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Homogeneous and Sensitive Detection of microRNA with Ligase Chain Reaction and Lambda Exonuclease-Assisted Cationic Conjugated Polymer Biosensing

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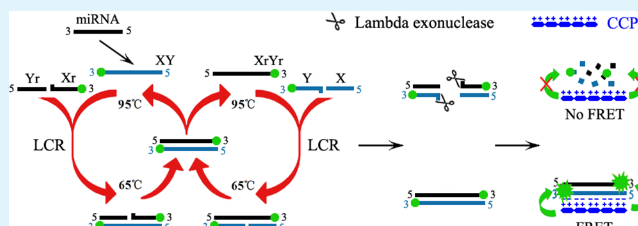
Supporting Information

ABSTRACT: A simple and homogeneous microRNA assay is developed by integration of ligase chain reaction (LCR) and lambda exonuclease-assisted cationic conjugated polymer (CCP) biosensing. LCR is utilized for exponential amplification of microRNA, and lambda exonuclease is introduced to degrade excess fluorescein-labeled probes in LCR for eliminating background signal. After addition of CCP, efficient fluorescence resonance energy transfer from CCP to fluorescein in LCR products occurs. The method is sensitive enough to detect 0.1 fM target microRNA and specific to discriminate one-base difference of microRNAs, which paves a new way for homogeneous microRNA detection and molecular diagnosis.



KEYWORDS: *ligase chain reaction, cationic conjugated polymer, lambda exonuclease, microRNA, homogeneous detection*

MicroRNAs (miRNAs) are a class of single-stranded, short (approximately 19–23 nucleotides), and non-coding RNAs that play important roles in gene regulation and cell function.[1,2] The expression levels of miRNAs are directly associated with human cancers, where miRNAs can act as either oncogenes or tumor suppressors.[3,4] Therefore, miRNA detection is crucial for cancer dignosis and therapy as well as drug screening. However, miRNA detection challenges many of analysis methods for the unique characteristics of miRNAs with small size, sequence similarity among family members of miRNAs, and low abundance in total RNA samples.[5,6] Up to now, many methods, such as northern blotting,[7,8] microarray,[9,10] polymerase chain reaction (PCR),[11−13] isothermal amplification techniques,[14−19] etc., have been developed for detection of miRNAs. Because of their low abundance in different parts of the tissue samples, sensitive amplification techniques are usually employed to detect miRNAs, such as PCR,[11−13] rolling circle amplification (RCA),[18,19] loop-mediated isothermal amplification (LAMP),[20] ligase chain reaction (LCR),[21−23] etc. Among these techniques, LCR possesses unique advantages for sensitive and specific miRNA detection. In LCR, two pairs of oligonucleotide probes are designed for performing ligation-based exponential amplification. Each pair of oligonucleotide probes consist of two probes that are adjacent and perfectly complementary to target miRNA or the ligated DNA strand. Upon hybridization to a target miRNA strand, the adjacent probes are ligated by a specific ligase to form a long DNA strand. Afterwards, the ligation products can serve as templates for the subsequent thermal cycles, leading to an exponential amplification process. Due to the high-efficiency of thermostable ligase, LCR exhibits better specificity than primer extension-based amplification, such as PCR, RCA, LAMP, etc. However, LCR for miRNA detection was generally limited by gel electrophoresis separation[21,22] or heterogeneous analysis process,[23] which brought about multiplex steps, high cost, and long analysis time. Therefore, development of LCR-based homogeneous miRNA detection has attracted widespread attention because of its simple procedures, short analysis time, and no requirement of separation steps.

Cationic conjugated polymers (CCPs) provide a versatile platform for homogeneous biosensing due to its excellent light harvesting and signal amplification abilities.[24−27] CCPs with a large number of repeated absorbing units can act as donor to transfer the excitation energy along the whole backbone of the CCP to fluorophore, therefore the signal of fluorophore is enhanced through fluorescence resonance energy transfer (FRET). Recently, we developed a homogeneous LCR strategy with CCP as indicator that was successfully applied to single nucleotide polymorphism (SNP) detection.[26] However, in the LCR, the excess and unreacted probes with 5′-fluorescein label must be digested by exonuclease I and exonuclease III for suppressing the background in FRET measurement. Meanwhile, the other adjacent probes for ligation reation were designed with phosphorothioate modification at its 3′-terminal bases to ensure the fluorescein-labeled LCR products resisting digestion of exonuclease I and exonuclease III, and achieving

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efficient FRET detection. As a result, the method utilized two exonucleases combining with multiple functionalized probes to carry out homogeneous LCR with CCP-based detection, which incurred complex steps and limited its development. Thus, it is desirable to further exploit simple and homogeneous LCR with CCP-based miRNA detection.

