChemical Reagent Co., Ltd. (Tianjin, China). The two enzymes, including the DSN enzyme and the Exo-III enzyme, were brought from the TaKaRaBiotechnology Co. Ltd. (Beijing, China). The Tris buffer (1 mol/L, pH = 8) and $MgCl_2$ were purchased from ThermoFisherScientific (China) Co., Ltd. (Shanghai, China). The

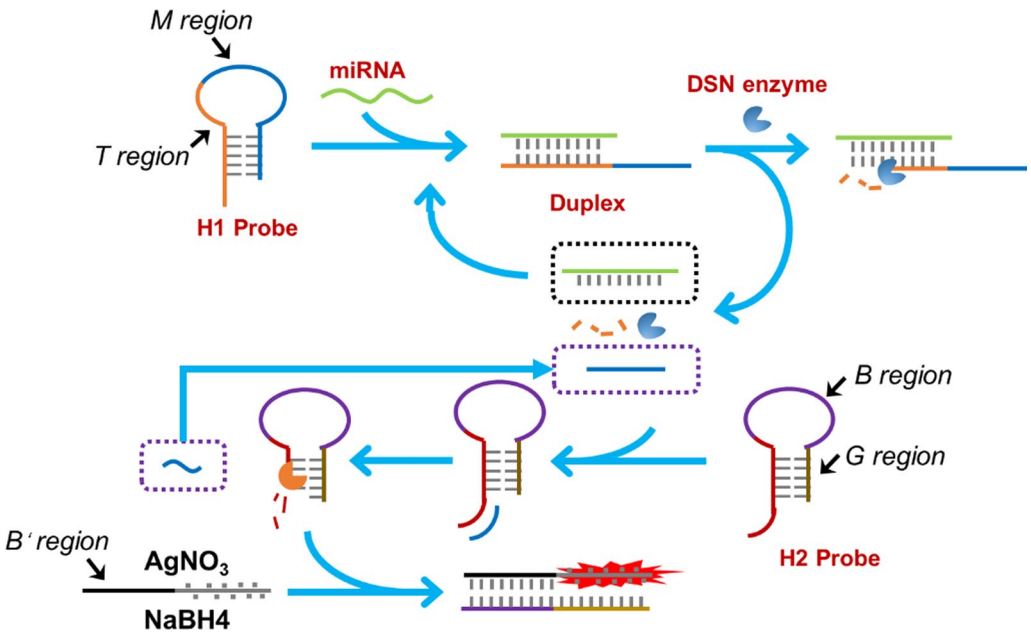


Fig. 1 The working mechanism of the proposed method containing DSN enzyme and Exo-III enzyme-assisted signal amplification and DNA-AgNCs-assisted signal generation

fluorescence spectrum was recorded by the Hitachi fluorescence spectrophotometer (Tokyo, Japan). The details of the nucleic acids used in the research are listed in Additional file 1: Table S1 and brought from Shanghai Sango biotech Co., Ltd. (Shanghai, China).

Preparation of dark AgNCs (silvernanoclusters)

The dark AgNCs were prepared according to former references through reducing $AgNO_3$ by $NaBH_4$. The experimental details of preparing AgNCs are shown in Supporting Information.

Construction of DSN enzyme and Exo-III-assisted signal amplification and fluorescent detection of target miRNA

Fluorescence assay to study the construction of the first signal recycle: First, 50 μL H1 probe (10 μM) was heated to 90 °C for 10 min and then naturally cooled to room temperature, keeping for 1 h to form its hairpin structures. The fluorescence signal of the H1 probe before and after assembly was detected. A 5 μL fluorescent H1 probe and 5 μL target miRNA were mixed in a tube containing a Tris buffer and incubated for 30 min. The fluorescence signal of the H1 probe when miRNA-21 existed or not was detected. A 5 μL fluorescent H1 probe, 5 μL target miRNA and 5 μL DSN enzyme were mixed and incubated for 30 min. The fluorescence signal was detected and compared with that when DSN was absent.

miRNA detection: 5 μL fluorescent H1 probe, 5 μL target miRNA and 5 μL DSN enzyme (6 U/L) were mixed and incubated for 50 min. Afterward, the mixture was heated to 70 °C for 10 min to degrade the DSN enzyme. 0.5 U Exo-III enzymes were subsequently added in the mixture and incubated for 30 min. According to former literature (Ma et al. 2021), the mixture was incubated at 50 °C for 100 min to perform the DSNSA reaction. Subsequently, 4 μL 10 μmol/L dark AgNCs and 5 μL 10 × PB are added into the above reaction solution. The fluorescence signal of the approach was detected by the fluorescence spectrophotometer.

