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Integration of T7 exonuclease-triggered amplification and cationic conjugated polymer biosensing for highly sensitive detection of microRNA

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ABSTRACT

A novel and highly sensitive method for detection of microRNA (miRNA) was developed by integration of T7 exonuclease-triggered amplification and cationic conjugated polymer (CCP) biosensing. First, a fluorescein-labeled probe was designed with the complementary sequence to the target miRNA. When target miRNA was absent in the solution, the fluorescence probe interacted with CCP through the strong electrostatic interactions, leading to the highly efficient fluorescence resonance energy transfer (FRET) from CCP to fluorescein. In the presence of target miRNA, the probe hybridized with the miRNA to form DNA/miRNA duplex hybrids. Then, T7 exonuclease digested cyclically the fluorescence probes in hybrids and triggered the enzyme amplification reaction, generating a large number of single nucleotides. Owing to the weak electrostatic interaction between CCP and the single nucleotide, the FRET from CCP to fluorescein would not take place, which effectively reduced the background and significantly enhanced the sensitivity and the dynamic range of miRNA detection. The linear range of the assay was 0.2–100 pM and the detection limit 0.08 pM was 58 times lower than that of the endonuclease-based assay. The method is simple, cost-effective, and with no need for the sophisticated instrument, and has broad application prospects for miRNA detection and early diagnosis.

1. Introduction

MicroRNAs (miRNAs) are a class of endogenous and small non-coding RNAs (about 20–24 nucleotides), which regulate gene expression at the post-transcriptional level [1,2]. MiRNAs are closely related to the normal function of eukaryotic cells and the dysregulation of miRNA is associated with diseases. Thus, miRNAs have been attracted immense interest as new biomarkers for predicting and treating diseases, especially cancer [3–5]. MiRNA detection is important for studying the biological functions of miRNAs, diagnosis and treatment of human diseases [6,7].

Up to now, various methods have been developed for detection of miRNAs [8–11]. Due to the low expression level of miRNAs in tissues and cells, miRNA detection generally relies on the sensitive amplification techniques, mainly including polymerase chain reaction (PCR) [12,13], rolling circle amplification (RCA) [14–16], loop-mediated isothermal amplification (LAMP) [17], exponential amplification reaction (EXPAR) [18,19], and nuclease-triggered cyclic amplification (NCA) [20–22]. PCR is now the gold standard for miRNA detection that needs the sophisticated equipment for high-accuracy controlling the thermal cycle temperatures. RCA, LAMP and EXPAR as the isothermal amplification techniques based on the primer extension reaction are increasingly developed for detection of miRNA with robustness, simplicity, and specificity. However, because of the short sequences of miRNAs, it is difficult to directly fulfill the isothermal amplification reaction for miRNA detection. Therefore, the sequences of miRNAs were lengthened by adding the adaptor sequence in the 5′ and 3′ terminals of miRNA. Alternatively, the specific probes, such as padlock probe and stem-loop probe, etc., were particularly designed to hybridize with miRNA for performing the amplification reaction [14–17]. These resulted in complex probe design and multi-step experimental process.

The NCA is based on the digestion of the probes by the specific nucleases. In NCA-based miRNA detection, the nucleases possess the characteristics for degradation of the DNA probe in DNA/miRNA hybrid and the weak activity for the miRNA in DNA/miRNA hybrids and single-stranded DNA [20–22]. In the presence of target miRNA, the specific probe can hybridize with miRNA to form DNA/miRNA duplex hybrid and is subsequently digested by the specific nuclease, while target miRNA is released and recycled to trigger NCA. Thus, NCA is used for detection of miRNA in terms of its simple probe design and isothermal reaction process. Currently, duplex-specific nuclease (DSN)

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as an endonuclease has been widely used in the NCA-based detection of miRNA because of its preference for cleaving DNA in DNA/miRNA hybrid by comparison with single-stranded DNA. Moreover, TaqMan and molecular beacon probes were employed for combining with the DSN-based NCA for miRNA detection in a homogeneous form [20–22]. However, the TaqMan and molecular beacon probes are costly and these methods are difficult to differentiate miRNAs with high homology that differ by single-base differences. Recently, our group developed a homogeneous miRNA detection method by combining DSN-induced NCA with cationic conjugated polymer (CCP) material based on its excellent light-harvesting and signal amplification abilities [23–25]. By the CCP-based fluorescence resonance energy transfer (FRET) properties, miRNA can be specially detected with the high sensitivity and specificity in a homogeneous fashion. However, the degradation products of DSN are the small fragments of polynucleotides, which can interact with CCP also through the electrostatic interaction. Thus, the nonspecific FRET from CCP to the fluorophore is high in the DSN-based NCA, which limits the sensitivity of miRNA detection and the application of the method.

T7 exonuclease (T7 Exo), as a special exodeoxyribonuclease, was recently reported for miRNA detection based on its characteristic of specially digesting DNA probe in DNA/RNA hybrid to the single nucleotide in 5′–3′ direction, while it is not active on the single stranded RNA or DNA [26,27]. Owing to the weak interaction between CCP and the single nucleotide, the FRET from CCP to fluorescence dye labeled in the single nucleotide is lower than that of the polynucleotide fragments from DSN, thereby reducing the background. Herein, we develop a novel miRNA detection assay by combining T7 Exo-triggered NCA with CCP for sensitive detection of miRNA.

