Analytical Methods

PAPER

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2014, **6**, 9477

An enzyme-free signal amplification strategy for sensitive detection of microRNA *via* catalyzed hairpin assembly

Zhenze Jiang,† Hui Wang,† Xiaobo Zhang, Chenghui Liu* and Zhengping Li

Received 13th September 2014
Accepted 14th October 2014

DOI: 10.1039/c4ay02142h

www.rsc.org/methods

In this work, a simple fluorescence turn-on assay is developed for a sensitive detection of microRNA (miRNA) through an enzyme-free signal amplification strategy. This design is based on the miRNA-catalyzed hairpin assembly (CHA). Two metastable hairpin DNA probes, one of which is double-labeled by a fluorophore/quencher pair with efficiently quenched fluorescence, are rationally designed to preclude unexpected hybridization events between themselves. Therefore, in the absence of miRNAs, the two types of hairpins could stably coexist in the solution with low background fluorescence because of the stringent design. When target miRNAs are added, they work as catalysts to trigger the self-assembly pathway of the two probes and initiate the cycling of CHA circuits, which results in greatly enhanced fluorescence signal. With the help of an efficient signal amplification of CHA and the low-background design of hairpin probes, at concentrations as low as 1 pM miRNA can be detected using this simple and low-cost protocol. High specificity and a wide dynamic range from 1 pM to 2 nM are also obtained. Therefore, this method may have great potential for miRNA-related biological studies.

Introduction

Since the identification of the first microRNA (miRNA), lin-14, numerous studies have been conducted on this 18–25 nt long noncoding RNA.[1] It has been revealed that miRNAs regulate several biological processes, including cell differentiation, proliferation and apoptosis, by regulating gene expression by mechanisms such as cleaving target mRNAs or inhibiting their translation.[2–5] Recent studies also show that abnormal levels of certain miRNAs are closely associated with the development of several diseases including cancers.[6–8] Therefore, sensitive detection of miRNA levels is essential for miRNA-targeted clinical diagnosis and drug development.[9]

Traditionally, northern blotting used to be the most widely utilized method for miRNA detection.[10–12] However, this protocol is time-consuming and requires a large amount of sample. Its sensitivity and specificity are also limited. Microarray-based methods have been quickly developed for miRNA analysis in the past few years.[13,14] However, the high expense and complicated procedures limit their wide application. In addition, they also suffer from low sensitivity due to small sample volume and cross-hybridization. Recently, enzyme-catalyzed nucleic acid amplification strategies have become more popular for miRNA detection. For instance, the RT-PCR method requires a little sample but performs with high sensitivity.[15,16] Nonetheless, the reverse transcription from miRNA to cDNA is rather difficult because of the small size of miRNA, which requires sophisticated sequence design. Although the application of isothermal amplification approaches, including rolling circle amplification (RCA), isothermal exponential amplification reaction (IEXPAR) and loop-meditated isothermal amplification (LAMP),[17–21] have further made great advances for miRNA analysis, most of these amplification strategies require the use of one or even more types of enzymes, which make the experiments complicated and costly. In this regard, enzyme-free signal amplification techniques are considered to be more attractive for miRNA analysis especially in complex biosamples.[22]

Recently, the research on DNA circuits based on the simple base-pairing rules has brought new ideas in nucleic acid amplification.[23–25] The entropy-driven catalyzed hairpin assembly (CHA) is one of the most versatile enzyme-free nucleic acid amplification strategies.[26–32] CHA typically requires a catalyst, usually single-stranded DNA (ssDNA), to trigger the reaction and requires a strand-exchange process to achieve the amplification. Pierce *et al.* have demonstrated that the DNA circuits can be adapted to ssDNA detection based on the target-initiated self-assembly of two metastable hairpin probes.[27,28] However, they also noticed that single-stranded DNA hairpins will form knots, links and, in particular, kissing hairpin complexes when complementary bases are exposed.[28] Because of the loop–loop kissing interaction between the complementary sequences of the two hairpin

Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an 710119, P.R. China. E-mail: liuch@snnu.edu.cn; Fax: +86 29 81530859

† Z. Jiang and H. Wang contributed equally to this work.

probes, a rather high background signal will be obtained, obviously decreasing the signal to noise ratio of this signal amplification assay. To solve this problem, Chen *et al.* made a few modifications in the sequence design of the hairpins to preclude such undesirable interactions.[29] They rationally designed two hairpin probes whose unpaired parts were not complementary with each other and successfully reduced the uncatalyzed reaction rate (from ~100 M^{-1} s^{-1} to <0.5 M^{-1} s^{-1}).[29,30] It should be noted that the design of such hairpins is rather stringent such that CHA is mainly preferable for the detection of short single-stranded nucleic acids. MiRNAs, the 18–25 nt RNA molecules, exactly meet such conditions. Thus, we believe the CHA-based amplification strategy will be more powerful and practical for miRNA analysis.

Herein, inspired by the pioneering design of Chen's group, we wish to report in this work a new fluorescence turn-on assay for a low-background detection of miRNA based on the miRNA-catalyzed CHA reaction between two types of rationally designed hairpin probes. This method offers several advantages. First, because CHA is simply driven by a base-pairing mechanism, it overcomes several drawbacks of enzyme-catalyzed reactions. Second, this method only requires an isothermal amplification, which largely simplifies the experiment procedures and cuts down on the expenses. Third, the elegantly designed hairpin DNA probes successfully avoid undesirable hybridization between themselves in the absence of miRNA catalyst. As a result, a low background is realized. Because of these distinct features of the proposed approach, miRNA can be clearly detected at concentrations as low as 1 pM, showing rather high sensitivity among the enzyme-free amplification assays for miRNA analysis.

Experimental

Materials and reagents

All of the nucleic acid molecules, including miRNAs and hairpin DNA probes, were custom synthesized by TaKaRa Bio Inc. (Dalian China), and the detailed sequences are listed below. Saline sodium phosphate (SSPE) buffer (20×, 200 mM phosphate buffer containing 3 M NaCl and 20 mM EDTA, pH 7.4) was purchased from Shanghai Sangon Biotechnology Co. Ltd. All the solutions used for miRNA analysis were prepared with RNAse-free water, which was obtained from TaKaRa Bio Inc. (Dalian China).

Detailed nucleic acids sequences (5′ → 3′) used in this work:

Probe 1. *FAM*-TAGGTTG(2*)-TATAGTT(3*)-CCATGTGTAGA(4*)-AACTATA(3)-CAACCTA(2)-*BHQ*-CTACCTCA(1)

Probe 2. CCATGTGTAGA(4*)-TAGGTTG(2*)-TATAGTT(3*)-TCTACACATGG(4)-AACTATA(3)

Let-7a. UGAGGUAG(1*)-UAGGUUG(2*)-UAUAGUU(3*)

Let-7f. UGAGGUAGUAGAUUGUAUAGUU

Let-7g. UGAGGUAGUAGUUUGUACAGU

Let-7i. UGAGGUAGUAGUUUGUGCUGU

Mir-143. GGUGCAGUGCUGCAUCUCUGGU

Mir-181. CAUCCCUUGCAUGGUGGAGGG

Standard procedures of the CHA-based miRNA assay

Probe 1 (P1) and Probe 2 (P2) were separately refolded in 5× SSPE (50 mM phosphate buffer containing 750 mM NaCl, pH 7.4) before use according to the following process (94 °C for 5 min, 80 °C for 5 min, 75 °C for 10 min, 70 °C for 10 min, 65 °C for 10 min, 60 °C for 5 min, 25 °C for 20 min). Typically, 50 nM of P1, 100 nM of P2 and serial dilutions of miRNA target were mixed in a final 150 μL of 4× SSPE buffer solution (40 mM phosphate buffer containing 600 mM NaCl, pH 7.4). The mixture was incubated at 35 °C for 2 h. Then fluorescence measurements were obtained on a LS-55 fluorescence spectrophotometer (PerkinElmer, USA) with a λ_{Ex} of 480 nm.

