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Double-strand Displacement Biosensor and Quencher-free Fluorescence Strategy for Rapid Detection of microRNA

Rong Liao, Kui He, Chunyan Chen, Changqun Cai*, Xiaoming Chen*

Key Laboratory of Environmentally Friendly Chemistry and Applications of Ministry of Education, College of Chemistry, Xiangtan University, Xiangtan, Hunan 411105, China

Corresponding author. Tel.: +86 15273219560; Fax: +86-731-5829-2251

E-mail addresses: cai_mao3@hotmail.com (C. Cai)

ABSTRACT: We describe a facile quencher-free fluorescence strategy for rapid detection of microRNAs (miRNAs) by using a novel double-strand displacement sensor. The sensor is designed with an outstanding 2-aminopurine (2-AP) fluorophore as a probe and a pre-designed cDNA, which can completely complement the target miRNA and partly complement the 2-AP probe. When the target miRNA is added, the cDNA can be competed off from the cDNA\2-AP probe duplex, thereby forming a cDNA\RNA heteroduplex. The free 2-AP probe induces an increase in the fluorescent signal. A limit of detection of 5 nM and a wide linear range from 5 nM to 1000 nM (R^2 = 0.9971) are achieved by this assay. The rapid detection strategy can be accomplished within 2h without expensive nanoparticles and complicated instruments for the whole procedure, thus offering a significant potential for clinical application.

Keywords: 2-aminopurine probe; double-strand displacement; miRNA; quencher-free

INTRODUCTION

MicroRNAs (miRNAs) are a class of endogenous, non-coding, single stranded ~22 nucleotides RNAs, it play vital regulatory roles in plants and animals by targeting mRNAs for cleavage or translational repression.[1] Recent studies have found that the aberrant expression of miRNAs are associated with cell development, stress adaptation, and several diseases, including cancer.[2,3] In addition, miRNAs have been evaluated as potential biomarkers for the diagnosis, prognosis, and treatment of diseases.[4,5] Therefore, an accurate and quantitative analysis of miRNA expression is critical to further understand the biological functions of miRNAs and the clinical diagnosis.

To date, some traditional methods have been reported; for example, Northern blotting is widely used to recognize miRNAs,[6] but this technique requires a large amount of samples and often fails to detect low-abundance miRNAs.[7,8] Microarray technologies are less expensive, but such methods exhibit low sensitivity and narrow dynamic range.[9,10] Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) covers a wide dynamic range and achieves high accuracy.[11] However, this method requires precise control of temperature, and the short length of miRNAs makes the experimental design very sophisticated.[12,13] To address the abovementioned

difficulties, several new strategies such as nanoparticle-based detection,[14,15] surface plasmon resonance,[16,17] electrochemistry[18,19], and molecular beacon (MB)-based detection[20-22] have been developed to improve the detection sensitivity and selectivity. Among these techniques, MB-based detection has elicited considerable attention.[23,24] In 2014, Burrows et al. employed this technique to detect miRNAs by using a double-strand displacement biosensor with a self-complementary fluorescent reporter;[25] this method was simple and rapid and did not require any additional hybridization or rinsing steps. The wide applications, as well as the exquisite sensitivity and selectivity of MBs for the detection of miRNAs have achieved great success. [20-22,24,25] However, MB-based detection requires site-specific labeling of each terminus of the hairpin with both a fluorophore and a quencher; hence, the labeling and purification become difficult, time-consuming and expensive. Furthermore, organic dyes present poor photostability, easy photobleaching, and short lifetimes.[26,27] As such, we sought to design a novel sensor that selects an outstanding fluorophore instead of organic dyes in probe, while combining stand displacement with quencher-free flourescence strategy to detect miRNAs rapidly and simply.

