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DSN enzyme recognition initiated rolling circle amplification (DiRCA) for accurate miRNA detection in immune response and immune repair after trauma

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Abstract

Recent studies have reported that miRNA plays an important role in immune response and immune repair after trauma. By regulating the expression of related target genes, miRNA regulates the production, proliferation, development and immune response of immune cells. Therefore, it is in urgent demand to develop an novel method for miRNA analysis. Rolling circle amplification (RCA), as an attractive isothermal signal amplification strategy, has been widely utilized in constructing miRNA detection assays. However, accurate and sensitive miRNA quantitative determination remains a huge challenge for RCA based approaches. Herein, we propose a DSN enzyme based signal cycle initiated Rolling Circle Amplification assay (DiRCA) for sensitive and accurate miRNA detection. In DiRCA, target miRNA unfolds hairpin structure probe in the detection scaffold, forming a RNA–DNA duplex. DSN enzyme is utilized to specifically digest the DNA sequence in RNA–DNA duplex, releasing miRNA to form a signal cycle; its capability to distinguish one base pair mismatch in RNA–DNA duplex endows DiRCA a high selectivity. Meanwhile, DSN enzyme based cleavage initiates RCA, transcribing G-rich sequences for signal generation. Based on the DSN assisted signal cycle and RCA, DiRCA shows a low limit of detection of 0.43 fM and a superior capability in selectively detecting mismatched miRNA sequences, showing a promising prospect in the early-diagnosis of disease.

Keywords Rolling circle amplification (RCA), microRNA (miRNA), Duplex-specific nuclease (DSN enzyme), Thioflavin T (ThT)

Introduction

The inflammatory response is the first stage of wound healing and requires the involvement of the immune system. In the process of wound inflammation, microRNAs (miRNAs) can regulate the differentiation and function of different types of macrophages by regulating key transcription factor signaling pathways, so that they can play a role in accelerating the growth of epidermal keratinocytes, increasing collagen deposition and avoiding neovascular injury in the process of wound healing, thus greatly speeding up wound healing. Non-coding miRNAs are endogenous small noncoding RNAs with the length of about 18–25 nt that paly essential role in RNA silencing and regulating the gene expression (Gebarowska et al.

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2021; Tafrihi and Hasheminasab 2019); they emerged as potential biomarkers for the diagnosis of a variety of diseases, such as cancers (Saliminejad et al. 2019; Aggarwal et al. 2020; Hayes et al. 2014). However, accurate and sensitive quantitative determination of miRNAs remains a huge challenge due to their low amount, short length, and high sequence homology among family members, highlighting the requirement of developing novel assays for sensitive, selective, and reliable miRNA detection.

The rapid advances of miRNA quantification techniques have been witnessed in recent years, facilitating the early diagnosis of diseases and personalized medicine (Jet et al. 2021; Ma et al. 2017). With Reverse Transcription-Polymerase Chain Reaction (RT-PCR) as an example, it can sensitively quantify low amount of miRNAs via efficient nucleic acids amplification (Chen et al. 2005; Cirillo et al. 2020; Hindson et al. 2013). Taking the advantages of high signal amplification efficiency, RT-PCR has been widely employed in fundamental medical researches and clinical diagnosis. However, RT-PCR method requires complicated primer design, tedious labor intensity, and cumbersome equipment for thermal recycle, that greatly hindered its further application. Isothermal nucleic acids amplification strategies have been emerged as alternative methods of RT-PCR, possessing the advantages of efficiently amplifying nucleic acids under constant thermal condition (Bodulev and Sakharov 2020; Reid et al. 2018). The isothermal nucleic acids amplification strategies such as rolling circle amplification (RCA) (Xu et al. 2021; Zhao et al. 2008), strand displacement amplification (SDA) (Gong et al. 2021a; Zeng et al. 2022a), loop-mediated isothermal amplification (LAMP) (Soroka et al. 2021), and recombinase polymerase amplification (RPA) (Li et al. 2018) have attracted abundant attention and are extensively utilized in various fields. Due to its superior capabilities of versatility and simplicity, RCA is widely exploited in constructing novel approaches for in vitro and in situ miRNA detection. For example, Ruixuan Wang et al. proposed a miRNA detection approach by integrating RCA based signal amplification and CRISPR-Cas9 assisted signal generation and demonstrated it feasibility in detecting miRNAs, including miRNA-21 and let-7a (Wang et al. 2020). However, the assays developed with only RCA for signal amplification mostly exhibited limit of detection (LOD) in pM level that could not meet the high requirement of trace amount of miRNA quantification. Many efforts have been made to improve the miRNA detection sensitivity of RCA based approaches, mainly based on attaching a signal recycle to RCA. For example, Xianxian Zhao et al. improved the detection sensitivity of RCA based approach by combining the trans-cleavage activity of CRISPR-Cas12a (Zhang et al. 2020). Nevertheless, the selectivity of the assay was determined by the RCA, and the false cyclization of padlock may cause error in results. The issues are widely existed in those approaches that signal cycles are attached to RCA (Zhang et al. 2018; Qiu et al. 2017). Therefore, novel strategies should be proposed to improve the accuracy of RCA based approaches.

