Amplified fluorescence determination of microRNAs in homogeneous solution with cationic conjugated polymers†

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Combining the miRNA-primed extension reaction and the optical amplification of a conjugated polymer, a sensitive, selective and homogeneous method for miRNA determination has been developed based on a fluorescence resonance energy transfer (FRET) mechanism.

MicroRNAs (miRNAs) are a class of small (19–23 nucleotides), endogenous regulatory RNA molecules, which are believed to be involved in the regulation of nearly 30% of all human genes.[1] Moreover, recent studies have pointed towards a connection between specific changes in miRNA expression and various diseases,[2] especially for various types of cancer.[3] In the past five years there has been a surge of interest in understanding the regulatory roles of miRNA in cellular processes. MiRNA assay plays a critical role in better understanding the biological function of miRNAs and has great potential in early disease diagnostics and new drug discovery.[4,5] Currently, northern blotting and microarray-based methods are most widely used for miRNA detection. However, the northern blotting methods are time-consuming and are not sensitive, therefore requiring large amounts of sample. The microarray-based method is attractive due to its high-throughput capability[6] and several RNA-labeling strategies have been investigated to improve the sensitivity of miRNA detection on the microarrays.[7] Nevertheless, the short length of miRNAs results in the risk of cross-hybridization and low specificity. Furthermore, to obtain better insight into the biological function of miRNAs needs *in situ* detection of miRNAs in specific tissues, in which washing steps can not be used and, therefore, homogeneous assays are desirable. More recently, a few homogeneous methods for miRNA detection have also been reported, including quantitative RT-PCR (reverse transcription polymerase chain reaction),[8] modified Invader assay,[9] and Ribozyme amplification method.[10] These methods are sensitive, however, they need sophisticated processes, locked nucleic acid-primer, and multiplex fluorescence-labeled DNA probes due to the short lengths of the miRNAs, resulting in high-cost and long analysis time. There is thus a requirement for rapid, sensitive, cost-effective and homogeneous methods for miRNA detection.

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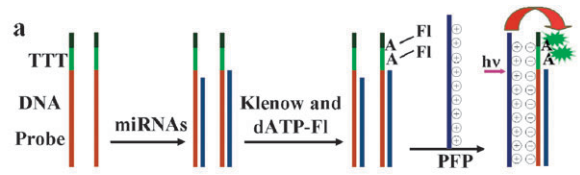
The main challenges for miRNA detection originate from the extremely short length and strong similarity among family members of miRNAs. It is important to note that the short length of miRNAs makes them ideal to be used as primers for primer extension reactions. The RNA-primed methods are very specific and robust because of the high specificity of DNA polymerase and easy optimization of the primer extension reactions.[11] However, RNA-primed methods still retain several challenges. One is their low sensitivity due to lack of signal amplification. Another is the need for separation steps to discriminate between the signals from the extended products and the non-extended labeled-dNTPs (deoxynucleotide triphosphates). In recent years, cationic conjugated polymers (CCPs) have been widely used to fabricate biological sensors for detecting DNA, proteins and single-nucleotide polymorphisms (SNPs).[12] CCPs, containing a large number of absorbing units, can transfer the excitation energy along their whole backbone to the fluorescent reporter, resulting in the amplification of the fluorescent signal. Moreover, based on the strongly electrostatic interactions between CCPs and nucleic acid strands, water-soluble CCPs can offer a unique platform for nucleic acid detection in a homogeneous solution. As demonstrated here, coupled with the miRNA-primed extension reaction, it is possible to use CCPs to design a simple, sensitive, homogenous, and cost-effective platform for the quantitative detection of miRNAs.

The miRNA assay is illustrated in Fig. 1a. To demonstrate our new strategy for miRNA assay, let-7 miRNA family members are used as targets because their sequences are very similar (Fig. 1b) and their expression levels are closely associated with cell development and human cancers.[3] Poly[(9,9-bis(6′-*N*,*N*,*N*-trimethylammonium)hexyl)fluorenylene phenylene dibromide] (PFP, see structure in Fig. S1†) is used as the CCP in the fluorescence resonance energy transfer (FRET) experiments. The designed DNA probe includes 22 base sequences from the 3′-terminus perfectly complementary to let-7a and 3′-TTTCTAgC-5′.[11] After hybridization between the probe and the target miRNAs, the fluorescein-labeled dATP (dATP-Fl) can be incorporated into the miRNAs by an extension reaction in the presence of Klenow DNA polymerase when the 3′-termimus of the miRNAs is complementary to the probe. Upon adding PFP, strong electrostatic interactions between the DNA/RNA hybrid and PFP makes the extended dATP-Fl close to PFP, leading to efficient FRET from PFP to the fluorescein label on the extended dATP because the absorption of the fluorescein (λ_{max} = 480 nm) overlaps with the emission of PFP ($\lambda_{ex}/\lambda_{em}$ = 380 nm/424 nm, see Fig. S2†). In contrast, the non-extended dATP-Fl has much weaker electrostatic interaction with PFP and thus keeps



b DNA Probe: 5'-Cg ATC TTT AAC TAT ACA ACC TAC TAC CTC A-3'
let-7a: 5'-UgAggUAgUAggUUgUAUAgUU-3'
let-7b: 5'-UgAggUAgUAggUUgUgUggUU-3'
let-7c: 5'-UgAggUAgUAggUUgUAUggUU-3'
let-7d: 5'-AgAggUAgUAggUUgCAUAgU-3'
let-7e: 5'-UgAggUAggAggUUgUAUAgU-3'