In this paper, we develop a novel approach for homogeneous detection of miRNA that makes full use of LCR and lambda exonuclease-assisted CCP biosensing technique. Lambda exonuclease is a highly processive exodeoxyribonuclease that selectively degrades the 5′-phosphorylated strand of double-stranded DNA (dsDNA) in 5′-3′ direction. The enzyme exhibits low activity on single-stranded DNA (ssDNA) and non-phosphorylated DNA.[28,29] To carry out homogeneous miRNA detection, lambda exonuclease is introduced to degrade the excess fluorescence-labeled 5′-phosphorylated probes after LCR to suppress the background signal. At the same time, fluorescein-labeled LCR products can not be degraded because the LCR products without 5′-phosphorylated modification are not the preferred substrates of lambda exonuclease. Eventually, the LCR products bind to CCPs through electrostatic interactions to initiate FRET. In this assay, only one lambda exonuclease is employed, and only fluorescein-labeled probe is required in each pair of probes in LCR, which reduce the complex probe modifications and the cost. The simple and sensitive method paves a new way for homogeneous miRNA detection and molecular diagnosis.

RESULTS AND DISSCUSSION

Figure 1 shows the principle of the homogeneous LCR and lambda exonuclease-assisted CCP biosensing technique for miRNA detection. Let-7a is chosen as the model miRNA. For LCR, two pair of probes, one pair of probes composed of X, Y and the other pair of probes composed of Xr, Yr, are designed and their sequences are shown in Table S1 in the Supporting Information. The sequences of let-7a at the 5′ and 3′ terminal are respectively complementary to the sequences at the 5′ terminal of probe Y and at the 3′terminal of probe X. First, after the hybridization at 39 °C between let-7a and probe X and Y, the probe X and Y are specifically ligated by T4 RNA ligase 2 to form ssDNA XY. Second, after thermal denaturation at 95 °C, the probe Xr and Yr, which are respectively complementary to probe X and Y, are added to the reaction solution and are ligated by thermostable ampligase at 65 °C to produce dsDNA XY/XrYr. Subsequently, by repeating the thermal cycles at 95 °C for 30 s and 65 °C for 30 s, the ssDNA XY and XrYr respectively serve as the templates to hybridize and ligate probe Xr and Yr, probe X and Y, resulting in exponential amplification of miRNA. As a result, a large amount of dsDNA XY/XrYr is produced by LCR. Thereafter, the excess and unreacted probes X and Xr, Y and Yr hybridize and form the 5′-phosphorylated dsDNA XXr and YYr, respectively, at 37 °C. By introduction of lambda exonuclease, which selectively degrades the 5′-phosphorylated strand of dsDNA, the dsDNA XXr and YYr with 3′-fluorescein modification are degraded to mononucleotides in a 5′-3′ direction. In contrast, the LCR products of the XY and XrYr can not be degraded because their 5′-non-phosphorylated sequences are not the preferred substrates of lambda exonuclease. Finally, by addition of CCP, positively charged CCP is close to the negatively charged fluorescein-labeled LCR products through strong electrostatic interaction, leading to efficient FRET from CCP to fluorescein. In contrast, the degraded mononucleotides by lambda exonuclease are far away from CCP because of the much weaker electrostatic interactions between mononucleotides and CCP. Accordingly, effective FRET from CCP to the fluorescein-labeled mononucleotides does not occur. As a result, it is possible to perform homogeneous miRNA detection via integration of LCR and lambda exonuclease-assisted CCP detection by monitoring FRET signal from CCP to fluorescein.