2. Material and methods

2.1. Materials and apparatus

T7 Exo was purchased from New England Biolabs (USA). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was obtained from Sigma. The cationic poly [(9,9-bis(6-N,N,N-trimethylammonium) hexyl) fluorenylene-phenylene dibromide] (PFP) used as the CCP in the FRET experiments was synthesized according to the procedure reported in the literature [28]. The synthetic oligonucleotides used in this assay were obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The sequences of the probes and miRNAs are listed in Table 1. All the oligonucleotides were HPLC-purified. All solutions were prepared in deionized and sterilized water. All other reagents were analytically pure grade without further purification.

The NCA reaction was performed in a 2720 thermal cycler (Applied Biosystems). A Hitachi F-4500 spectrofluorometer (Tokyo, Japan) was used to measure the fluorescence spectra.

Table 1
Sequences of probes and miRNAs (5′–3′) used in the experiments.

miRNAs and probes	Sequence[a]
Let-7a	UGAGGUAGUAGGUUGUAUAGUU
Let-7b	UGAGGUAGUAGGUUGU**G**U**G**GUU
Let-7c	UGAGGUAGUAGGUUGUAU**G**GUU
Let-7d	AGAGGUAGUAGGUUG**C**AUAGU
Let-7e	UGAGGUAG**G**AGGUUGUAUAGU
Let-7f	UGAGGUAGUAG**A**UUGUAUAGUU
Let-7g	UGAGGUAGUAG**U**UUGUA**C**AGUU
Let-7i	UGAGGUAGUAG**U**UUGU**GCU**GUU
Probe-7a	FAM-AACTATACAACCTACTACCTCA[b]

[a] The underlined bases in let-7 miRNAs family are that differ from those in let-7a.
[b] FAM is carboxyfluorescein.

2.2. T7 Exo-triggered NCA reaction

The T7 Exo-triggered NCA reaction was performed in a 10 μL mixture containing 1 × NEBuffer 4 (50 mM potassium acetate, 20 mM Tris-acetate (pH = 7.9), 10 mM magnesium acetate and 1 mM DTT), 50 nM probe-7a, 5 nM let-7a, 8 U Ribolock RNase Inhibitor. The reaction mixture was first heated at 65 °C for 3 min, and then annealed at 30 °C, and subsequently 2 μL T7 Exo (10 U/μL) was added into the reaction mixture. After that, the reaction mixture was incubated at 30 °C for 2 h for performing the NCA reaction in a 2720 thermal cycler.

2.3. CCP fluorescence measurement

After finishing the NCA reaction, 4 μL NCA products and 4 μL 7.5 μM PFP were diluted by 192 μL 25 mM HEPES to 200 μL. The mixture was incubated for 5 min and then to perform fluorescence measurement with the excitation wavelength at 380 nm. The spectra were recorded between 400 nm and 650 nm.

2.4. Real sample analysis

Human cervical carcinoma cell (HeLa cell) was cultured in DMEM medium supplemented with 10% FBS, and the cells were cultured at 37 °C in a humid atmosphere with 5% CO_2. Cells were collected in the exponential phase of growth and they were harvested for extraction at last. The extraction of miRNA samples according to the kit of RNAiso for Small RNA (TaKaRa, Dalian, China). Then the extracted sample was used to quantitative detection of sample analysis.

3. Results and discussion

3.1. Principle of the assay for miRNA detection

Scheme 1 shows the principle of T7 Exo-triggered NCA combining with the CCP biosensing for miRNA detection. The fluorescence-labeled probe is designed with the fully complementary sequence to the target miRNA. As shown in Scheme 1A, when target miRNA is absent in the reaction solution, the fluorescence-labeled probe itself cannot be digested by T7 Exo. Therefore, the probe can interact with CCP through the strong electrostatic interactions, leading to the highly efficient FRET from CCP to fluorophore. In the presence of miRNA, the probe hybridizes with the miRNA to form DNA/miRNA duplex hybrids (Scheme 1B), where after the fluorescence-labeled probe in hybrids can be digested to the single nucleotides in the 5′–3′ direction by T7 Exo. At the same time, the target miRNA is released and can bind to the new fluorescence-labeled probe again. Accordingly, the probe is cyclically digested by T7 Exo, generating a large number of single nucleotides. By introduction of CCP, unefficient FRET from CCP to fluorophore would be observed because of the weak electrostatic interaction between CCP and the single nucleotides. Thus, the miRNA can be detected on the basis of the FRET from CCP to fluorophore.

To verify the principle of the assay, we first selected let-7a as the model miRNA and designed the corresponding FAM-labeled probe (probe-7a) with the complementary sequence to let-7a. As shown in Fig. 1, by addition of CCP to the reaction solution containing probe-7a only, the fluorescence intensities at 423 nm and 526 nm respectively coming from CCP and FAM are considerable, suggesting that CCP binds to probe-7a through the strong electrostatic interaction and the efficient FRET from CCP to FAM occurs. In contrast, in the presence of target let-7a, the fluorescence intensity at 423 nm was remarkable and no fluorescence peak at 526 nm was observed. This is attributed to the fact that the probe-7a can hybridize with let-7a to form DNA/RNA hybrid duplex and be subsequently digested to the mononucleotides by T7 Exo. The mononucleotides with poor charge are far away from CCP, leading to unefficient FRET from CCP to FAM. As a result, the homogeneous miRNA detection can be performed by combining T7 Exo-triggered