Results and discussion

Characterization of the DNA-templated AgNCs

The characteristics of AgNCs to emit fluorescence signal when received excitation is fundamental for outputting results and determines the establishment of the approach. To investigate the fluorescent characteristic of AgNCs, we recorded the excitation and emission spectrum. The excitation spectrum (blue line) in Fig. 2 suggested excitation light at 560 nm can stimulate AgNCs to generate the most obvious fluorescent response, and the emission spectrum (red line) indicated AgNCs emitted enhanced signal at 630 nm. Therefore, emission spectrum was recorded with excitation at 560 nm, and excitation spectrum was recorded for 630 nm emission wavelength.

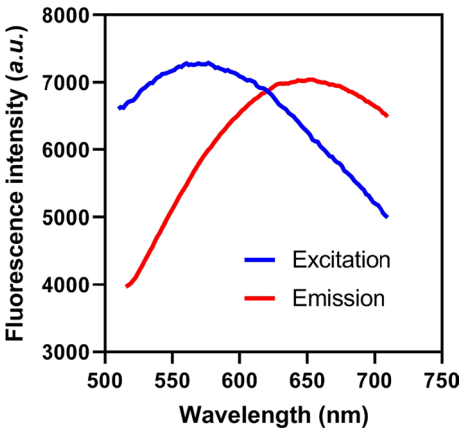


Fig. 2 Excitation spectrum (blue curve) and emission spectrum (red curve) of DNA-templated AgNCs. Emission spectrum was recorded with excitation at 560 nm, and excitation spectrum was recorded for 630 nm emission wavelength

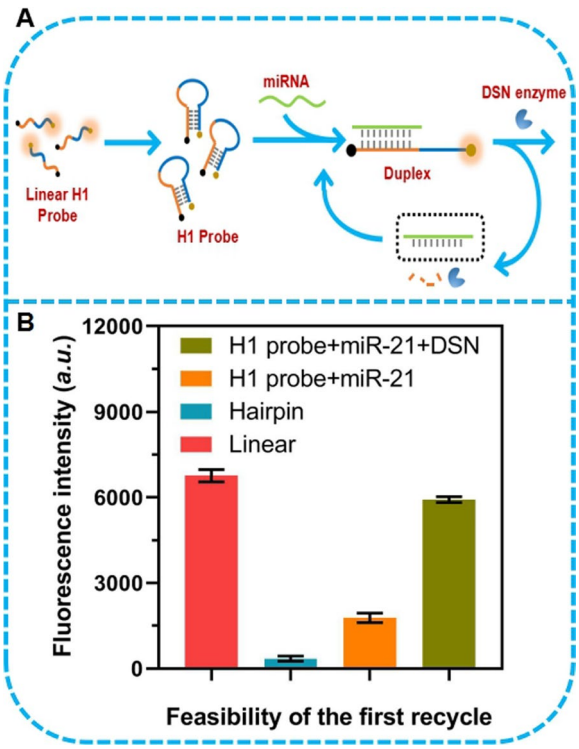


Fig. 3 Construction of the first signal recycle. **A** Illustration of the fluorescence assay to study the first signal recycle. **B** Fluorescence intensity of FAM labeled H1 probe on different experimental conditions

Construction of DSN enzyme-assisted signal recycles

A fluorescence assay was conducted to study the construction of DSN enzyme and Exo-III enzyme-assisted signal recycles. As illustrated in Fig. 3A, the two terminals of H1 probe were labeled with FAM and BHQ-2, respectively. In the state of hairpin structure, FAM signal was quenched by BHQ-2. From the result in Fig. 3B, the obtained fluorescence intensity of H1 probe after assembly was significantly lower than that before the assembly, indicating linear H1 probe was successfully assembled to the hairpin structure. Upon the addition of miRNA-21, which was partially complementary with stem section in H1 probe, FAM signal reappeared, implying a portion of H1 probes were unfolded by miRNA. Notably, the phenomenon that approximate 20.12% FAM signal recovered when miRNA-21 was added could be explained by that not all H1 probes were unfolded and concentration of H1 probe was much higher than miRNA-21. When mixed with DSN enzyme, the obtained fluorescence was 3.2 times higher than that when it was absent, suggesting a greatly enhanced fluorescence signal and the formation of the first signal recycle.

