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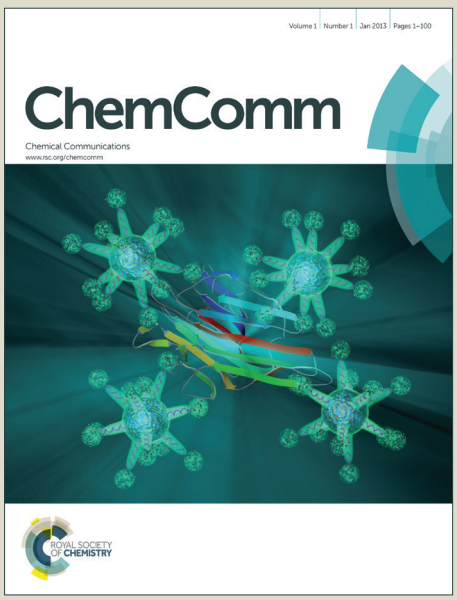

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ARTICLE TYPE

Rapid detection of microRNA based on p19-enhanced fluorescence polarization

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Via a molecular caliper p19 protein, we have developed an amplified fluorescence polarization method for rapid microRNA detection. This proposed assay has several intrinsic features including rapidity, simpleness, and accuracy.

MicroRNAs (miRNAs) constitute a large family of endogenous, non-protein coding RNA molecules (~22 nucleotides), which play a critical role in a wide range of physiologic and pathologic processes.[1] They have emerged as key post-transcriptional regulators via incorporating into an active RNA-induced silencing complex (RISC) and targeting specific messenger RNAs (mRNAs) to repress the translation or induce the degradation of mRNAs. It is predicted that mammalian miRNAs can regulate ~50% of all protein-coding genes.[2] Tremendous studies have revealed that miRNAs are involved in diseases, genetic disorders, and immune system function. Specially, miRNAs expressions are frequently dysregulated in the initiation and development of a variety of human cancers and highlight their importance in cancers by acting as both oncogenes and tumor suppressors.[3] Based on these findings, miRNAs have become promising biomarker candidates for cancer classification and prognosis.[4] Thus, developing sensitive, selective, rapid and quantitative detection method for miRNAs is of great significance in understanding biological functions of miRNAs, early diagnosis of cancers, as well as discovery of potential drug targets.

In contrast to nucleic acid detection, miRNAs detection is still difficult and challenging due to their small size, sequence homology among family members, susceptible degradation, and low abundance in total RNA samples. Recently, various enzyme-assisted signal amplification methods have been developed and applied to specific miRNA detection. For example, some nuclease such as RNase H,[5] DNase I,[6] and duplex-specific nuclease [7] have been employed as cleavage enzymes in linear miRNA recycling-oriented amplification. Many exponential amplification methods based on multi-enzyme combination have been developed, such as target-primed branched rolling-circle amplification (BRCA) using ligase and phi29 DNA polymerase,[8] exponential amplification reaction (EXPAR) using DNA polymerase and nicking endonuclease,[9] and hairpin-mediated quadratic enzymatic amplification (HQEA) using nicking endonuclease, lambda exonuclease, and Bst polymerase.[10] Although these methods have shown their respective advantages, they are all time-consuming due to enzyme-catalyzed reaction, which couldn't meet the requirement of rapid detection such as "on the spot" testing in some cases. In this work we have devoted our ongoing effort to develop new miRNA detection method with the outstanding features of short assay time, simple handling procedure and adoptability to point-of-care testing.

The fluorescence polarization assay is a widely utilized technique for rapid and quantitative analysis of diverse molecular interactions and enzyme activity in clinical and biomedical application. Our group have applied fluorescence polarization assay in detection of nucleic acid,[11] metal ion,[12] and protein activity.[13] Fluorescence polarization offers a lot of merits including the homogeneous format, speed, accuracy, reproducibility, and automated high-throughput capability. Especially, it works well in studying the protein-nucleic acid interactions.[14] A 19-kDa protein (p19) expressed from the Carnation Italian Ringspot Virus (CIRV) plant can function as an effective suppressor of RNA silencing, by binding and sequestering the small double-strand RNA (dsRNA) to prevent their incorporation into RISC and thus to turn off the silencing pathway.[15] In principle, the p19 protein acts as a dimer and binds the minor groove of dsRNA via hydrogen-bonding and electrostatic interactions between the β-sheet surface of the p19 protein homodimer and the sugar-phosphate backbone of the dsRNA.[16] It behaves like a molecular caliper and binds dsRNA in a size dependent, sequence independent manner. Preferentially, p19 protein displays specificity upon binding 19~21 nt long dsRNA with nanomolar affinity, which also reported to bind miRNA with high affinity.[16b, 17] By taking advantage of the protein-RNA interactions of the p19 protein with small miRNA, herein we have developed an amplified fluorescence polarization assay to quantify the miRNA.