Duplex-specific nuclease (DSN enzyme) can selectively degrade DNA in DNA-RNA hybrids and distinguish single base mismatches in DNA-RNA hybrid (Wu et al. 2020; Gong et al. 2021b). Inspired by its superior capability in distinguishing single base mismatches, we propose in this research a DSN enzyme based signal cycle initiated Rolling Circle Amplification assay (DiRCA) for sensitive and accurate miRNA detection. In this assay, DSN enzyme selectively degrade the miRNA-DNA duplex endows a high accuracy and induces the subsequent RCA based signal amplification. With the transcribed G-rich amplification products can be specifically recognized via efficient binding between G-quadruplexes sequences and the fluorescent dye Thioflavin T (ThT).

Experimental condition

Material and reagents

The sequences used in this research are listed in Additional file 1: Table S1 (details of the oligonucleotides used in this research). All the oligonucleotides were synthesized and purified from Sangon Biotechnology Co., Ltd (Shanghai, China). DSN enzyme, T4 DNA ligase, and phi29 enzyme were brought from Sigma life science Co., Ltd (Jiangsu, China). Diethyl pyrocarbonate (DEPC) water, Deoxyribonucleoside 5′-triphosphate mixture (dNTPs) mixture were provided by Tiangen Biotech. Co. Ltd. (Beijing, China). The other reagents in this research were in analytical-reagent grade.

Construction of detection scaffold

For the construction of detection scaffold, 2 μL obtained hairpin sequences (2 μM) were firstly heated to 90 °C for 10 min and were gradually cooled to room temperature. 2 μL assembled hairpin structure sequences were then mixed with 2 μL ssDNA sequences (2 μM) and incubated at 30 °C for 20 min.

Feasibility of DSN enzyme based signal cycle

Fluorescence assay to study the miRNA based disassociation of hairpin structure probe 2 μL detection scaffold was mixed with 2 μL miRNA-21 (2 μM) and the mixture was incubated for 15 min at room temperature. The fluorescence signals were recorded by Hitachi fluorospectro photometer F-7000 (Tokyo, Japan).

Fluorescence assay to study DSN enzyme based signal cycle 2 μL detection scaffold was mixed with 2 μL miRNA-21 (2 μM) and 2 μL DSN enzyme. The mixture

was incubated for 30 min at room temperature. The fluorescence signals were recorded by Shimadzu RF-5301PC fluorescence spectrometer.

Detection of miRNA-21

For the detection of miRNA, 2 μL detection scaffold was mixed with 2 μL miRNA-21 (2 μM) and 2 μL DSN enzyme. The mixture was incubated for 30 min at room temperature. Afterwards, the mixture was heated to 75 °C for 5 min to degrade the DSN enzyme. 2 μL T4 DNA ligase (1 U/L) was then added in the mixture and was incubated for 30 min. The mixture was then heated to 65 °C for 5 min to degrade T4 DNA ligase. 2 μL phi 29 enzyme, 2 μL dNTP, 1 μL ThT (5 μM) and 2 μL NEB buffer were then added in the mixture. The obtained mixture was incubated at room temperature for 120 min. The ThT signal was recorded by Shimadzu RF-5301PC fluorescence spectrometer after the reaction was finished.