Optimization of experimental condition

For a better detection performance, we then optimized the experimental parameters. Considering the crucial role of DSN enzyme and Exo-III enzyme in establishment of signal amplification process, we firstly optimized the concentration of DSN enzyme and Exo-III enzyme. From the result in Fig. 4A, the obtained fluorescence signal of the approach increased with the concentration of DSN enzyme ranged from 1 U/L to 6 U/L. When the sensing system was added with more than 6 U/L DSN enzyme, no more significant signal enhancements were observed, indicating that a saturation has been reached with 6 U/L DSN enzyme. Thus, 6 U/L DSN enzyme was utilized in the following experiments. With constant 6 U/L DSN enzyme, we then studied the concentration of Exo-III enzyme. As shown in Fig. 4B, the optimized DSN enzyme incubation time was determined 60 min. The optimizing result of Exo-III enzyme concentration was determined 0.5 U/L, and Exo-III enzyme incubation time was determined 40 min as shown in Additional file 1: Fig. S1.

Fluorescence measurement of the approach when detecting miR-21

We then tested the detection performance of the approach under the obtained optimized experimental conditions. Before investigating the sensitivity, synthesized miRNA was diluted to different concentrations by DEPC water. The fluorescence spectrum generated by 1 fM ~ 1 nM miRNA-21 was recorded (Fig. 5A). Correlation analysis in SPSS that the fluorescent value at 630 nm showed a linear response to the logarithmic concentrations of miRNA ranging from 1 fM ~ 1 nM. The correlation equation between fluorescent value and the amounts of miRNA was determined as $Y = -904.4 * \lg C - 2442$, covering more than 7 orders of magnitude (Fig. 5B). The limit of detection (LOD) was calculated 245 amol (3σ, $n = 11$), which is comparable or even better than some of the former established miRNA detection strategies (Wang et al. 2020; Zou et al. 2019). To the best of our knowledge, the remarkably improved sensitivity of the approach could be possibly derived from the high fluorescence emission capability of AgNCs and the Exo-III and DSN enzyme-assisted signal amplification.

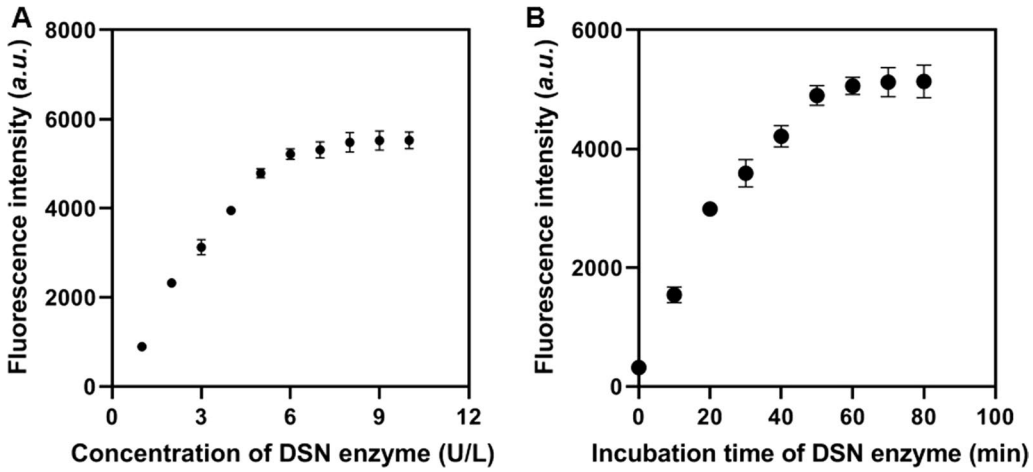


Fig. 4 Optimization of experimental conditions. **A** Fluorescence signal of the approach with different concentrations of DSN enzyme. **B** Fluorescence intensity of the approach with different incubation time of DSN enzyme

