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A G-quadruplex DNA-based, Label-Free and Ultrasensitive Strategy for microRNA Detection

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MicroRNAs (miRNAs) have been considered to be potent biomarkers for early disease diagnosis and for cancer therapy. The rapid and selective detection of miRNAs without reverse transcription and labelling is highly desired. Herein, we report a simple and label-free miRNA detection method that is based on the Duplex-Specific Nuclease (DSN)-Assisted simple target miRNA recycling procedure. The interaction of the G-quadruplex DNA structure with N-methyl mesoporphyrin IX (NMM) led to a label-free signal output. Under the optimised conditions, this method allowed for simple, rapid, and sequence-specific detection of miR-141 over a dynamic range from 1 fM to 100 nM with a linear range from 1 pM to 100 nM. Moreover, our method offered an excellent capacity to discriminate between miRNA family members with just one mismatched nucleotide. This simple and label-free strategy holds great potential in applications in biomedical research and in early clinical diagnostics.

MicroRNAs (miRNAs) are a group of endogenously expressed, short, single-stranded, noncoding and evolutionally conserved RNAs of approximately 18–25 nucleotides (nt)[1]. These RNAs began to attract attention as it became clear that they play key roles in various physiological and pathological processes through post-transcriptionally targeting mRNAs for either degradation, translational repression, or both[2,3]. Recent years have witnessed a considerable increase in our understanding of the regulation of gene expression by miRNAs. MiRNAs are involved in crucial physiological processes, including development, differentiation, apoptosis and proliferation[1,4–8], and they have also been implicated in various pathological conditions, including cancer, cardiovascular disorders, diabetes, and neurological disorders[9–14], via their regulation of the expression of a diverse array of genes.

The unique characteristics of miRNAs, including their small size, low abundance, and sequence similarity among family members[2], make their detection very challenging. To date, several strategies have been developed for profiling miRNAs, such as some widely used traditional methods including Northern blotting[15], quantitative real-time PCR (qRT-PCR)[16,17] and microarray-based hybridization[18,19], but they all have some limitations of low selectivity or insufficient sensitivity, or time-consuming amplification process. Consequently, numerous novel approaches, including electrochemical devices[20,21], molecular beacons[22–24], nanoparticle sensors[25,26] and microfluidic chip[27], have been developed to improve the detection selectivity and sensitivity. Moreover, some recent detection strategies combined these methods. For example, Ju group and Mulchandani group have reported highly innovative strategies combining electrochemical method coupled with nanoclusters[28,29]. Nevertheless, these methods still need labeling or time-consuming pretreatment procedure. So, there is still an urgent desire to develop new strategies that can simply detect target miRNAs without labeling and reverse transcriptional amplification.

Instead of the indirect detection methods relying on the target miRNA amplification, Ye *et al* and Zhou *et al* reported two ingenious designs based on the duplex-specific nuclease (DSN) inducing miRNA recycling for direct miRNA detection[30,31]. However, these highly sensitive methods need labeling and the latter requires a several-hour pretreatment process. Herein, we take advantage of DSN enzyme to propose a rapid and label-free signal-amplifying mechanism employing the combination of NMM and G-quadruplex as a signal output unit (Figure 1). First, we introduced the conformational interaction between the G-quadruplex DNA and NMM with a considerable fluorescence change to realize the goal of being label-free; Second, we employed the specific recognition of