Results and discussion

The working mechanism of DiRCA for miRNA detection

The analytical procedures of the DiRCA are illustrated in Fig. 1. In DiRCA, a detection scaffold is constructed by hybridizing the toehold section in the hairpin structure probe with a single-strand DNA (ssDNA) sequence. In detail, the **d′** section is complementary with **d** section, **c** section is C-rich section, and **b′** is partially complementary with target miRNA. In the present of target miRNA, it can specifically bind with **b′** section in detection scaffold and gradually unfold the hairpin structure, forming a miRNA-DNA duplex. The DSN enzyme, as a member of DNases family derived from the Kamchatka crab, is capable of degrading dsDNA or DNA in DNA/RNA hybrids while leaving the single strand intact, and is used to specifically identify the miRNA-DNA duplex. DSN enzyme selectively recognizes the miRNA-DNA duplex and degrades the DNA sequence, releasing miRNA for a next signal recycle and exposing the **d** section. The superior capability of DSN enzyme in distinguishing single base mismatches endows the method a high selectivity to target miRNA. The **d** section in detection scaffold hybridizes with the **d′** section to form a circular padlock under the assistance of T4 DNA ligase. With circular padlock as template, a long ssDNA product is extended on the 3′ terminal of the ssDNA (primer) under the assistance of phi29 enzyme. The amplified ssDNA products can assemble to G-quadruplex that can be accurately identified by commercial fluorescent dye ThT, allowing high sensitive and accurate detection of miRNA.

Construction of detection scaffold and feasibility of DiRCA

The assembly of detection scaffold determined the analytical performance of DiRCA. The construction of detection scaffold commonly follows the procedures of heating the mixture to 90 °C and cooling it to room temperature. Considering the structure of detection scaffold, the hairpin structure was firstly assembled via quenching and the hairpin structure sequence localized on ssDNA sequence