Results and discussion

Principle of the CHA-based MiRNA assay

Fig. 1 illustrates the working principle of the CHA-based miRNA assay. Let-7a is selected as a proof-of-concept target miRNA in this study. According to the sequence of let-7a, two metastable stem-loop DNA hairpins (P1 and P2) are rationally designed, which can stably coexist in the solution in the absence of let-7a catalyst. Domain 1, 2 and 3 sequences of P1 are designed to be complementary to let-7a. The 5′-end of P1 is labeled with fluorescein (FAM), and its complementary nucleotide on the stem is labeled with BHQ to efficiently quench the fluorescence of FAM. Domain 4 is a bit longer than the other domains to ensure the inherent stability of P2. Domain 4 contains 11 nt, whereas Domain 3 only contains 7 nt. Thus, the hairpin structure of P2 will form a 4–4* stem region instead of a 3–3* stem region. Noticeably, P1 and P2 here do not contain any unpaired domains that are complementary to each other. Therefore, the spontaneous hybridization of the two hairpins is efficiently prevented, which ensures an extremely low fluorescence background.[29] Domain 1, the 3′-terminal sticky end of P1, acts as the toehold to trigger the CHA reaction in the presence of let-7a

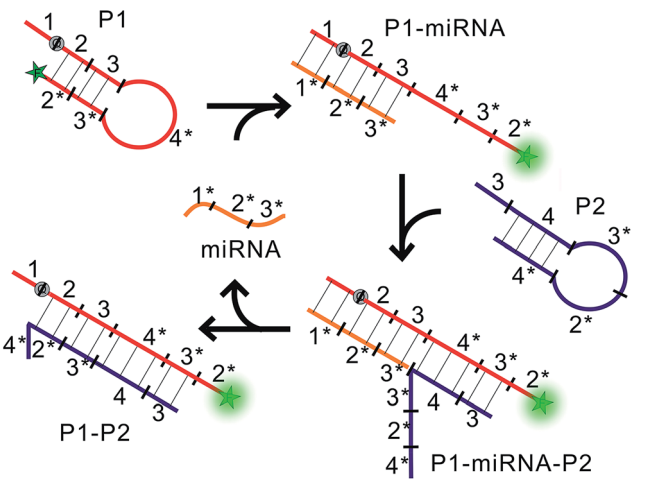

Fig. 1 Schematic illustration of the CHA-based miRNA assay for the detection of let-7a. Domains are marked by numbers and their complementary parts are denoted by asterisks. Q represents for quencher (BHQ) and F represents for fluorophore (FAM).

catalyst. Let-7a will first hybridize with the toehold (Domain 1) of P1 and open the hairpin through an unbiased strand–displacement interaction, which leads to strongly recovered fluorescence of FAM. Moreover, the newly released sticky Domain 3* of P1 will further hybridize with the exposed 3′ sticky sequence of P2 (Domain 3) to initiate another toehold-mediated strand displacement reaction, which displaces let-7a into the solution again. Then, the released let-7a may become the catalyst again to trigger another round of CHA. In this manner, each let-7a molecule can specifically trigger various cycles of self-assembly between P1 and P2, which will lead to greatly amplified fluorescence. Therefore, by recording the enhanced fluorescence signal, sensitive detection of let-7a can be realized.