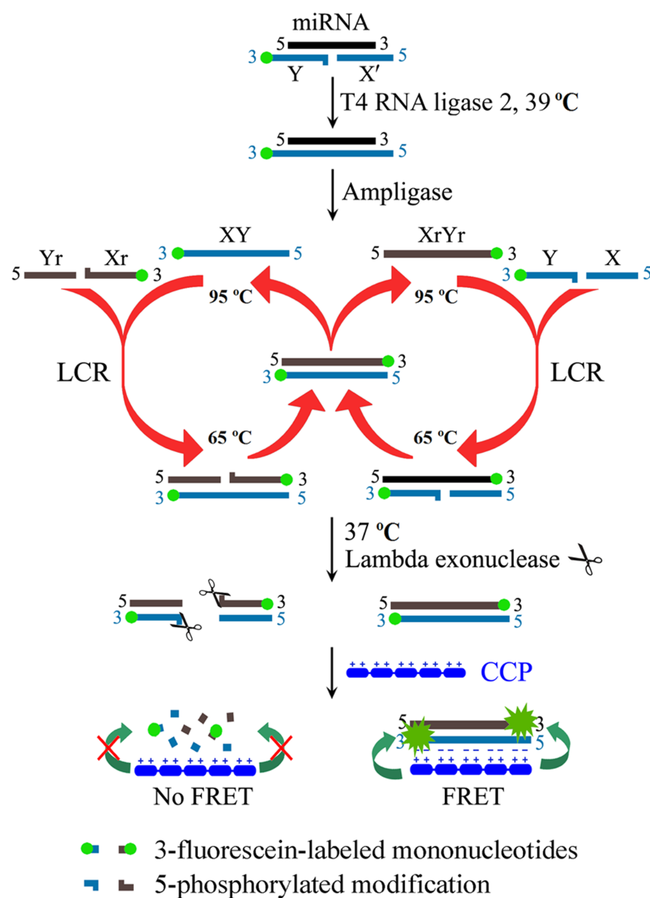

Figure 1. Principle of LCR and lambda exonuclease-assisted CCP biosensing for miRNA detection.

The homogeneous miRNA detection assay was further demonstrated by the fluorescence spectra of CCP and different LCR products with and without the treatment by lambda exonuclease. In a previous report, our group demonstrated that the probe X modified with two ribonucleotides at its 3′-terminus could greatly enhance the ligation efficiency of T4 RNA ligase 2 with probe Y phosphorylated at its 5′-terminus by using miRNA as the template.[12] Thus, in this assay, the probe X was modified with two ribonucleotides at its 3′-terminus, referred as probe X′, for ligating the probe Y with 5′-phosphorylated modification in the ligation reaction catalyzed by T4 RNA ligase 2. Figure 2 shows the fluorescence spectra of CCP, fluorescein, and LCR products in different assays. Without lambda exonuclease treatment, blank and let-7a gave rise to equal FRET from CCP to fluorescein because all of the ligated and unligated probes in LCR could interact with CCP through strong electrostatic force. In contrast, with lambda exonuclease digestion of LCR products, efficient FRET from CCP to fluorescein was significantly generated from let-7a, whereas only a limited FRET from CCP to fluorescein occurred from blank. These were attributed to the facts that no LCR took place in blank solution and the unligated probes were