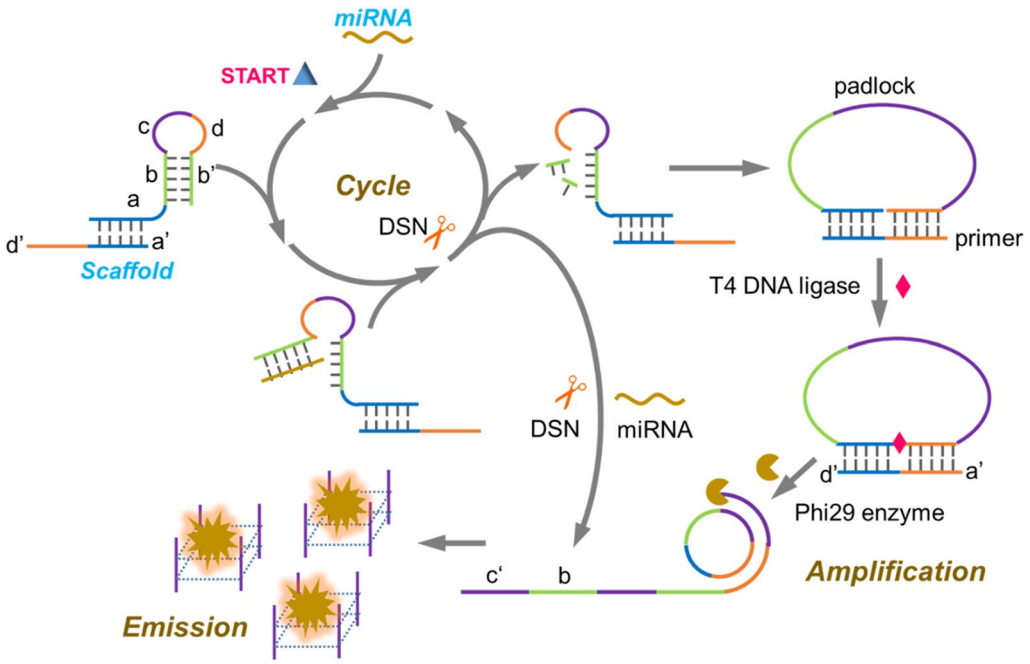

Fig. 1 The working mechanism of the established approach

at room temperature. A fluorescence assay was employed to assess the construction of detection scaffold by labeling FAM moiety on the 5′ terminal of ssDNA and its corresponding quenching moiety BHQ on the 3′ terminal of hairpin sequence, respectively (Fig. 2A). The result in Fig. 2B showed a gradually decreased fluorescence signal after the hairpin structure sequences (1 μM) was assembled on the ssDNA sequence (1 μM). Upon the addition of miRNA (1 fM), the fluorescence signals recovered, indicating the hairpin structure was unfolded by target miRNA. In addition, a greatly elevated fluorescence signal was observed upon the addition of DSN enzyme, indicating the formation of signal cycle. PAGE analysis was then used to verify the RCA process (Additional file 1: Fig S1, PAGE analysis of DiRCA). The feasibility of DiRCA was demonstrated through recording the ThT signals when several experimental components existed or not. As shown in Fig. 2C, the ThT signals showed no significant differences with the blank group, and only when all essential components existed in the sensing system, a greatly enhanced fluorescence signal was observed, demonstrating the feasibility of DiRCA.

Optimization of experimental conditions

The analytical performance of DiRCA is generally affected by several experimental conditions, including the concentration of DSN enzyme, incubation time, and amount of T4 DNA ligase and phi29 enzyme. To obtain the optimized experimental conditions, DiRCA is performed to quantify 1 nM target miRNA. As shown in