Optimization of experimental conditions

First, as is known to us, the ionic strength will influence both the hybridization of nucleic acids and the stability of hairpin structures. In order to reconcile this conflict, the effect of ionic strength on the performance of the CHA-based miRNA assay was investigated at room temperature (25 °C) by varying the concentration of NaCl in phosphate buffer (pH 7.4). The results are shown in Fig. 2. It can be observed that when the Na^+ concentration increases from 200 to 600 mM, the fluorescence signal produced by 100 pM let-7a tends to increase slowly. Afterward, the fluorescence response will decrease again when further increasing the Na^+ from 600 to 800 mM probably due to the elevated Tm value of hairpin probes at high ionic strength. Moreover, one can also observe in Fig. 2 that the ionic strength only shows negligible influence on the fluorescence of blank controls without let-7a. Therefore, 4× SSPE buffer (40 mM phosphate buffer containing 600 mM NaCl, pH 7.4) is selected as the reaction media for this method.

Second, high reaction temperature will accelerate the let-7a-catalyzed CHA reaction between the hairpin probes. Nonetheless, enhanced temperature will also disrupt the metastable states between the two stem-loop hairpins, which may lead to undesirable high background. Therefore, we further optimized the reaction temperature by testing temperatures from 25 °C to 50 °C, at intervals of 5 °C. As shown in Fig. 3a, both the fluorescence signals produced by 100 pM let-7a and the blank control increase gradually with increasing reaction temperature. The highest ratio of the fluorescence produced by let-7a to that by the blank control (denoted as *S*/*N*) is obtained at 35 °C (Fig. 3b). Thus, 35 °C is used as the optimal temperature in subsequent experiments.

Third, according to the design principle shown in Fig. 1, a high P2/P1 ratio may also be favorable for accelerating the signal output of the proposed miRNA assay. The chance of probes meeting each other will certainly increase if more P2 is added. This will lead to the augmentation of signal output but also at the risk of increasing background signal. Therefore, the effect of P2/P1 ratio was studied by testing different P2 concentrations of 50 nM, 100 nM, 150 nM and 200 nM with a P1 concentration fixed at 50 nM. As shown in Fig. 4, the signals produced by let-7a and the blank control both gradually increase, whereas the *S*/*N* ratio reaches the maximum with a P2 concentration of 100 nM. Therefore, the combination of 50 nM P1/100 nM P2 is used for subsequent analytical purposes.

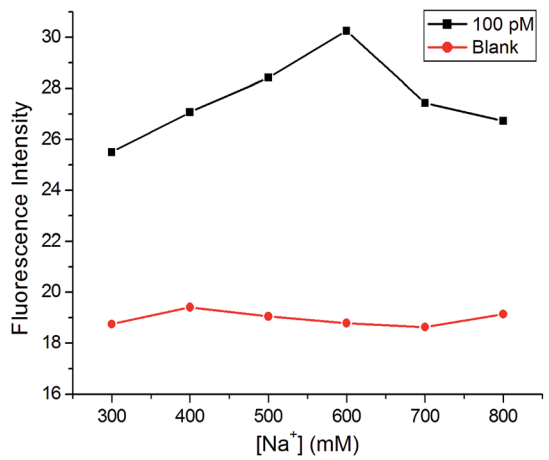

Fig. 2 Optimization of NaCl concentration. Experimental conditions: P1, 50 nM; P2, 50 nM; let-7a, 100 pM; CHA reaction was performed at 25 °C for 2 h.