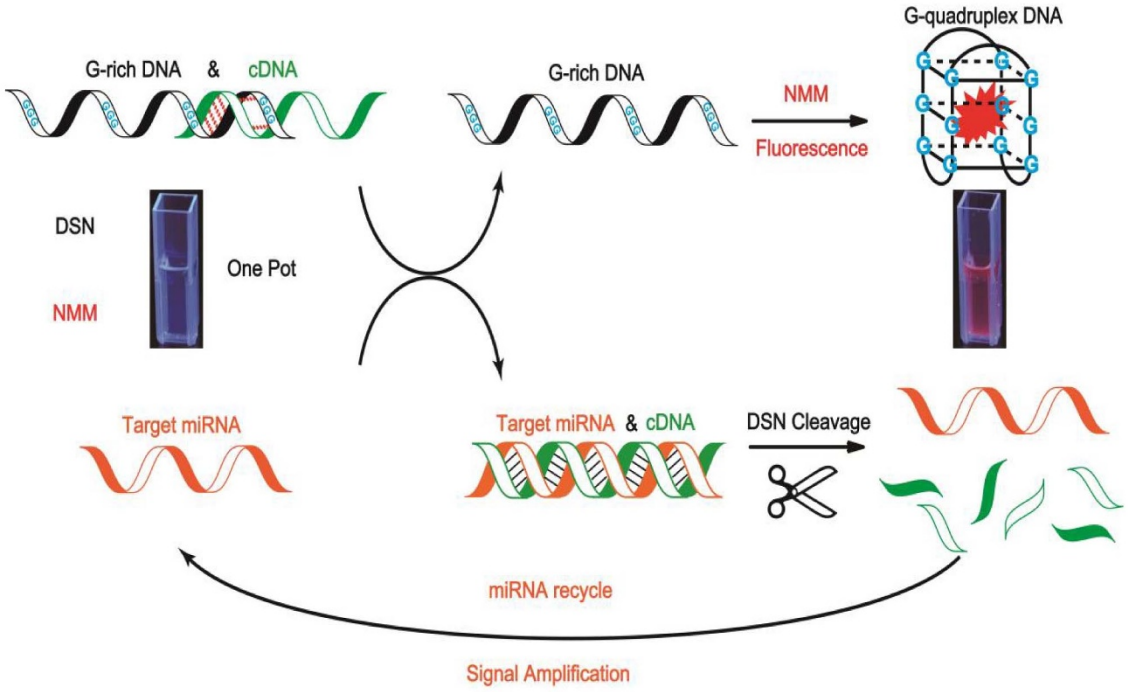

Figure 1 | **Schematic representation of the direct detection of miRNAs based on our strategy.**

the DSN for DNA/RNA to work on the rapid signal amplification issue. These properties will be key in simple miRNA detection.