Fig. 1 (a) Schematic representation of the miRNA assay. (b) Nucleic acid sequences used in the assay. The deep red line, green line and deep green line in the DNA probe represent the sequence 3′- ACTC CAT CAT CCA ACA TAT CAA, TTT and CTAgC-5′, respectively. The blue line represents the miRNAs. The different bases compared to let-7a in other miRNAs are marked in red.

far away from PFP. Therefore, in view of the change of FRET signal, the target miRNAs can be detected in homogeneous solution without the requirement of any separation steps.

To perform the miRNA extension reaction with 2.5 unit Klenow DNA polymerase, Fig. 2 compares the emission spectra observed upon adding PFP. As expected, no obvious FRET signal at 535 nm can be observed for the control experiment in the absence of target miRNA. Meanwhile, let-7d and let-7e only produce extremely low FRET signals because their sequence length is shorter, with one base at the 3′-terminus, compared to let-7a, resulting in an inefficient extension reaction. For let-7a, let-7b, and let-7c, the bases at the 3′-termini are the same and complementary to the designed DNA probe immediately downstream of the sequence of 3′-TTTCTAgC-5′. The extension reactions occur in the presence of dATP-Fl and Klenow DNA polymerase and sizable FRET signals appear at 535 nm. let-7c and let-7b, respectively contain one and two different bases compared to let-7a and, therefore, have one and two mismatched bases with the DNA probe. Although the FRET signals of let-7a, let-7c, and let-7b gradually decrease in terms of the increase of the mismatched

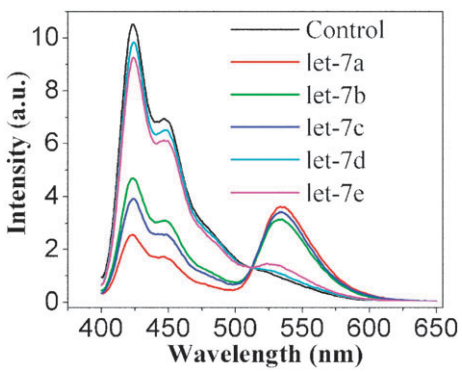


Fig. 2 Emission spectra of a solution containing PFP and miRNA extension products by using 2.5 unit Klenow DNA polymerase to perform the extension reactions. The final concentration: [each miRNA] = 1.0×10^{-8} M, [DNA probe] = 2.0×10^{-8} M, [dATP-Fl] = 3.0×10^{-8} M, [PFP] = 4.0×10^{-7} M in RUs (monomer repeat units). The excitation wavelength is 380 nm.

bases, the results in Fig. 2 show poor selectivity for detection of miRNAs containing the same base at their 3′-termini.

In view of a better insight into the biological function of individual miRNA, to discriminate one-base difference between miRNA molecules is very important for miRNA assays. Based on a systematic investigation of the influence of experimental conditions on the selectivity for miRNA detection, it was found that the amount of Klenow DNA polymerase is the critical factor affecting the selectivity. By using 1.0 unit Klenow DNA polymerase to perform the miRNA extension reactions, as depicted in Fig. 3a, let-7a, let-7b, and let-7c can be discriminated by measuring the FRET signals at 535 nm. Furthermore, the FRET ratio ($I_{535\ nm}/I_{424\ nm}$) produced by let-7a, let-7b, let-7c, let-7d, and let-7e is 0.471, 0.086, 0.149, 0.026, and 0.021, respectively (Fig. 3b). That is to say, the selectivity factor for detection of let-7a and let-7c by using the DNA probe complementary to let-7a is greater than 3 : 1. Worth mentioning here is that let-7a and let-7c only differ by one base near the 3′-end (base A in let-7a, G in let-7c) and the interaction energy between T–A and T–G is similar.[13] Therefore, it is difficult to discriminate between let-7a and let-7c by all hybridization-based methods, in which only the fluorescence correlation spectroscopy method gives 3-fold difference between let-7a and let-7c.[14] Thus, the proposed FRET method can be characterized with high selectivity. On the other hand, one can also see from Fig. 3a that the fluorescence intensity at 535 nm using 380 nm excitation produced by 1.0×10^{-8} M let-7a is approximately 5 times larger than that obtained from 3.0×10^{-8} M dATP-Fl at 524 nm (maximum emission wavelength of dATP-Fl) by direct excitation at the fluorescein absorption maximum (480 nm). The result implies that the fluorescence signal can be greatly amplified by FRET of PFP, indicating a miRNA assay with high sensitivity. In these experiments, let-7a is perfectly complementary to the DNA probe. Therefore, the dATP-Fl can be easily incorporated at the 3′ terminus of let-7a in the presence of Klenow DNA polymerase. let-7b contains two mismatched bases at the fourth and sixth base from its 3′ terminus and let-7c contains one mismatched base at the fourth base from its 3′ terminus. Millar *et al.* have demonstrated that the mismatched bases can result in weaker binding of the duplex DNA to the polymerase domain.[15] We suppose that the mismatched bases have the same effect in the RNA/DNA duplex, which will decrease the