Fig. 1 outlines the working principle of the proposed method for specific miRNA detection using p19-enhanced fluorescence polarization. Briefly, the reaction system consists of a dye-labeled RNA probe and p19 protein. The RNA probe is designed to be complementary to target miRNA. In the presence of a target miRNA, the miRNA-specific RNA probe hybridizes with the target miRNA to form a duplex. Based on the Perrin equation, the fluorescence polarization (FP) value of dye-labeled molecule is proportional to its rotational relaxation time at constant temperature and solution viscosity, which in turn depends on its molecular volume (molecular weight). If a dye-labeled molecule is small, it rotates faster and hence has a smaller FP value. Conversely, the larger dye-labeled molecule has bigger FP value due to its slow rotation.[12] Thus, the newly formed dsRNA duplex

has a larger FP value than that of the RNA probe due to the enlargement of the molecular weight. It should be noted that fluorescence polarization assay is not suitable for analyzing the molecular interaction between the small molecules such as the nucleic acids hybridization in this case because of the small change in FP value (ΔFP). Upon the addition of p19 protein, the miRNA-RNA probe duplex associates with p19 protein, which results in a significant increase of the FP value. This phenomenon provides a basis for quantitative analysis of miRNA via the introduction of p19 protein to amplify the fluorescence polarization signal on small molecules interaction.

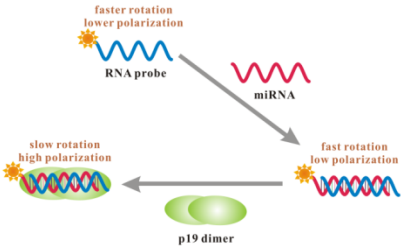

Fig. 1 Schematic illustration of the proposed p19-enhanced fluorescence polarization assay for miRNAs analysis.

To demonstrate the feasibility of the proposed method, we selected liver-specific miR-122 as a model to optimize the experimental conditions. MicroRNA-122 comprises ~70% of the total miRNAs found in the human liver[18] and is commonly deregulated in liver fibrosis and heparocellular carcinoma. First, we studied the binding ability of p19 protein upon different substrates including ssRNA, ssDNA, dsRNA, dsDNA, and RNA-DNA heteroduplex. Two FITC-labeled probes (named as Probe-1 and Probe-2, see the sequence information in Table S1 in ESI) complementary to the target (miR-122 or DNA-122) were prepared. The sequences of Probe-1 and miR-122 were the same as that of Probe-2 and DNA-122, except that the ribonucleotides and uridine in Probe-1 and miR-122 were replaced with deoxyribonucleotides and thymine in Probe-2 and DNA-122, respectively. As shown in Fig. 2A and 2B, when p19 protein was added to Probe-1 (ssRNA) or Probe-2 (ssDNA), the FP value remained unchanged. When a target miR-122 or DNA-122 was added, there was a small increase in FP value because of the formation of dsRNA, dsDNA, or RNA-DNA duplex. When p19 protein was added to dsRNA or RNA-DNA duplex, there was a significant increase in FP value due to the formation of the larger complex which tumbled much slower in solution and the emitted light remained polarized on a significantly longer time scale. The ΔFP value was approximately 4-fold higher with p19 protein than that of dsRNA or RNA-DNA duplex without p19 protein. Moreover, it is observed that the p19 protein preferentially bound dsRNA compared with RNA-DNA duplex, and it hardly bound with dsDNA. These results were well consistent with the reported data[19] that p19 protein does not bind to ssRNA, ssDNA, or dsDNA, and has a better binding ability with dsRNA than RNA-DNA duplex. Thus, we chose RNA probe as sensing element in the following experiments.

The length of a dsRNA substrate has a significant effect on p19 binding. We selected three sets of dsRNA duplex with paired nucleotides of 19 bp, 20 bp and 22 bp, which were in the form of Probe-1/miR-122, Probe-3/miR-122, and Probe-4/miR-122, respectively. As shown in Fig. 2C, the 19-bp Probe-1/miR-122 duplex achieved the highest ΔFP value, which was in good accordance with literature data.[19] Thus the best RNA probe for miR-122 detection in this work was chosen Probe-1. We then continue to investigate the response time between p19 protein and its substrate dsRNA. Fig. 2D shows that once added p19 protein to the mixture of dsRNA, the FP value of the solution rapidly increased to a maximum value within 3 min. The rapid reaction rate perfectly satisfies our initial aim of "on the spot" testing for miRNA analysis.

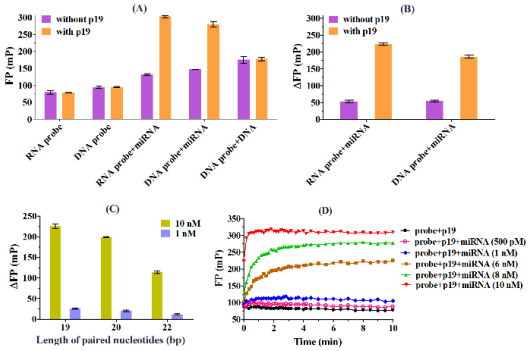

Fig. 2 (A) Investigation of the binding ability of p19 protein upon different substrates including ssRNA (RNA probe, Probe-1), ssDNA (DNA probe, Probe-2), dsRNA (RNA probe+miRNA), RNA-DNA heteroduplex (DNA probe+miRNA), and dsDNA (DNA probe+DNA). In this data, miRNA refers to miR-122, DNA refers to DNA-122. (B) The ΔFP values of the solution of dsRNA and RNA-DNA heteroduplex in the absence and the presence of p19 protein, respectively. (C) Investigation of the binding ability of p19 protein upon dsRNA duplexes with different lengths of paired nucleotides between RNA probe and miR-122. (D) Real-time FP measurement of reaction solution containing Probe-1 and p19 protein in the presence of miR-122 from 500 pM to 10 nM. ΔFP value is calculated by subtracting the background FP value of probe solution from the FP value of sample solution.