Results and Discussion

As previous mentioned, considering the trace amount of the target miRNA, signal amplification methods with cyclic utilization of miRNA are preferable. DSN enzyme exhibits a strong preference for DNA duplexes (dsDNA and DNA in DNA-RNA heteroduplexes) but is practically inactive towards single-stranded DNA or single- or double-stranded RNA. Meanwhile this enzyme shows a wonderful ability to discriminate between perfectly and non-perfectly matched (up to one nucleotide mismatch) short duplexes[32,33]. Thus, in our strategy, a cDNA strand which is completely complementary to the target miRNA and partly complementary pairing with G-rich DNA was designed first. Then this cDNA can be competed off from the cDNA\G-rich DNA duplex to form a cDNA\RNA heteroduplex and release the G-rich oligonucleotides when the target-miRNA was introduced. Subsequently, the cDNA strand of the formed cDNA\RNA heteroduplex can be cleaved by DSN enzyme to release trace amounts of the target miRNA into the next cycle, which make the released G-quadruplexes' accumulated resulting in signal amplification. On the other hand, a label-free detection strategy based on nucleic acid level is also our goal to achieve. Recent research progress has demonstrated that, G-quadruplex DNA, a specific type of G-rich nucleic acid sequence[34], can be remarkably recognized by NMM with high selectivity, unlike the triplex, duplex or single-stranded forms of DNA[35,36]. The fluorescence intensity of NMM exhibits a considerable increase upon binding to G-quadruplex DNA[37,38], which can be utilized as a signal reporter[22,39].The conformation of released G-rich oligonucleotides would change into G-quadruplex DNA with the presence of 2.0 mM K^+. Then, the NMM remarkably recognises and electively binds to the G-quadruplex DNA, resulting in a significant enhancement in the fluorescence signal. In this work, we selected miR-141, an epithelial-associated miRNA expressed in a wide range of common human cancers, as a target to optimise the experimental conditions. Since the differences of G-rich DNA sequences used in our method can result in large changes in signal intensity, a series of them were screened to find a suitable sequence to ensure a relatively large gap in the fluorescent intensity ratio. Meanwhile, the efficiency of the hybridization of target miRNA\cDNA and the G-rich DNA release also should be considered. According to the sequence of the target miRNA, the designed G-rich DNA candidates (GR1 – GR6) were listed in Table S1. As the screening results presented in Figure 2A, we selected GR1 (red) to synthesise the probe. Next, the time-dependent fluorescence changes were measured to optimize the recycling step. As shown in Figure 2B, after 40 min, the fluorescent intensities were approaching stable; therefore, the most effective reaction time of 40 min was used in all subsequent assays. Furthermore, the optimum reaction temperature of the isothermal signal amplification was screened. DSN enzyme is stable over a wide range of pH (from 4 to 12) in the presence of 5 mM Mg^{2+} and the suitable temperature range for its activity is 55–65°C[34]. To determine the optimum temperature for our reaction system, we carried on the microRNA detection experiments at 30, 40, 50, 60, 70, 80°C, respectively. As shown in Figure 2C, reaction at 60°C gave the best result and 60°C was chosen as the optimum temperature. Finally, the optimum concentration of the DSN in our reaction was studied. To investigate the influence of the amount of DSN enzyme, the fluorescence response was measured upon the addition of 0.02 U, 0.04 U, 0.08 U, 0.1 U, 0.2 U, and 0.5 U DSN in presence of 100 nM miR-141, respectively. The control sample was treated in the same manner without miR-141. The results showed a dramatic increase at $\lambda = 610$ nm in the fluorescence emission spectrum when the amount of DSN enzyme was increased from 0.01 U to 0.5 U. Figure 2D, in which F_0 and F were the NMM fluorescence intensity at $\lambda = 610$ nm in the absence and presence of miR-141 respectively presented the fluorescence ratio value (F/F_0-1) affected by the amount of DSN enzyme. Although higher amounts of DSN enzyme would result in higher fluorescence signals, the background would also increase. The highest change (F/F_0-1) was observed when the concentration of DSN enzyme was 0.2 U. Thus, 0.2 U DSN was considered to be optimum concentration for our method (Figure 2D).

Next, we investigated the sensitivity of this method under the optimum conditions. The fluorescence intensities upon the addition of various concentrations of miR-141 (from 1 fM to 100 nM) were