In this work, 2-aminopurine (2-AP) was selected as a fluorophore in probe to construct a miRNA sensor. As a fluorescent analogue of adenine, the fluorescence of 2-AP is susceptible to its local environment. While the base or nucleotide of 2-AP is highly fluorescent in a solution, its fluorescence is slightly quenched in single stranded DNA and highly quenched in double stranded DNA because of the base-stacking interaction.[28-30] Therefore, we took advantage of the trait of 2-AP to develop a strand displacement and quencher-free fluorescent method for miRNA detection (Scheme 1). In this work, miRNA-122 was selected as a template because it is the most abundant miRNA in the liver and is involved in hepatocellular carcinoma (HCC) development and hepatitis C virus infection.[31] A recent discovery demonstrated that miRNA-122 is significantly downregulated in hepatocellular carcinoma.[32] A cDNA strand that is completely complementary to the target miRNA and partly complementary to the paring with the 2-AP probe was designed. The 2-AP probe formed a hybrid with the cDNA, and the fluorescence was significantly quenched first. Afterward, this cDNA can be competed off from the cDNA\2-AP

probe duplex to form a cDNA\RNA heteroduplex and release 2-AP probe by adding miRNA-122, thereby significantly enhancing the fluorescence signal. The proposed sensing protocol is rapid and simple, and it does not require expensive nanoparticles and complicated procedures. The displacement biosensor behaves as a signal-off type biosensor, and the fluorescence quenching of the 2-AP probe results from its stacking interaction with adjacent bases without any additional quenchers. Therefore, this detection platform offers significant potential for further applications in early cancer diagnosis and clinical analysis.

MATERIALS AND MERHODS

Materials. The 2-AP probe, cDNA, miRNA, diethylpyrocarbonate (DEPC)-treated water, 1× TE (10 mM Tris, 1 mM EDTA) buffer, 50× TAE concentrated solution (2 M Tris-acetic acid; 100 mM EDTA), and 10× TM (500 mM Tris, 80 mM $MgSO_4$) buffer were purchased from Shanghai Sangon Co., Ltd. (Shanghai, China). The sequences of the oligonucleotides and miRNAs are listed in Table 1.

Table 1. Sequences of 2-AP probe, cDNA and miRNA[α]

note	sequence(5'-3')
2-AP probe	CAAAC GCACC A***TGGA GTGTG <u>A</u>CAA***
cDNA	CA AACAC CAT***TG TCACA CTCCA***
miRNA-122	UGGA*G UGU*GA CAAUG G*UG*U*U* U*G*
miRNA-21	UAGCU UAU*C*A GACUG AUG*U*U* GA
miRNA-26a	UU*C*A*A GUAAU CCAGG AUAGG C*U
miRNA-141	CAUCU UC*CAG UACAG UGUU*G GA

[α] The underlined base of 2-AP probe indicates the 2-AP substitution. The bold italic region of 2-AP probe indicates the binding sequence of cDNA. The bold italic region of cDNA indicates the binding sequence of 2-AP probes. And the asterisk behind base of miRNAs represents the binding base of 2-AP probe.

Fluorescent miRNA Assays. The 2-AP probe and cDNA were prepared in 1× TE buffer. MiRNA-122 was prepared in DEPC-treated water. The 1 μM 2-AP probe was incubated with 1 μM cDNA in 10× TM buffer solution (50 mM Tris, 8 mM

$MgSO_4$, pH 7.5) at 40 ^oC for 60 min. Up to 20 μL of various concentrations of target miRNAs were added, and the sample was incubated at 40 ^oC for 60 min. Finally, the resultant reaction solution was subjected to the fluorescence measurement by using a spectrofluorophotometer (RF-5301pc, SHIMAPZU, Japan). The excitation wavelength of 300 nm and the emission wavelength of 367 nm were used for the fluorescence measurement. The excitation and emission slits were set at 5.0 and 10.0 nm, respectively.

Gel Electrophoresis Analysis. Reaction products were analyzed by 2% agarose gel in 50× TAE buffer (40 mM Tris-acetic acid; 2 mM EDTA) followed by electrophoresis for 30 min at 120 V. The gel was stained by Du red and scanned by a GelDoc XR System (Bio-Rad, USA).