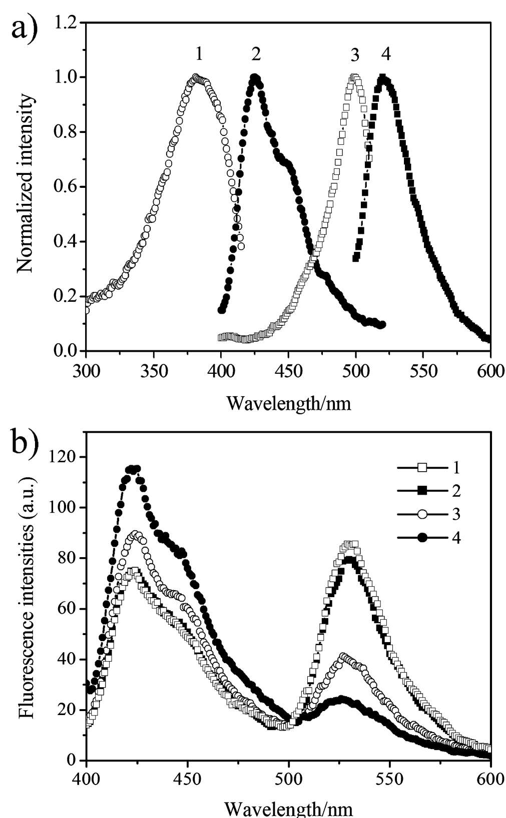

Figure 2. Fluorescence spectra of CCP, fluorescein and different LCR products. (a) Curves 1 and 2 are the exciation and emission spectra of CCP, respectively. Curves 3 and 4 are the exciation and emission spectra of fluorescein, respectively. The final concentration of fluorescein was 20 nM; CCP was 0.15 μM in RUs. (b) Curves 1 and 2 are the fluorescence spectra of LCR products of let-7a and blank without the treatment by lambda exonuclease, respectively. Curves 3 and 4 are the fluorescence spectra of LCR products of let-7a and blank with the treatment by lambda exonuclease, respectively. Fluorescence measurements were carried out as described in the Experimental Section in the Supporting Information. The final concentration of let-7a was 10 fM; CCP was 0.15 μM in RUs.

degraded to mononucleotides by lambda exonuclease, resulting in ineffective FRET from CCP to fluorescein. FRET efficiency, defined as the ratio of fluorescence intensity at 525 nm to that of at 420 nm ($I_{525\ \mathrm{nm}}/I_{420\ \mathrm{nm}}$), was used to measure the content of miRNA. As shown in Figure 2b, the significantly difference of the FRET efficiency from let-7a and blank indicates the good potential of this method for miRNA detection.

To demonstrate the specifity of the proposed stragety for miRNA detection, we selected four miRNAs including let-7a, let-7c, let-7f, and miR221. Although let-7a, let-7c, and let-7f come from let-7 family with one base difference, their expression levels are different and are closely associated with cell development and human cancer. Thus, to make great efforts to discriminate let-7 family miRNAs with similar sequences is of great importance in better understanding their biological functions. As shown in Figure 3, under the same experimental conditions, the relative FRET efficiencies for let-7c, let-7f and miR221 are approximately 3-fold, 13-fold, and 44-fold lower than that of let-7a, respectively. These results indicate the high specifity of our assay for miRNA detection. Furthermore, it is clearly observed that one mismatched base at the central region of let-7f provides high discrimination ability

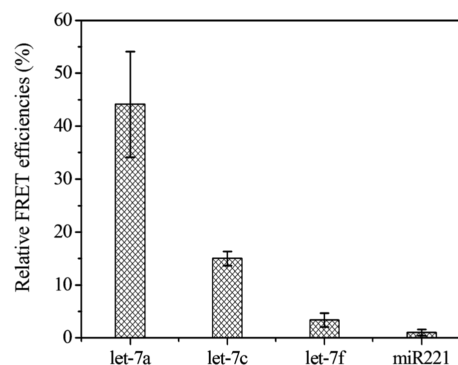

Figure 3. Comparison of the relative FRET efficiencies for let-7a, let-7c, let-7f, miR221. The ligation reactions catalyzed by T4 RNA ligase 2 was performed at 39 °C. The final concentrations of let-7a, let-7c, let-7f, miR221 were all 10 fM. Sequential detection was carried out as described in the Experimental Section in the Supporting Information. Error bars were estimated from three replicate measurements.

as compared with that of at the 3′-teminal region of let-7c, which suggests our proposed assay can be used to discriminate miRNA family members with high specificity.

Under the optimum experimental conditions, the dynamic range and sensitivity of the miRNA detection were investigated by a synthetic let-7a as target miRNA. Figure 4 shows the