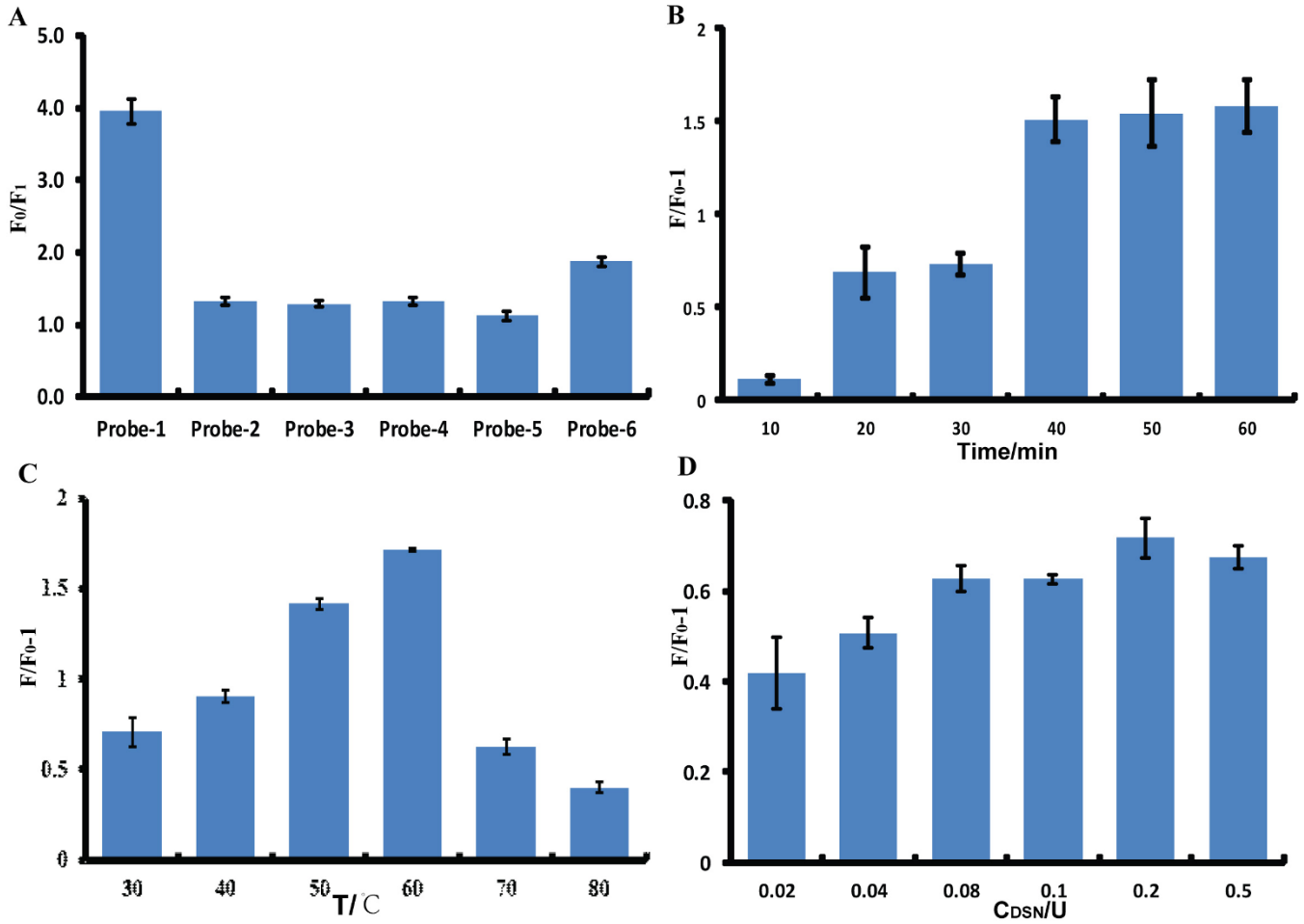

Figure 2 | Optimisations of our method reaction conditions. (A) Screening tests of the probe for miR-141. (B) Reaction time titration tests in the recycle step. The concentrations of MiR-141 and probe are 10 nM and 100 nM, respectively, with 0.2 U DSN enzyme in a 0.2 mL system. (C) Bars represent the fluorescence ratio values of (F/F_0-1) on different reaction temperature, with 0.2 U DSN for 40 min in a 0.2 uL system. (D) Bars represent the fluorescence ratio values of (F/F_0-1) upon the addition of different amounts of DSN enzyme. F_0 and F are the fluorescence signals in the absence and the presence of miR-141, respectively; F1 is the fluorescence signal in the presence of G-quadruplex.

measured. Figure 3A presented the emission spectra of the measuring result. As expected, a gradual increase in the fluorescent peak at 610 nm was clearly observed as the concentration of miR-141 increased from 0 to 100 nM. Figure 3B illustrated the changes in fluorescence intensity (F/F_0-1) in responding to the different miR-141 concentrations. The (F/F_0-1) value is linearly dependent