RESULTS AND DISCUSSION

Principle of miRNA Assays. The principle of miRNA assay is illustrated in Scheme 1. A 2-AP probe was designed with one substitution of 2-AP molecule for adenine in a single-stranded DNA. Previous reports indicated that the fluorescence of 2-AP in double-stranded DNA was much lower than that in single-stranded DNA because of the additional base-stacking interaction.[28-30] Similarly, the fluorescence of the cDNA\2-AP probe duplex was lower than that of 2-AP probe. The cDNA strand was completely complemented with miRNA-122 and partly complemented with 2-AP probe. In the presence of the target miRNA, the cDNA can be competed off from the cDNA\2-AP probe duplex to form a cDNA\RNA heteroduplex. Meanwhile, the 2-AP probe was released, thereby significantly enhancing the fluorescence signal. To demonstrate the feasibility of the proposed method for miRNA-122 detection, analyses were performed through fluorescence measurement (Figure 1A) and agarose gel electrophoresis analysis (Figure 1B). As shown in Figure 1A, the fluorescence intensity in the red line was lower than that in the black line. The black line represented the fluorescence emission spectra of the 2-AP probes (1 μM). The red line was obtained by fluorescence detection from the reaction solution of 1 μM cDNA and 1 μM 2-AP probes. The results showed that the cDNA and 2-AP probes can form the cDNA\2-AP probe duplex, which led to the decrease of fluorescence intensity. While

miRNA-122 was added into the cDNA\2-AP probe duplex solution (500 nM), the fluorescence intensity increased with the characteristic emission peak of 367 nm (blue line), indicating that partial 2-AP probes were released in the solution. The above results were further confirmed by agarose gel electrophoresis analysis (Figure 1B). As shown in Figure 1B, one band was under 25 bp in lane 1 (1 μM 2-AP probe), and the other band was above 25 bp in lane 2 (1 μM cDNA and 1 μM 2-AP probe). However, no light band was found in lane 2 at the position of the 2-AP probe. This finding indicated that the cDNA and 2-AP probes absolutely formed the cDNA\2-AP probe duplex. Two bands appeared in lane 3, in which one band was in the same position as that of the 2-AP probe (lane 1), and the other band was in a similar position to that of lane 2. Thus, with the addition of target miRNA, the cDNA can be competed off from the cDNA\2-AP probe duplex to form a cDNA\RNA heteroduplex, and the 2-AP probe was released simultaneously. Furthermore, the current displacement biosensor behaves as a signal-off-type biosensor (Figure 1).

Optimization of Reaction Conditions. To achieve the optimum assay performance, sensing conditions were optimized including the reaction time and work temperature. First, time-dependent fluorescence changes were measured to optimize the hybridization of the cDNA and 2-AP probes. Both the concentrations of the 2-AP probes and cDNA were then fixed at 400 nM. After 60 min, the fluorescence intensity was approaching to stability (Figure 2); therefore, 60 min was selected as the most effective reaction time for the cDNA hybridized with the 2-AP probes. In the presence of miRNA-122, this cDNA can be competed off from the cDNA\2-AP probe duplex to form a cDNA\RNA heteroduplex with the release of the 2-AP probes. As shown in Figure 3, the stand-displacement reaction rate was rapid and basically stable at 60 min; consequently, 60 min was also selected as the most effective reaction time for all subsequent assays. The whole detection procedure was rapid and can be completed within only 2 h. Furthermore, the reaction temperatures to increase the detection sensitivity were compared through miRNA detection experiments at 30 ^{o}C, 40 ^{o}C, 50 ^{o}C, and 60 ^{o}C. As shown in Figure 4, the highest fluorescence intensity ratio (F/F_0-1, where F_0 and F are the fluorescence signal in the absence and the presence of miRNA, respectively) was observed when the temperature was 40 ^{o}C. Therefore, 40 ^{o}C was

selected as the optimum reaction temperature.

Sensitivity and Selectivity of the Assay. Under optimum conditions, miRNA-122 was detected to evaluate the sensitivity of the proposed strategy. Both the concentrations of the 2-AP probes and cDNA were fixed at 1 μM upon incubation for 60 min at 40 ^{o}C. The fluorescence intensities upon the addition of different concentrations of miRNA-122 (0 nM to 1000 nM) were also measured. Figure 5 presented the emission spectra of the measurements; as expected, a gradual increase in the fluorescent peak at 367 nm was clearly observed as the concentration of miRNA-122 increased from 0 nM to 1000 nM (a-k). Figure 6 illustrates the changes in the fluorescence intensity (F/F_0-1) in responding to the different miRNA-122 concentrations. A relatively wide dynamic range was obtained from 0 nM to 1000 nM miRNAs. The (F/F_0-1) value was linearly dependent on the miRNA-122 concentrations in the ranges of 5 nM to 1000 nM, with a correlation equation of (F/F_0-1) = $8.4298 \times 10^{-4}X + 0.06048$, where Y is the fluorescence of (F/F_0-1), and X is the concentration of miRNA-122 (R^2 = 0.9971). The limit of detection was estimated to be 5 nM. The preliminary results indicated that the liner range was wide, and the proposed method was sensitive for the multiplex detection of miRNAs.