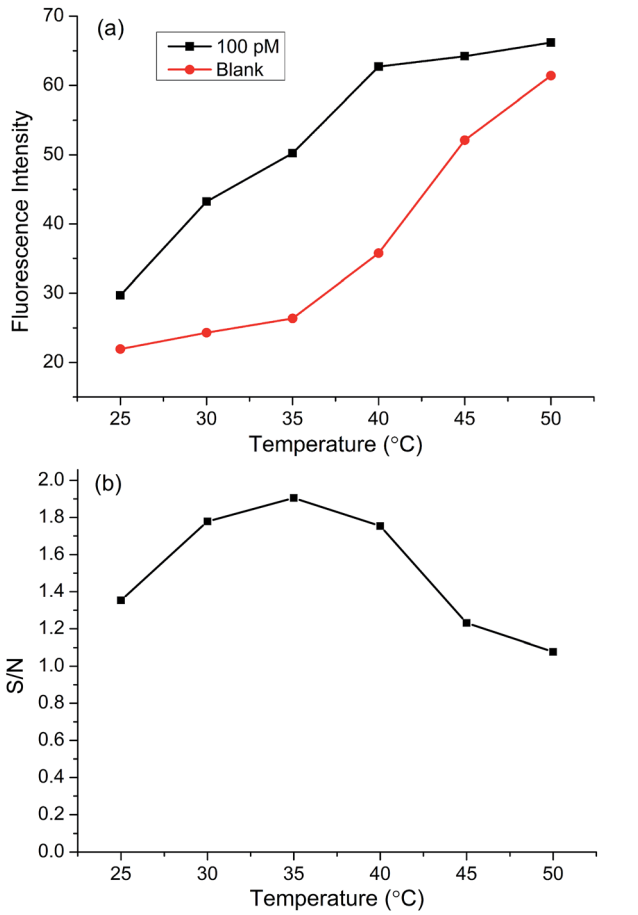

Fig. 3 Optimization of the reaction temperature. Experimental conditions: P1, 50 nM; P2, 50 nM; let-7a, 100 pM; CHA reaction was performed in 4× SSPE at different temperature for 2 h. *S*/*N* refers to the ratio of the fluorescence signal produced by 100 pM let-7a to that by the blank control.

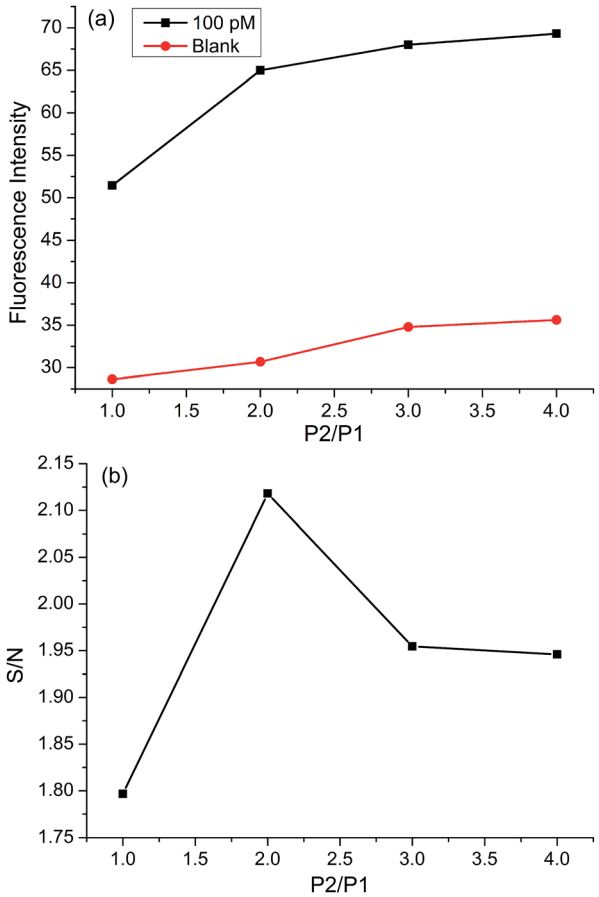

Fig. 4 Effect of P2/P1 ratios on the proposed assay. P1, 50 nM; let-7a, 100 pM; CHA reaction was performed in 4× SSPE at 35 °C for 2 h. *S/N* refers to the ratio of the fluorescence signal produced by 100 pM let-7a to that by the blank control.