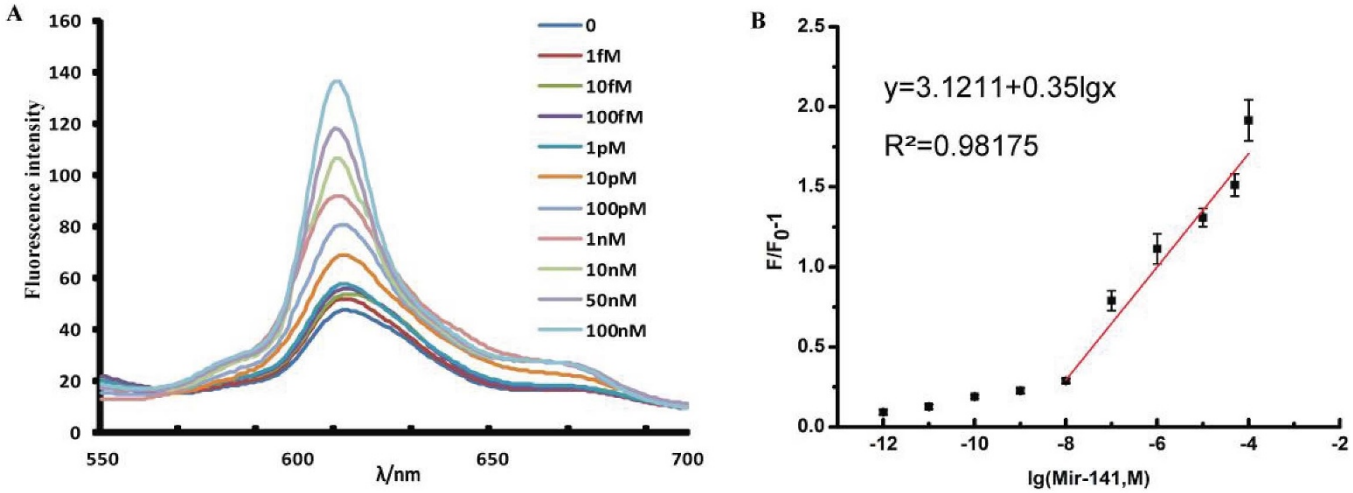

Figure 3 | (A) Fluorescence emission spectra (excitation at 399 nm, emission at 610 nm) upon the addition of miR-141 (0 fM, 1 fM, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 50 nM, 100 nM). Inset: (B) Scatter plot of (F/F_0-1) as function of the concentrations of miR-141, where F_0 and F are the NMM fluorescence signals in the absence and presence of miR-141, respectively.

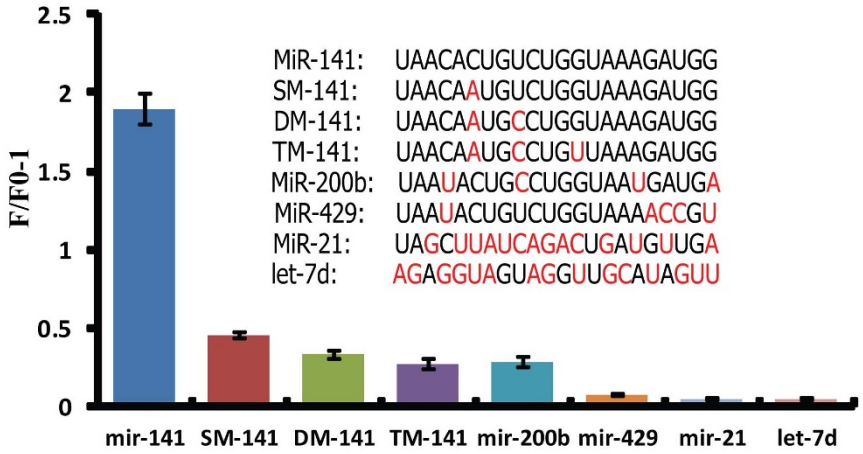

Figure 4 | Specificity of our method. Bars represent the fluorescence ratio ($F/F_0 - 1$) upon the addition of different miRNA targets. Inset: Fluorescence emission spectra for the different miRNAs targets. Inset: Sequences of miR-141, miR-429, miR-200b, miR-21, andlet-7d. The bases that differ from those in miR-141 are indicated in red.

on the logarithm (lg) of miR-141 concentration in the ranges 10 pM−100 nM, with a correlation equation of $(F/F_0-1) = 3.12 + 0.35\,\lg(\text{miRNA})$, where Y is the fluorescence ratio (F/F_0-1) and X is the concentration of miR-141 (regression coefficient $R^2 = 0.9818$). The limit of detection of miR-141 based on 3σ was approximately 1fM synthetic miRNA under pure conditions. The preliminary results indicate that the proposed method has great potentials and sensitivity for the multiplex detection of miRNAs.