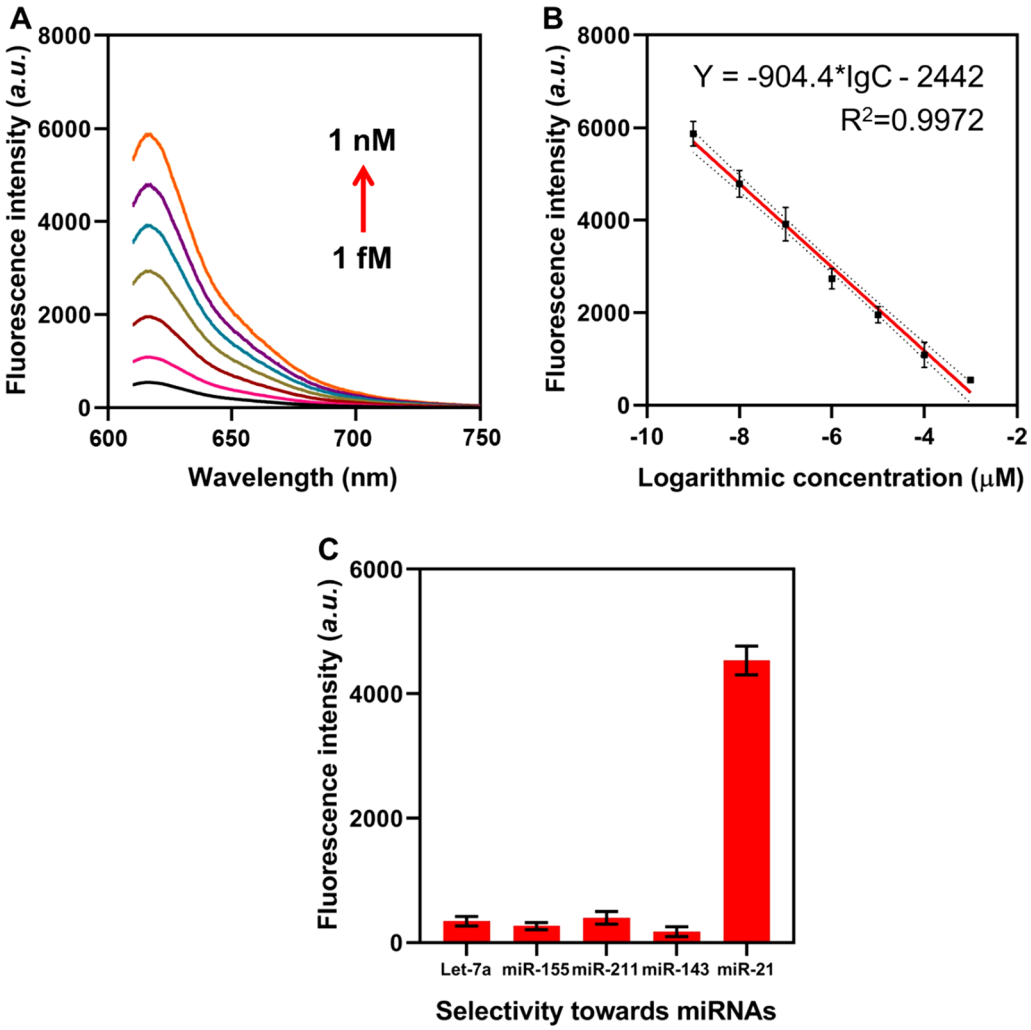


Fig. 5 Detection performance of the approach. **A** Fluorescence spectrum of the approach when detecting different concentrations of miRNA-21. **B** Correlation equation between the obtained fluorescence intensities and logarithmic concentrations of miRNA-21. **C** Fluorescence intensity of the approach when detecting different miRNAs

Selectivity of the approach toward miRNA-21 was verified through comparing the recorded fluorescence signal when detecting miRNA-21 and some other miRNAs that have different mismatches with miRNA-21. The result in Fig. 5C showed that fluorescence value aroused significantly when detecting miRNA-21 compared with control group (without miRNAs). On the contrary, the fluorescence signals showed neglectable differences with the control groups when detecting other miRNAs of the same concentration with target miRNA-21 (100 pM), indicating a high specificity of the approach. To study the repeatability of the approach, the established approach was applied to detect 10 technique duplicates and the variable coefficient (CV) was calculated. The low CV in Additional file 1: Fig S2 indicated small differences between the 10 samples detection results and a high repeatability.

Conclusion

We depicted here a novel label-free and sensitive miRNA detection approach through integrating DSN enzyme and Exo-III enzyme-assisted signal amplification, and DNA-templated AgNCs for signal generation. The approach eventually exhibited a wide detection range, covering 5 orders of magnitude. The low LOD of the approach could be possibly explained by high signal emission capability of AgNCs and dual signal recycles. The excellent selectivity of the approach for miRNA 21 detection holds great promise for practical application in biosensing and clinical assay.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40543-022-00335-4.

Additional file 1. Table S1. Details of the nucleic acids used in this research. **Fig S1**. Optimization of incubation time. (A) Fluorescence signal of the approach with different concentrations of Exo-III enzyme. (B) Fluorescence intensity of the approach with different incubation time of Exo-III enzyme. **Fig S2**. Fluorescence intensity of the approach when detecting 10 technical duplicates.

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Author contributions
CM is the supervisor of the team in all research steps including designing, data analysis and manuscript writing. HL, as the first author, has the main role for experimental data collection, data gathering, preparation of results, and data analysis. CZ assists the data analysis. All authors read and approved the final manuscript.

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Availability of data and materials
Almost all details of experimental data are presented in the article or additional file.

Declarations

Ethics approval and consent to participate
The manuscript does not contain clinical or trial studies on patients, humans, or animals.

Competing interests
The authors declare that they have no competing interests.

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