The high sequence similarity among family members is a distinctive characteristic of miRNAs, and to detect such characteristic is always challenging for any miRNAs detections. Previous research has evaluated miRNA-21 and miRNA-26a as a potential biomarkers in HCC.[33] In the present study, a series of contrast experiments was performed using miRNA-21, miRNA-26a, and miRNA-141 to evaluate the specificity of the proposed miRNA assay. As shown in Figure 7, nearly negligible fluorescence changes were observed in the addition of miRNA-21, miRNA-26a and miRNA-141 compared with the blank samples. This result indicated that the proposed strategy exhibits good selectivity for miRNA detection.

Detection of miRNA-122 in Serum Samples. To test whether the assay can be applied for detection of miRNA in real biological samples, experiments were carried out in human serum samples. Various concentrations of miRNA-122 were added in 1000-fold dilution of human serum samples. The human serum was obtained from the Hospital of Xiangtan University and diluted 1000-fold with deionized water. The

experinmental results were shown in Table 2. The data exhibited good recovery, thereby indicating that the developed assay offered great potential for specific detecting of miRNA in biological fluids.

Table. 2 Results for the Determination of the miRNA in 1000-fold Dilution of human serum.[β]

samples	the concentration of miRNA ($nmol \cdot L^{-1}$)			
	Spiked	Measured	Recovery (%)	RSD (%)
Human serum	0.00	(n=3)		
	50.00	46.65	93.30	2.32
	400.00	397.31	99.26	2.10
	1000.00	1007.45	100.74	2.05

[β] Condition: cDNA and 2-AP probes dosages: 1 μM, temperature: 40 ^{o}C

CONCLUSION

A novel sensor combined by double-strand displacement with quencher-free fluorescent method to detect of miRNA-122 is developed in this study. In contrast to conventional MBs, the 2-AP probe presents good photostability, and it is quenched through its stacking interaction with the adjacent bases without the involvement of any additional quenchers. The fluorescence sensor also shows good sensitivity and selectivity for miRNA-122 detection. A limit of detection of 5 nM and a wide linear range from 5 nM to 1000 nM ($R^2 = 0.9971$) are achieved by this assay. Furthermore, this method reduces the costs, and the operation can be accomplished within only 2 h without sophisticated instrumentation. Considering these advantages, we believe that this strategy will be applied in early diagnosis of diseases.

AUTHOR INFORMATION

Corresponding Authors

*Tel.: (86)15273219560. Fax: +86 7315292251. E-mail: cai_mao3@hotmail.com (C. Cai)

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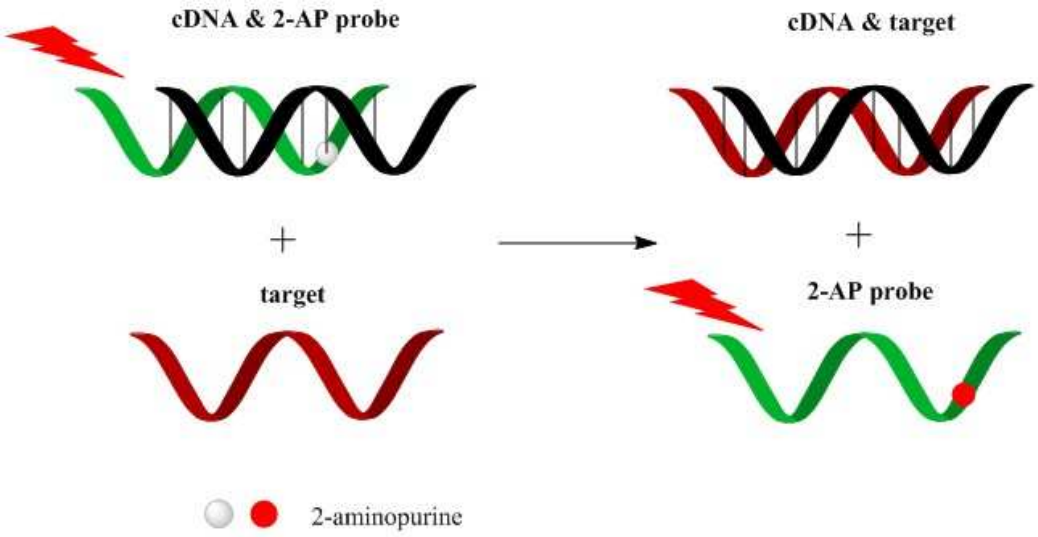
Scheme 1. Schematic of Detection Mechanism