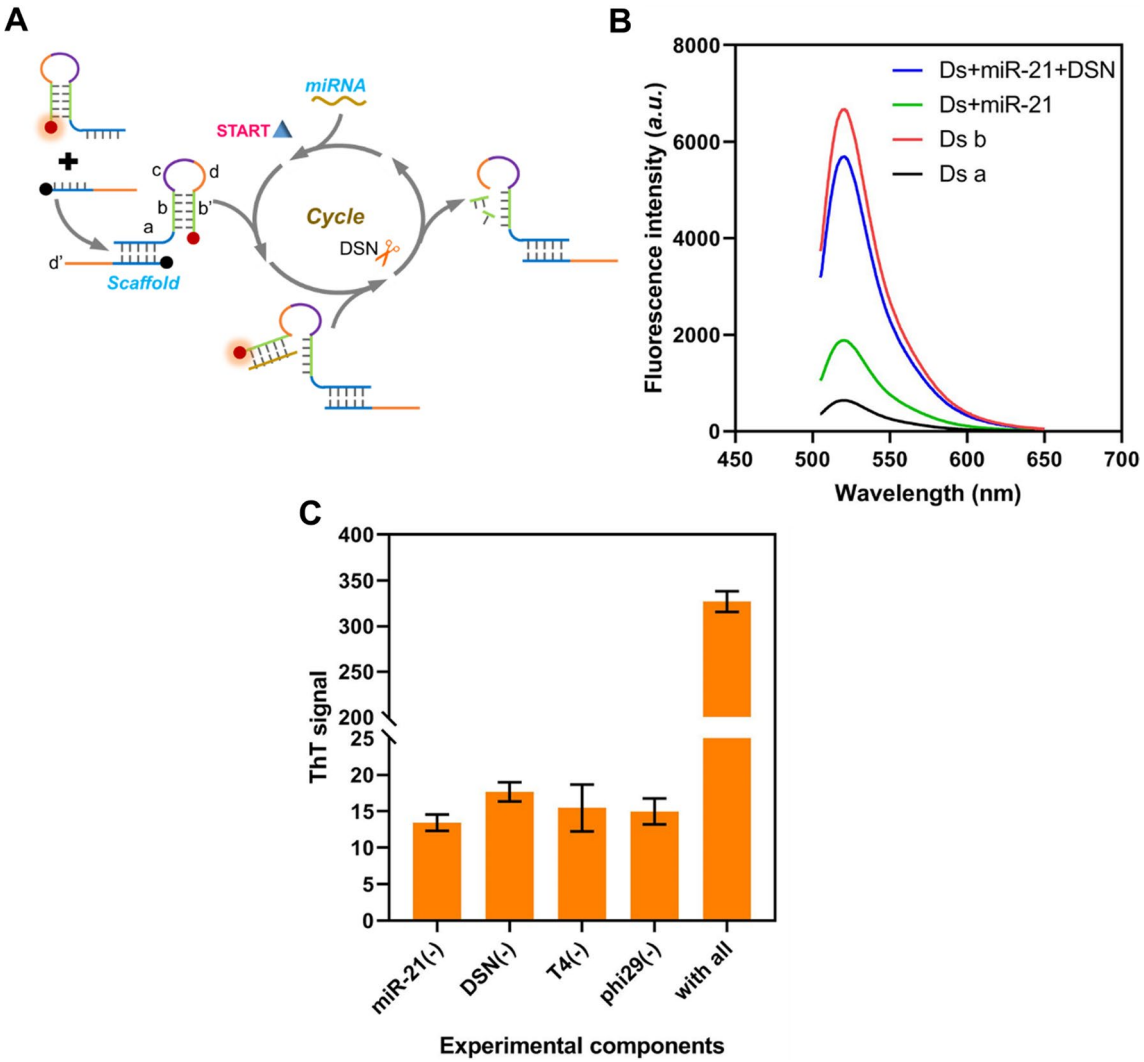

Fig. 2 Construction of detection scaffold and feasibility of DiRCA. **A** illustration of fluorescence assay to test the construction of detection scaffold. **B** fluorescence spectrum of FAM labeled detection scaffold. **C** ThT signals of DiRCA when each of experimental component existed or not