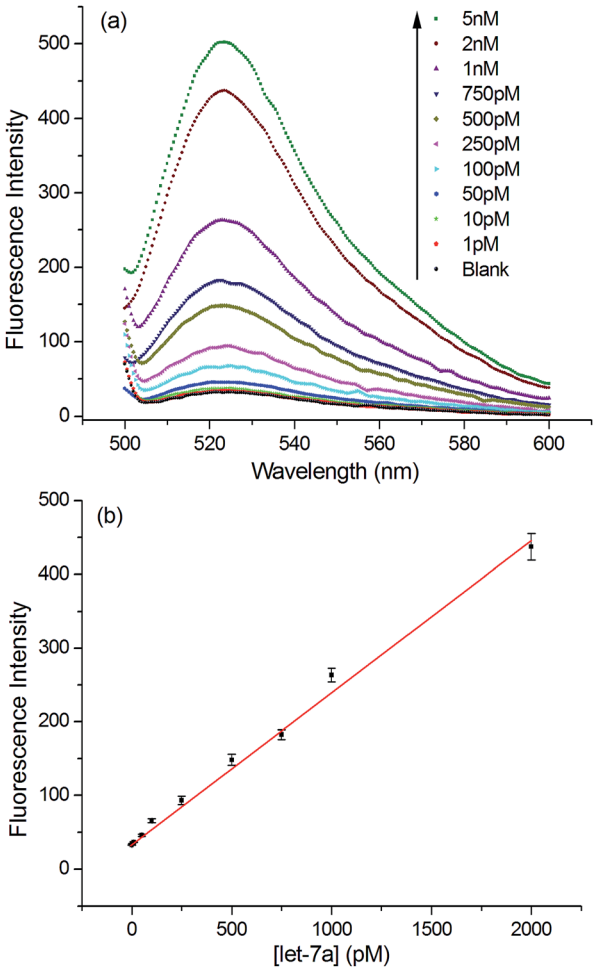

Fig. 5 (a) Fluorescence spectra of the CHA-based assay in the presence of varying concentrations of let-7a. (b) Relationship between the fluorescence intensity recorded at 523 nm and let-7a concentration. Error bars were calculated from three repetitive measurements.

Analytical performance of the CHA-based assay for let-7a analysis

Under the optimized experimental conditions mentioned above, the analytical performance of this CHA-based assay for the detection of let-7a was investigated. Fig. 5a exhibits the fluorescence responses of different let-7a concentrations by the use of the proposed CHA strategy. It is observed that the fluorescence signals gradually increase as the concentrations of let-7a vary from 1 pM to 5 nM. The dependence of the fluorescence intensities recorded at 523 nm on let-7a concentrations is plotted in Fig. 5b. It can be observed that the concentration as little as 1 pM let-7a can be clearly discriminated from the blank control, and the fluorescence intensities (I_F) are linearly proportional to the let-7a concentrations in the range of 1 pM to 2 nM. Correspondingly, the linear regression equations are $I_F = 0.20\ C_{let\text{-}7a} + 38.91$ ($R^2 = 0.9942$). It is worth noting that, although this CHA-based protocol is simple and cost-effective, the sensitivity is superior or at least comparable to that of many enzyme-catalyzed signal amplification assays for the detection of let-7a (with a detection limit typically in the range of 0.4–60 pM).[33–35]

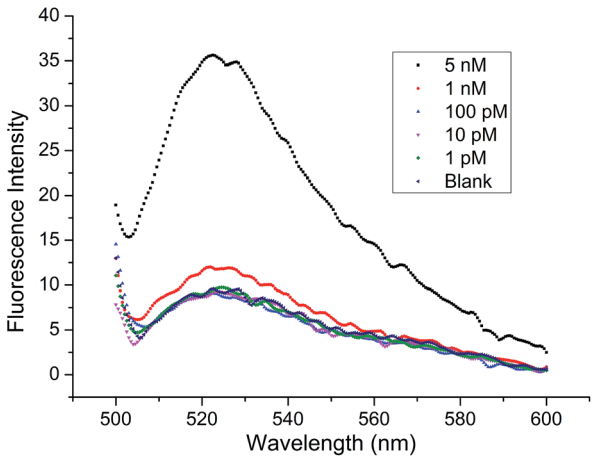

Fig. 6 Fluorescence spectra of the reaction system using only P1 as the probe in the presence of varying concentrations of let-7a. P1 concentration: 50 nM.