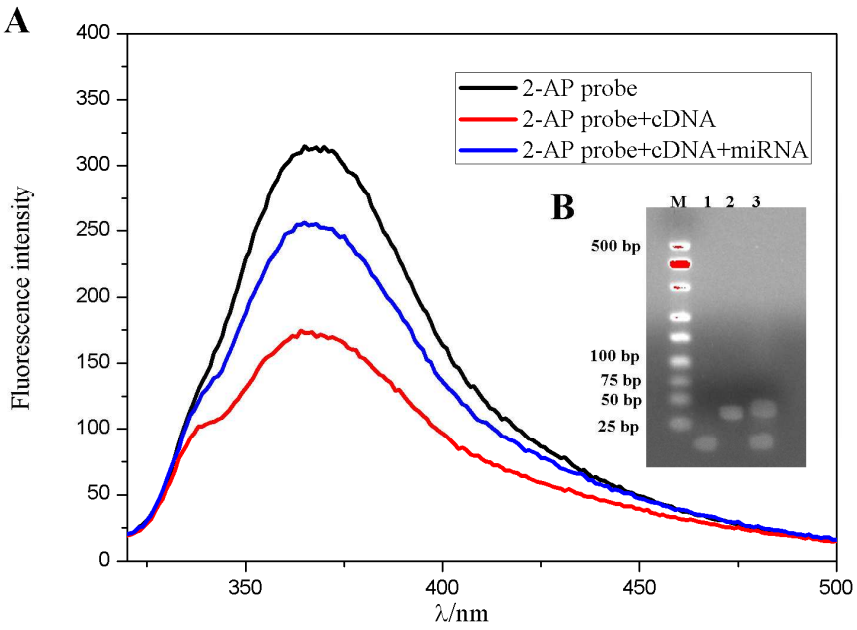


Figure 1. (A) Fluorescence emission spectra of reaction products in the presence of 2-AP probe (black line), 2-AP probe + cDNA (red line), 2-AP probe + cDNA + miRNA-122 (blue line). The concentration of the 2-AP probe is 1 μM, cDNA and miRNA-122 are 500 nM. (B) Agarose gel electrophoresis images of reaction products. Lane M, DNA marker (25-500 bp); lane 1, 1 μM 2-AP probe; lane 2, 1 μM 2-AP probe and 1μM cDNA; lane 3, 1 μM cDNA and 1 μM miRNA-122 with 1 μM 2-AP probe.

