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Dual signal amplification strategy for specific detection of Circulating microRNAs based on Thioflavin T

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ABSTRACT

Circulating microRNAs (c-miRNAs) are emerging as new non-invasive biomarkers for human cancers diagnosis and prognosis. Herein, in this study, an ideal biosensing system with highly sensitivity and excellent selectivity for rapid and facile detection of c-miRNAs has been developed. The sensing system mainly consists of two unlabeled hairpin probes (HP1 and HP2) and a circular probe. Upon binding with the target miRNA, HP1 is opened, which serves as a toehold to hybridize with HP2. Since HP1-HP2 duplex is more stable than HP1-miRNA duplex, the target miRNA can be displaced from HP1, and again binds with a new HP1 to initiate another reaction cycle. Moreover, the newly formed HP1-HP2 duplex can be further used as a primer to initiate rolling circle amplification (RCA) with the circular probe, producing extremely long single-stranded DNA molecules with repetitive sequence units which can form into a large number of G-quadruplexes. These G-quadruplexes can bind with ThT, resulting in a significantly enhanced fluorescent signal. This newly developed sensing system has great potential to be applied in biochemical research and clinical diagnosis based on the high-performance of circulating miR-21 detection in this study.

1. Introduction

microRNAs (miRNAs) are a class of short (18–24 nt), endogenous, non-coding RNA molecules that regulate the expression of over 60% target genes through sequence-specific hybridization to the 3′ untranslated region (UTR) of messenger RNAs [1,2]. It has been demonstrated that miRNAs are involved in various biological processes, such as cell proliferation, differentiation, stress resistance, and cell death [3–5]. There is now compelling evidence that miRNAs regulate all aspects of the so-called "hallmarks of cancer" that enable tumor growth and metastatic dissemination [6]. Recently the discovery of circulating microRNAs (c-miRNAs) in cancer patients holds great promise for the use of miRNAs as distinctive, non-invasive cancer biomarkers [7]. c-miRNAs are a group of miRNAs, which can be readily detected in plasma [8], serum or whole blood [9]. Despite some interesting and exciting findings, the field of c-miRNAs has been hindered by measurement-associated inconsistency and irreproducibility. Several traditional methods have been utilized for miRNAs detection, including northern blotting [10], microarrays [11], and real-time PCR (Q-PCR) [12,13]. However, these miRNAs detection techniques have irreparable limitations, such as low sensitivity, low selectivity, and labor-intensive steps. The unique characteristics of miRNAs, including their short length, low abundance, and sequence homology among the miRNAs family, also make them difficult to analyze [14]. In order to improve the sensitivity, specificity, and simplicity of miRNAs assay, a variety of new strategies have been developed, such as electrochemical sensors [15,16], immunosensors [17], nanopore sensors [18,19], and sequence-based amplification [20,21]. Among these methods, rolling circle amplification (RCA) has attracted much attention in miRNAs detection due to its sensitivity, good specificity and simplicity [22,23].

Rolling circle amplification (RCA) is an isothermal enzymatic process where a short DNA/RNA primer is amplified to form a long single stranded DNA/RNA using a circular DNA template and special DNA/RNA polymerases. The RCA product is a concatemer containing tens to hundreds of tandem repeats that are complementary to the circular template [24,25]. The power, simplicity, and versatility

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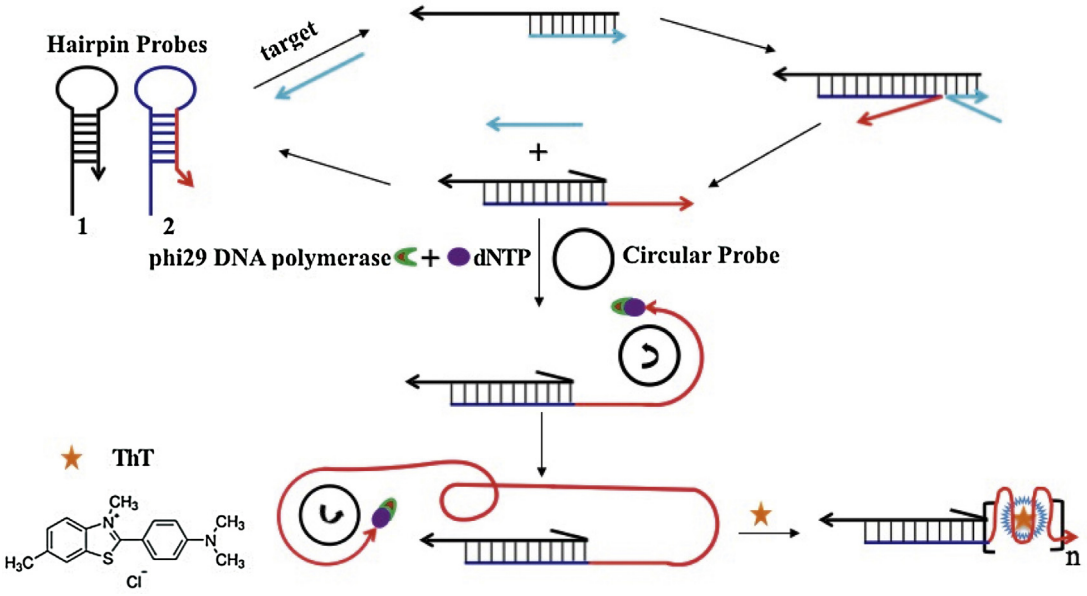
Table 1
Sequences of the oligonucleotides used in this study.[a]