The high sequence similarity between family members is a distinctive characteristic of miRNAs, which is always challenging for any miRNAs detections. The miR-141 belongs to the miR-200 family, which contains five members including miR-200a, miR-200b, miR-200c, miR-141, and miR-429. Three members of the miR-200 family (miR-141, miR-200b, and miR-429), miR-21, and let-7d as well as artificial synthesized single-base-mismatched miR-141 (SM-141), double-base-mismatched miR-141 (DM-141) and three-base-mismatched miR-141 (TM-141). The let-7d is conservative among human cells, and the miR-21 is highly over expressed in breast tumours. Seven artificially synthesised target miRNAs were analysed at the same concentration of 10 nM; Figure 4 presented a comparison of the fluorescence signal responses towards different miRNAs targets. As expected, the signal ratio ($F/F_0 - 1$) from miR-141 was a much stronger response than those from the other miRNAs. These results demonstrate that the specificities of the proposed method are sufficiently high to discriminate between the two miR-200 family members, which could even be easily discriminated from the SM-141, DM-141 and TM-141 signals.

Conclusions

Taken together, we developed a combination strategy to detect miRNAs. Specifically, the interaction of G-quadruplex and NMM as a label-free fluorescent reporter was coupled with the DSN-based signal-amplifying strategy to detect miRNAs. This method can rapidly and selectively quantify target miRNAs, avoiding complicated procedures or sophisticated instrumentation. Further, this method also reduces the costs and operating time without labeling and reverse transcriptional to amplify signals. The strategy is reliable, convenient, highly sensitive, and selective for multiple miRNAs. We expect that this general strategy will find potential uses early for disease diagnosis.

Methods

Synthesis of miR-141 probe. The probe was composed of designed G-rich oligonucleotides (GR) that can partly hybridised with the cDNA (blue) and with the cDNA that is completely complementary to target miR-141. The two constituents were mixed in 1×DSN buffer (100 nM each). Then, the mixture was incubated at 95°C for 10 min and cooled to room temperature. After these steps, the probe can be used in the subsequent detection steps.

Our Method for miRNAs Detection. First, a volume of 200 μL reaction mixture containing 1×DSN buffer (50 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$, 1 mM DTT), 0.2 U DSN (dissolved in 25 mM Tris-HCl, pH 8.0; 50% glycerol), 20 U RNase inhibitor, 100 nM probe and miRNA target, was incubated in a thermal cycler at 60°C for 40 min. Subsequently, the reaction mixture was added 5 μL 0.06 mM NMM and 4 μL 100 mM KCl, and incubated at room temperature for 30 min to make the NMM bind to the formed G-quadruplexs sufficiently. Following that, the reaction mixture can be taken to measure fluorescence signal.

Fluorescent miRNAs assays. Concentration titration tests of the target miR-141 was performed by adding 1fM to 100 nM target miR-141 to 100 nM probes in 1×DSN buffer, which contained 50 mM Tris-HCl, 5 mM $MgCl_2$ and 1 mM DTT at pH 8.0. The mixtures were then incubated at 60°C for 40 min to ensure reaction completion and signal stabilisation. Subsequently, 2 mM KCl and 1.5 μM NMM were added to the mixtures, which were then mixed followed by astatic culture at room temperature for 30 min. The changes in the fluorescence signal ratios were calculated using the formula $Y = F_0/F\text{-}1$, where F_0 and F are the fluorescence intensities at 610 nm (maximum emission wavelength) in the absence and presence of miR-141, respectively.

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Author contributions

J.Z. and W.W. conceived the idea and directed the work. J.Z. and W.W. designed the experiments. L.Y. and Y.Y.Y. performed the in vitro fluorescence tests. The data analyses were performed by L.Y. and L.P. All authors contributed to writing the manuscript and reviewed this manuscript.

Additional information

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