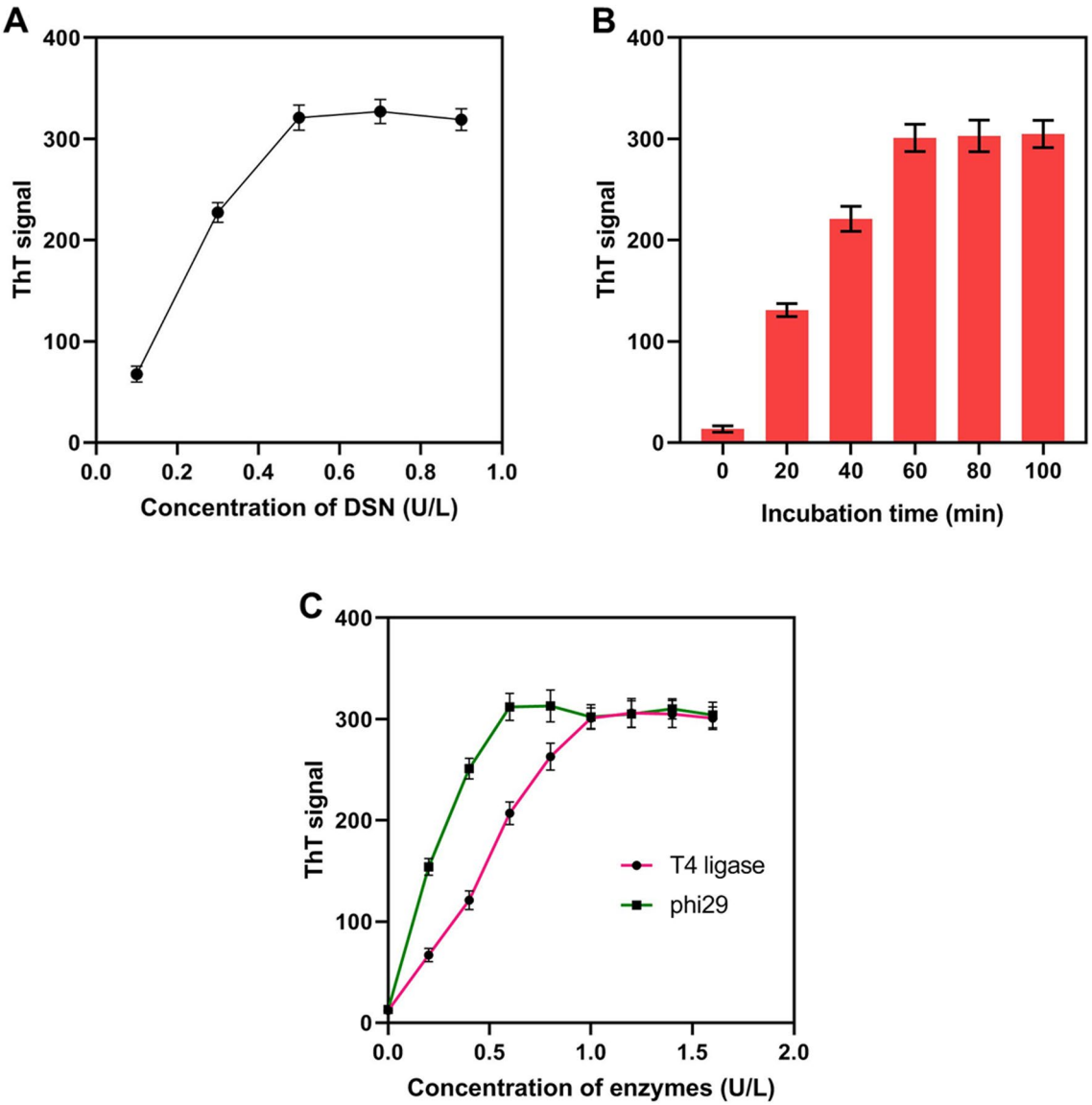

Fig. 3 Optimization of experimental conditions. ThT signal of DiRCA with different concentrations of DSN enzyme (**A**), incubation time (**B**), and concentrations of T4 DNA ligase and phi29 enzyme (**C**)

Fig. 3A, the ThT signals gradually increased with concentrations of DSN enzyme ranged from 0.1 to 0.5 U/L, and no more elevation was observed when the system was added with more DSN enzyme. The result in Fig. 3B indicated the ThT signals peaked at 60 min, and thus 60 min was selected for the following experiments. Meanwhile, the incubation time, and amount of T4 DNA ligase and phi29 enzyme were determined 60 min, 1 U/L, and 0.6 U/L (Fig. 3C).

Analytical performance of DiRCA

After optimizing the experimental parameters, we then tested the detection performance of DiRCA, including sensitivity and specificity. To investigate the sensitivity, DiRCA was utilized in detecting different concentrations of miRNA-21 and the ThT signal was recorded. As shown in Fig. 4A, the ThT signals elevated with the concentrations of miRNA-21 ranged from 1 fM to 1 nM. A linear correlation between ThT signals and the miRNA concentrations on the logarithmic scale were obtained (Fig. 4B). The equation was displayed as $Y = 73.62 \times \lg C - 46.03$, with a correlation coefficient of 0.993. In addition,