Oligonucleotides	Sequence (from 5′ to 3′)
Hairpin Probe1 (HP1)	**TCAACATCAGTCTGATAAGCTA**CGATGTGTAGATAGCTTATCAGACT
Hairpin Probe2 (HP2)	**TAAGCTATCTACACATGGTAGCTTATCAGACT**CCATGTGTAGAGGT
Circular Probe (CP)	**TACACA**ATCTCGACTAGTCAGACCCTAACCCTAACCCTAACCCTACAACATGTCTTTGAT**ACCTC**
microRNA21 (miR-21)	UAGCU UAUCA GACUG AUGUU GA
Single-base mismatched miR-21 (M1)	UAGCU UAUCA GAC**C**G AUGUU GA
Two-bases mismatched miR-21 (M2)	UAGC**A** UAUCA GAC**C**G AUGUU GA
Three-bases mismatched miR-21 (M3)	UAGC**A A**AUCA GAC**C**G AUGUU GA
Four-bases mismatched miR-21 (M4)	**A**AGC**A A**AUCA GAC**C**G AUGUU GA
Five-bases mismatched miR-21 (M5)	**A**AGC**A A**AUCA GAC**C**G AUGU**C** GA
miR-21 with one additional base (A1)	***T***UAGC UUAUC AGACU GAUGU UGA
miR-21 with two additional bases (A2)	***TT***UAG CUUAU CAGAC UGAUG UUGA
miR-21 with three additional bases (A3)	***GTT***UA GCUUA UCAGA CUGAU GUUGA

[a] In HP1 and HP2, the underlined letters indicate the sequences complementary to each other to form the stems of the hairpin probes, respectively. In HP1, the boldface letters indicate the sequences complementary to the target miRNA (miR-21). In HP2, the boldface letters indicate the sequences complementary to HP1. In CP, the boldface letters indicate the sequences complementary to HP2, and the underlined letters indicate the sequences to form a G-quadruplex, which can bind with ThT and light up fluorescent signal. In M1, M2, M3, M4, M5, the underlined-boldface letters indicate the mutation sites of miR-21. In A1, A2, A3, the letters shown in bold italics indicate the additional bases of miR-21.



Scheme 1. Schematic illustration of dual signal amplification strategy for specific detection of circulating microRNAs based on Thioflavin T. (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)

of the nucleic acid amplification technique have made it an attractive tool for biomedical research [26,27] and nanobiotechnology [28]. Recently, target catalyzed hairpin assembly (CHA) was developed for DNA nanostructure organization and also as a promising signal amplification strategy for DNA detection on account of its significant advantages such as simple, cost-effective, isothermal and high sensitive [29,30]. Therefore, we examined the combination of these two amplification strategies (CHA-RCA) to detect c-miRNAs.

G-quadruplexes can be formed from guanine-rich DNA and RNA sequences, usually effectively induced by Na+/K+ [31,32], small molecules, or certain cationic dyes [33,34]. Recently, Mohanty et al. [35] reported for the first time that water-soluble Thioflavin T (ThT) was a G-quadruplex specific fluorescent indicator among other DNA forms including single-strand, duplexes or triplexes. What's more, it has a weakly fluorescence by itself, while exhibit great fluorescence enhancement upon binding with G-quadruplex DNA structure under physiological salt conditions. The special structural selectivity of ThT for G-quadruplexes may improve the specificity of sensing.

Spurred on by all the above findings, herein, for the first time, we have developed a simple, specific and label-free biosensing system that ingeniously combines CHA and RCA for rapid and facile detection of c-miRNAs. The dual signal amplification strategy not only improves the sensitivity of the biosensing system but also provides a new way for c-miRNAs detection. As proof of concept, the detection of miR-21 is demonstrated in this study. The assay does not involve any chemical modification, which is simple and cost-effective. With the significant dual signal amplifications, the detection limit of the newly developed sensing system is lower than previous reported ThT-based methods. Importantly, the sensing system offers high selectivity for the determination between perfectly matched miRNA and single-base mismatched miRNA. Moreover, this sensing system has a good detection performance in real biological samples, which holding a great potential for further applications in the clinical diagnosis of cancers.

2. Experiments

2.1. Chemicals and reagents

All of the chemicals and reagents used in this study were analytical grade and used without further purification. The