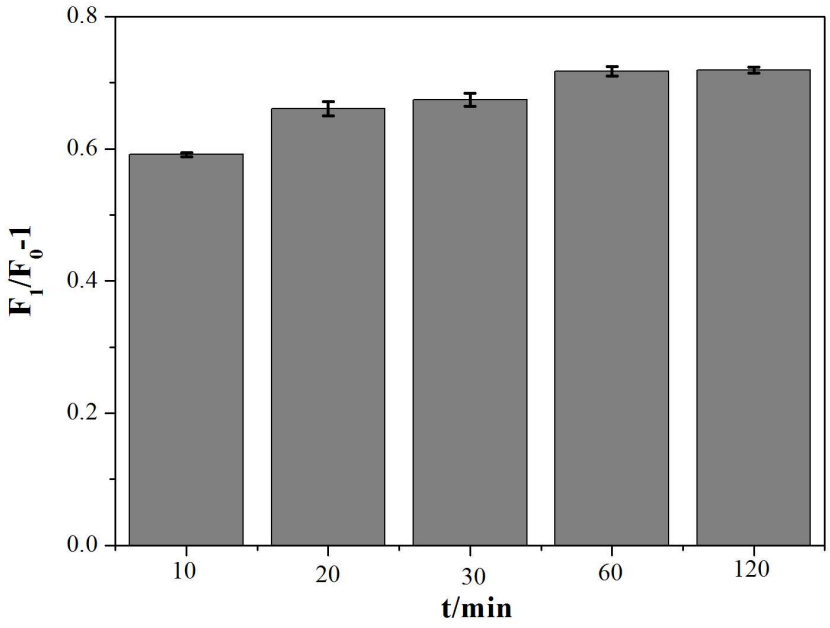


Figure 2. Variance of the fluorescence ratio value of F_1/F_0 -1 with the reaction time, F_0 and F_1 are the fluorescence signal in the presence and the absence of cDNA, respectively. The error bar represents the standard deviation of three measurements.

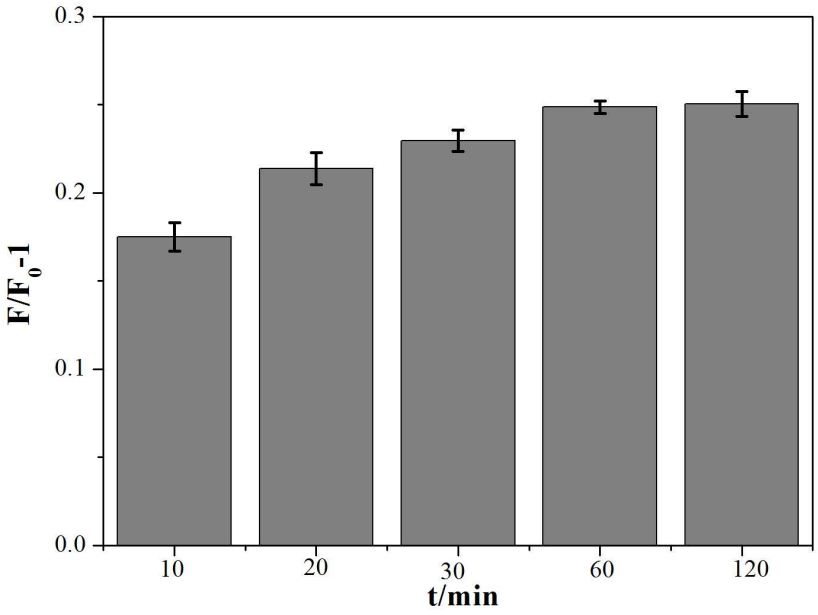


Figure 3. Variance of the fluorescence ratio value of F/F_0-1 with the reaction time, F_0 and F are the fluorescence signal in the absence and the presence of miRNA-122, respectively. The error bar represents the standard deviation of three measurements.

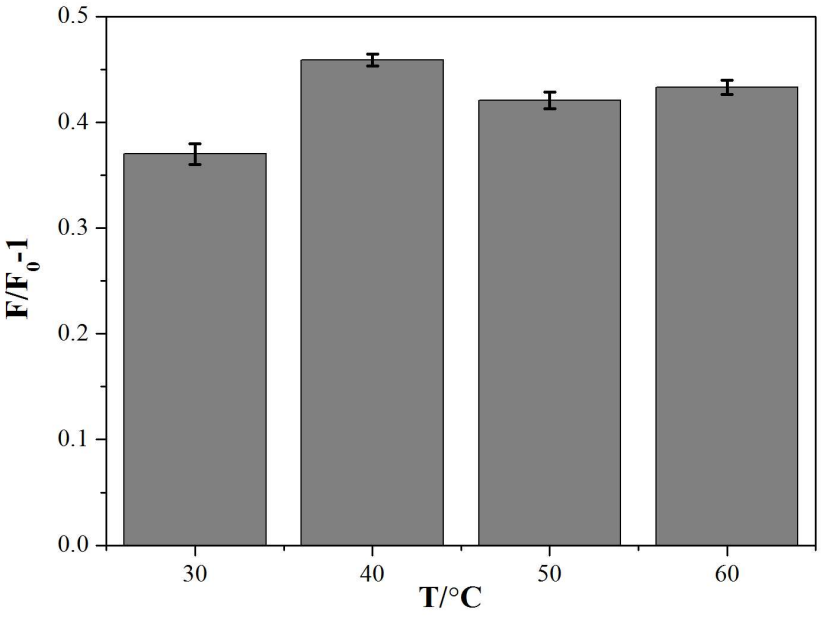


Figure 4. Bars represent the fluorescence ratio value of F/F_0-1 on different reaction temperature. F_0 and F are the fluorescence signal in the absence and the presence of miRNA-122, respectively. The error bar represents the standard deviation of three measurements.