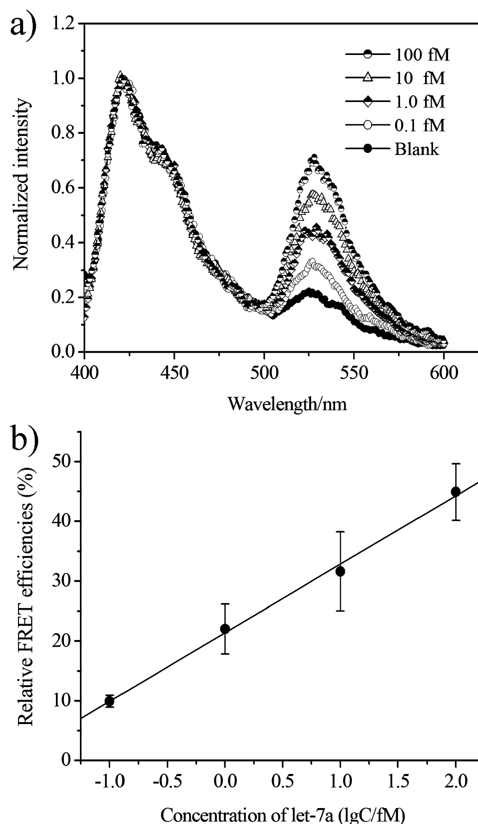

Figure 4. (a) Fluorescence spectra of solution containing CCP and LCR products of let-7a with varying concentrations (0, 0.1, 1, 10, and 100 fM). (b) Calibration curve of let-7a by using LCR with CCP-based detection. The relative FRET efficiency was the net intensity produced by let-7a, where the background signal had been subtracted for each value. Sequential detection was carried out as described in the Experimental Section in the Supporting Information. Error bars were estimated from three replicate measurements.

relationship between the relative FRET efficiencies and the concentration of let-7a. With the increase of the concentration of let-7a, the FRET efficiencies increased significantly. The assay had a wide dynamic range of 4 orders of magnitude with a limit of determination of 0.1 fM. There was a good linear correlation between the relative FRET efficiencies and the logarithm (lg) of let-7a concentration in the range of 0.1 fM to 100 fM. The correlation equation was $I_{525\ nm}/I_{420\ nm}$ = 21.48+11.57 lg(C/fM) and the correlation coefficient was 0.9983. The high sensitivity of the proposed method might be attributed to the two-step amplification from the LCR exponential amplification and CCP signal amplification.

To demonstrate the feasibility of the proposed method, let-7a in total RNA extracted from human lung was validated by the integration of LCR and CCP-based assay. In LCR, ribosomal RNA from *E. coli* was chosen as the control experiment due to the complex compositions of the human lung total RNA sample. Figure 5 shows that the FRET

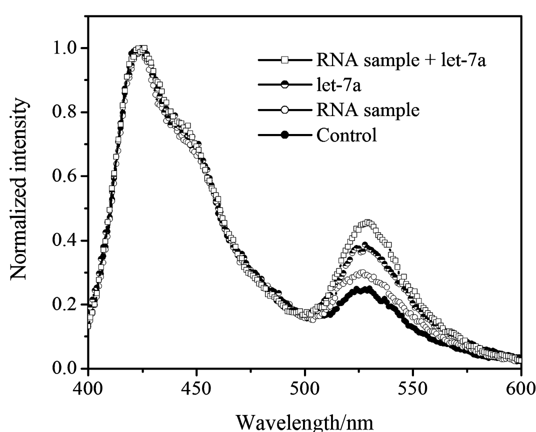

Figure 5. Fluorescence spectra of solution containing CCP and LCR products of human lung total RNA sample. In LCR, ribosomal RNA for control experiment was 20 ng; Human lung total RNA was 2 ng. The spiked synthetic let-7a in total RNA sample was 20 fg, which equaled 15 fM in 200 μL of reaction solution. Sequential detection was carried out as described in the Experimental Section in the Supporting Information.

efficiencies of the total RNA sample and synthetic let-7a increase significantly as compared with control experiment, respectively. Moreover, the FRET efficiency of the total RNA sample with addition of the synthetic let-7a is obviously higher than that of the total RNA sample or the synthetic let-7a used only. These results suggest that the miRNA from human lung total RNA sample is let-7a, indicating the feasibility of the LCR with CCP-based homogeneous assay for detection of miRNAs in real sample.

In conclusion, we have developed a homogeneous, highly sensitive and specific assay for detection of miRNA by integration of LCR and lambda exonuclease-assisted CCP biosensing. High sensitivity and specificity of the assay for miRNA detection is attributed to the high-efficiency ligase-based LCR and the signal amplificaiton ability of CCP. After LCR, a unique step of digestion of excess fluorescein-labeled probes by lambda exonuclease is introduced to eliminate background so as to achieve efficient FRET from CCP to fluorescein in a homogeneous fashion. The design of the probes in LCR is simple enough to match the requirement of lambda exonuclease. The method is simple, cost-effective, and paves a new way for homogeneous miRNA detection as well as molecular diagnosis.

■ ASSOCIATED CONTENT

Supporting Information

The sequences of the oligonucleotides and miRNAs used in this study, detailed experimental procedures, and optimization of the experimental conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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