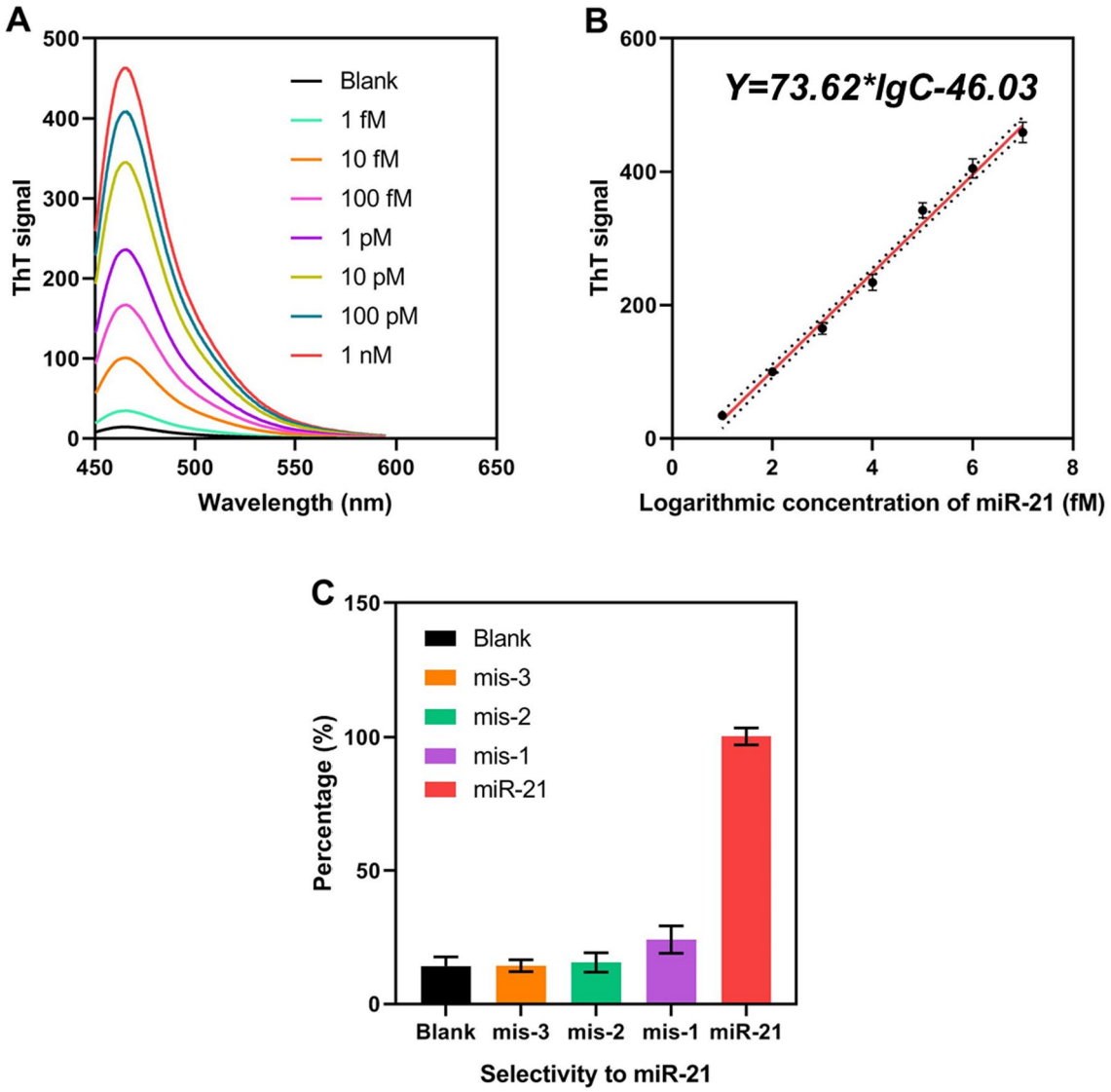

Fig. 4 Analytical performance of DiRCA. **A** fluorescence spectrum of DiRCA when detecting different concentrations of miRNA-21. **B** correlation equation between obtained ThT signal and logarithmic concentrations of miRNA-21. **C** percentage of recorded ThT signal of DiRCA between mismatched miRNAs and target miRNA-21

comparing DiRCA to linear RCA amplification for the construction of the miRNA detection platform, DiRCA achieved a significantly improved signal amplification efficiency, exhibiting a limit of detection as low as 0.43 fM (calculated on the basis of the 3σ rule) (Zeng et al. 2022b; Li et al. 2022).