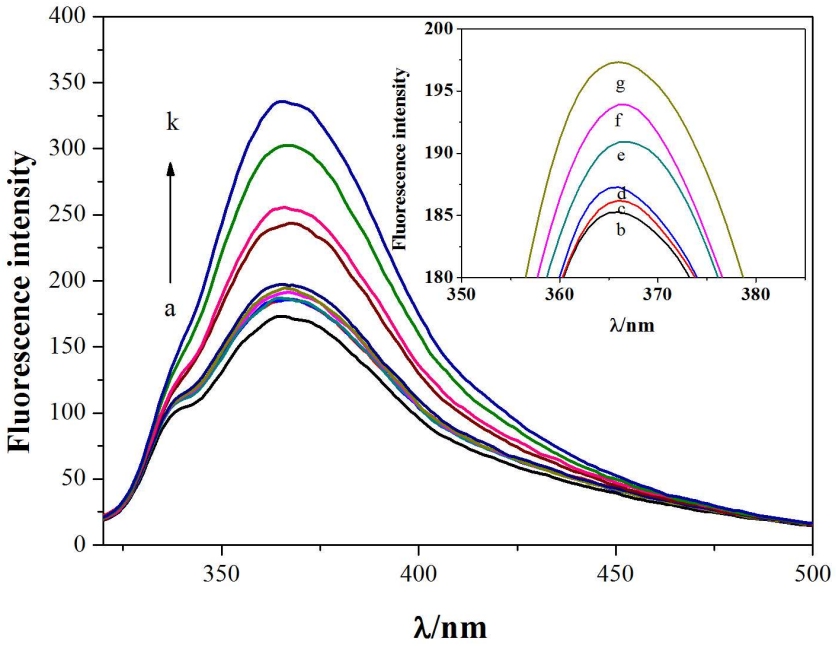


Figure 5. Fluorescence emission spectra of 2-AP and cDNA (1 μM) upon addition of target miRNA with different concentration: (a)0 nM; (b)5 nM; (c)10 nM; (d)25 nM; (e)50 nM; (f)75 nM; (g)100 nM; (h)400 nM; (i)500 nM; (j)750 nM; (k)1000 nM. Inset: a zoomed-in view of the data for low concentrations of target miRNA (b-g).

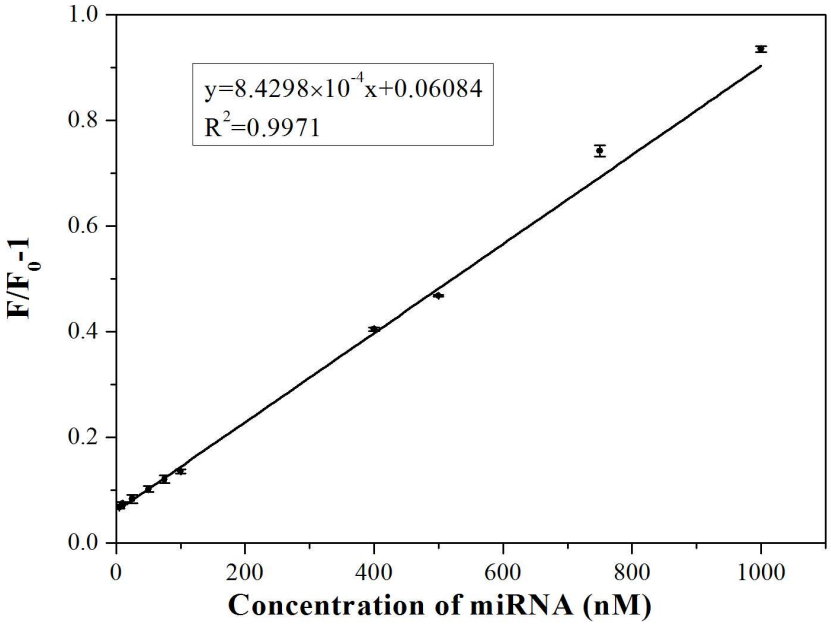


Figure 6. Scatter plot of (F/F_0-1) as function of the concentrations of miRNA-122 (5-1000 nM). Where F and F_0 are the fluorescence signals in the presence and absence of miRNA-122, respectively. The error bar represents the standard deviation of three measurements.