In order to obtain a better binding performance for p19 protein and dsRNA, we optimized the binding conditions including reaction buffer, probe concentration, and p19 protein concentration (See the detailed information in ESI). Under the optimum experimental conditions, the target miR-122 was detected quantitatively in a range from 10 pM to 10 nM by measurement of the fluorescence polarization of the reaction buffer containing Probe-1 and p19 protein. As shown in Fig. 3A, the ΔFP values were linearly dependent on the amount of miR-122 in the range from 10 pM to 10 nM. The correlation equation obtained is Y = 22.86X + 3.760, with a high correlation coefficient of 0.9950. The limit of detection based on 3σ/S is approximately 8.5 pM. As a control, the sensitivity of sensing system in the absence of p19 protein was also studied (Fig. 3B). It's clearly observed that our proposed method improves by ~2 orders of magnitude over the direct fluorescence polarization assay on miR-122 detection without p19 protein.

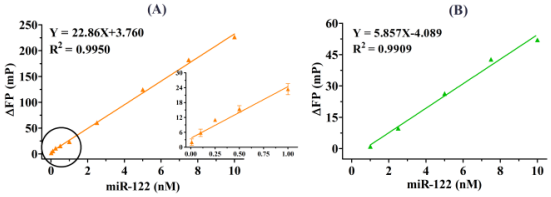

Fig. 3 Sensitivity investigation for miR-122 detection in the presence and absence of p19 protein. (A) Plot of ΔFP values as a function of miR-122 concentrations in the range from 10 pM to 10 nM in the presence of p19 protein. Inset: Plot of ΔFP values as a function of miR-122 concentrations from 10 pM to 1 nM. (B) Plot of ΔFP values as a function of miR-122 concentrations from 1 nM to 10 nM in the absence of p19 protein.

To evaluate the specificity of our proposed method for miR-122 analysis, we selected three hepatocellular carcinoma-related miRNAs (miR-221, miR-223, and miR-21),[20] five members of miR-200 family, and eight members of let-7 family as competing stimuli (see the sequence information in Table S1 in ESI). Fig. 4 exhibits the comparison data of ΔFP values towards different miRNAs targets and mixture samples. Only the FP values of sample containing miR-122 changed greatest, whereas those produced by other tested miRNAs were barely changed. It is worthy pointing out that the mixture samples showed the similar signal change as that of miR-122 alone. The mixture samples were prepared by adding all tested seventeen miRNA targets together at two concentrations of 10 nM and 1 nM, respectively. These results clearly suggest that the proposed miRNA detection approach had high sequence specificity. In addition, we spiked different amounts of miR-122 in the cell lysis buffer and cell lysates with known concentrations to test the accuracy of the proposed method. The result is summarized in Table S2 and Table S3 (see in ESI), which shows a good accuracy and reliability with recoveries from 94.47 to 103.05% and coefficients of variance (CV) from 5.43 to 12.45%.

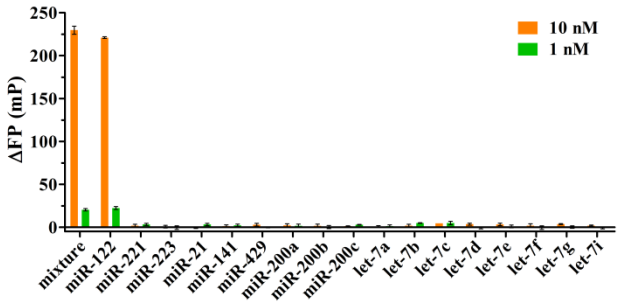

Fig. 4 Selectivity investigation of the proposed method using different miRNAs and the mixture sample made from all tested miRNAs. The miRNAs were tested at two concentrations of 10 nM and 1 nM, respectively.

In summary, we have developed a universal miRNA detection assay based on p19-enhanced fluorescence polarization. By pairing up the small miRNA recognition capability of p19 protein with the powerful fluorescence polarization, this assay offers the following advantages. First, this assay is very simple with one-step homogeneous procedure. Second, the isothermal process in the rapid "mix-and-measure" format within 3 min is well suitable for "on the spot" testing. Third, it exhibits a high reproducibility due to the intrinsic ratiometric feature of the fluorescence polarization, which avoids the drawbacks of the fluorescence intensity-based assays in the problem of reproducibility caused by photo bleaching and non-uniform emission of the fluorophore. Based on the above distinctive features, we believe that the proposed assay would have great potential as a routine tool for miRNA analysis in molecular disease diagnosis and biomedicine.

Notes and references

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