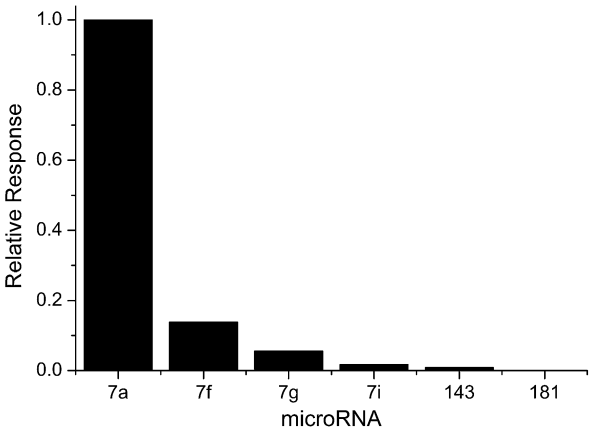

Fig. 7 Specificity evaluation for the CHA-based miRNA assay. All miRNAs are controlled at the same concentration of 100 pM and the let-7a-produced fluorescence response is normalized to be 1.

To verify that the fluorescence responses are indeed the result of let-7a-triggered CHA cycling but not from the one-step reaction with only P1, additional experiments were carried out using only P1 as the hairpin probe, which is similar to the traditional design of molecular beacons. As shown in Fig. 6, in such cases, let-7a can be detected only when its concentration is higher than 1 nM, indicating that the sensitivity is about 3 orders of magnitude lower than that of our CHA-based pathway. These results clearly suggest that without the P2-assisted CHA cycling, one let-7a molecule can only open a single P1 probe to recover the fluorescence of one FAM molecule, and thus the sensitivity is relatively low. In contrast, for the CHA-based assay, each let-7a molecule can catalyze numerous cycles for the opening of P1, which efficiently results in amplified fluorescence signal, and thus significantly improved sensitivity.

Specificity evaluation of the proposed MiRNA assay

The specificity of the proposed miRNA assay was further investigated. Two random miRNAs (mir-143 and mir-181) and several let-7 family members (let-7f, let-7g, and let-7i) were selected for the specificity evaluation. As can be seen from Fig. 7, only let-7a induces a great fluorescence signal, whereas the other miRNA molecules fail to generate significant fluorescence responses. It is worth noting that there are only 1-base, 3-base and 5-base differences between let-7a and let-7f, let-7g, and let-7i, respectively. These highly homologous sequences produce only 13.86% (let-7f), 5.60% (let-7g), and 1.76% (let-7i) nonspecific signals, suggesting that the CHA-based assay can clearly discriminate a one-base difference among the miRNA targets with rather high specificity.

Conclusions

In conclusion, an isothermal and enzyme-free signal amplification strategy for miRNA detection is developed in this work based on the target-catalyzed self-assembly of two elegantly designed hairpin probes. By rationally designing the unpaired domains of two hairpins to not be complementary to each other, background signal originating from the spontaneous interaction between the two hairpin probes is efficiently suppressed. With the help of an efficient signal amplification of CHA and the low-background design of hairpin probes, concentrations of let-7a as low as 1 pM can be detected using this simple and low-cost protocol, and the sensitivity is about 3 orders of magnitude higher than that of traditional molecular beacon-based assay without the CHA amplification. Therefore, due to the simple enzyme-free and isothermal characteristics, this proposed design provides a new method for the versatile, low-cost and sensitive detection of miRNAs in the field of biological research and clinical diagnosis.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (21335005, 91127035 and 21105020), the Natural Science Foundation of Shaanxi Province (2014JQ2058) and the Fundamental Research Funds for the Central Universities (GK201402051, GK201303003).

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