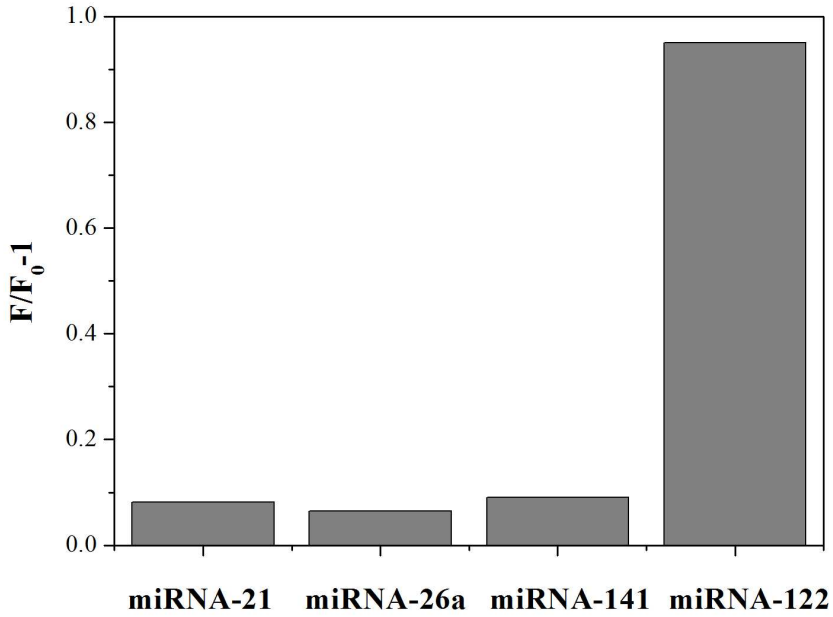
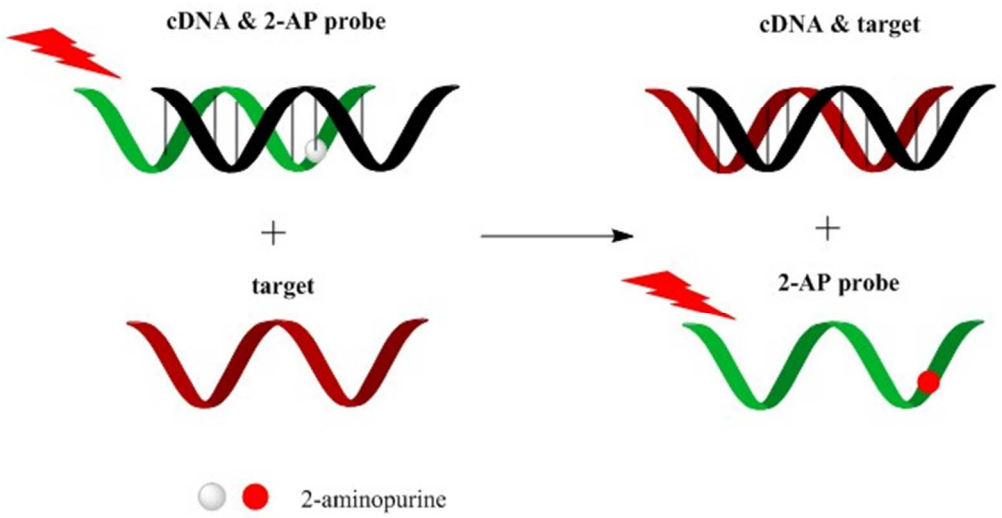


Figure 7. Selectivity of the proposed method. Bars represent the fluorescence ratio value of F/F_0-1 upon the addition of different miRNAs, F_0 and F are the fluorescence signal in the absence and presence, respectively.



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