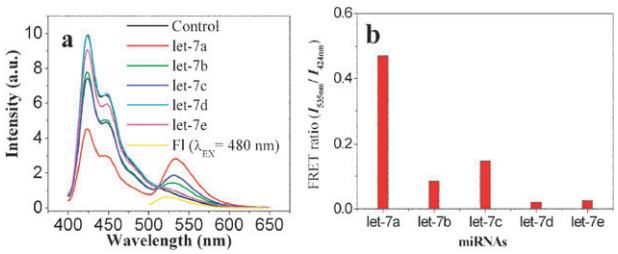


Fig. 3 (a) Emission spectra of the solution containing PFP and miRNA extension products by using 1.0 unit Klenow DNA polymerase to perform the extension reactions. The yellow line is the emission spectrum of 3.0×10^{-8} M dATP-Fl with excitation at 480 nm. (b) Representation of the FRET ratio ($I_{535\ nm}/I_{424\ nm}$) produced by various miRNAs. The experimental conditions were the same as those given in Fig. 2 except for the amount of Klenow DNA polymerase.

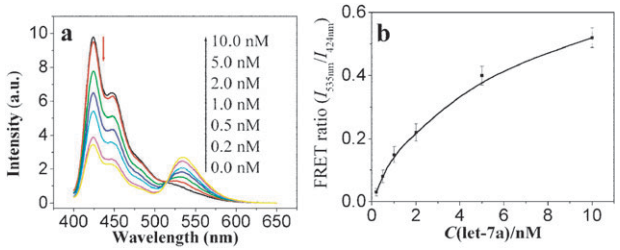


Fig. 4 (a) Emission spectra of the extension products produced by let-7a at various concentrations upon adding PFP. (b) FRET ratio ($I_{535\ nm}/I_{424\ nm}$) as a function of let-7a concentration. The FRET ratios are the average values of five-times repeated measurements. The final concentration: [DNA probe] = 2.0×10^{-8} M, [dATP-Fl] = 3.0×10^{-8} M, [PFP] = 4.0×10^{-7} M in RUs, Klenow: 1.0 unit. The excitation wavelength is 380 nm.

activity of the DNA polymerase and the incorporation efficiency of dATP-Fl on the 3′ terminus of let-7b and let-7c. Therefore, in the presence of an appropriate amount of the DNA polymerase let-7a, let-7b and let-7c can be easily recognized by monitoring the FRET signals (Fig. 3). However, the incorporation efficiency of dATP-Fl on the 3′ terminus of let-7b and let-7c would be improved when a large amount of the DNA polymerase exists, so that let-7a, let-7b and let-7c produce almost the same FRET signals (Fig. 2). According to the mechanism demonstrated above, by monitoring the FRET signals, the miRNA type would be accurately determined using different DNA probes, respectively complementary to various miRNAs.

To investigate the dynamic range for miRNA determination, the emission spectra of a series of extension products with various concentrations of let-7a were measured upon adding PFP with the excitation at 380 nm (Fig. 4a). Fig. 4b shows the FRET ratio ($I_{535\ nm}/I_{424\ nm}$) as a function of let-7a concentration. There is a good correlative relationship between the FRET ratio and the concentration of let-7a in the range of 0.2–10 nM. Therefore, the concentration of the miRNA would be quantitatively determined by measuring the FRET ratios. The detection limit (3σ, $n = 11$) was estimated to be 46 pM (corresponding to 9.2 fmol in a 200 μL sample). A series of seven repetitive measurements of 0.2 nM let-7a were used to evaluate the precision, and the relative standard deviation (RSD) was 3.8%.

In summary, we have demonstrated that water-soluble CCPs can be designed to directly determine miRNAs in a homogeneous solution with high sensitivity and selectivity. Due to the optical amplification by the CCPs, the sensitivity achieved by a common spectrofluorimeter with xenon lamp excitation is much higher than that of standard northern blot and microarray-based methods,[4] and is comparable to that of ribozyme amplification[10] and quantum dot-based fluorescence detection methods.[7e] By controlling the amount of DNA polymerase, the proposed method can discriminate one-base difference between miRNA molecules. Moreover, the homogeneous platform for miRNA assay eliminates the use of labeling probes and the requirement of isolation steps, which greatly simplifies the assay procedure and results in rapid and low-cost detection. In principle, multiplex detection of miRNAs can be easily realized by introducing different DNA probes, respectively complementary to different miRNA targets because the miRNA extension reactions can be performed under uniform conditions. Therefore, the proposed miRNA assay may open up a new possibility for routine miRNA analysis and molecular diagnostics.

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