Specific determination of target miRNA is a crucial indicator in evaluating analytical performance of DiRCA. Therefore, DiRCA was employed in distinguishing target miRNA-21 from a collection of mismatched sequences (mis-1, mis-2, and mis-3 that have 1, 2, or 3 base pairs mismatched with miRNA-21, respectively) under the same experimental conditions. The result is expressed as the percentage between the recorded ThT signal and the signal obtained by detecting the target sequence. As shown in Fig. 4C, the ThT signals of DiRCA when detecting mis-1, mis-2, mis-3, were 24%, 15%, and 14%, respectively. The detective results of DiRCA when detecting mis-2 and mis-3 showed no significant differences with the control group, indicating DiRCA efficiently distinguished 2 more mismatched base pairs. Meanwhile, the signal percentage of DiRCA when detecting mis-1 was also enough for distinguishing target miRNA-21.

Table 1 Recovery rate of DiRCA for miRNA-21 detection

miRNA-21	Serum (%)	Calculation	Recovery rate (%)
100 fM	5	101.21	101.21
100 fM	10	102.3	102.3
10 pM	5	9.965	99.65
10 pM	10	9.889	98.89

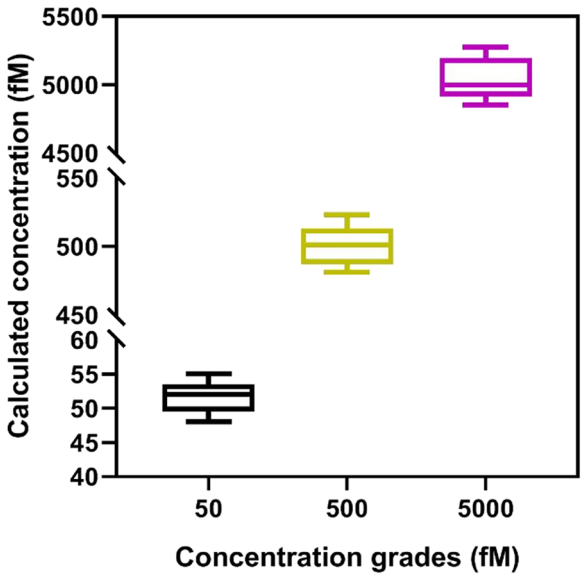

Fig. 5 Calculated concentration of target miRNA by DiRCA

Practical application potential of DiRCA

To investigate the potential of DiRCA for detecting miRNA-21 in real samples, a recovery assay was firstly performed. In the recovery assay, DiRCA was utilized to detect 100 fM and 10 pM miRNA-21 sequences, which were originally diluted in solutions containing 5% and 10% artificial serum. The recovery rates of DiRCA ranged from 98.89 to 102.3%, indicating a high detective stability and accuracy of DiRCA in complicated experimental conditions (Table 1). In addition, miRNA-21 sequences were diluted to 3 concentration grades, and DiRCA was utilized to detection the concentration of miRNA. The result in Fig. 5 showed DiRCA could specifically distinguish the different concentration grades, indicating its capability in analyzing miRNA expression level in clinical practice.

Conclusion

In this research, a DSN enzyme based signal cycle initiated rolling circle amplification assay has been established for sensitive and accurate miRNA detection. In DiRCA, target miRNA unfolds hairpin structure probe in detection scaffold to induce the DSN enzyme assisted signal cycle. The superior capability of DSN enzyme to distinguish single base mismatches in DNA-RNA hybrid endows the method a high selectivity that even identified one base pair mismatch. The DSN enzyme based digestion of DNA sequence in DNA-RNA hybrid initiated RCA for signal amplification. Based on DSN enzyme assisted signal cycle and RCA, DiRCA exhibited a low limit of detection of 0.43 fM and a high accuracy. In all, DiRCA shows a promising prospect in sensitive and accurate miRNA detection, and thus contributing to the early-diagnosis of diseases.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40543-023-00383-4.

Additional file 1. Table S1. Details of the oligonucleotides used in this research. **Fig S1.** PAGE analysis of DiRCA.

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Author contributions

YG and DZ is the supervisor of the team in all research steps including designing, data analysis and manuscript writing. YM and JZ, as the co-first authors, have the main role for experimental data collection, data gathering, preparation of results, and data analysis. LZ, TL, HZ, and MX assist 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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