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A hyperbranched transcription-activated CRISPR-Cas12a signal amplification strategy for sensitive microRNA sensing

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A CRISPR-Cas12a-based strategy is developed towards sensitive microRNA sensing, in which the hyperbranched rolling circle amplification and transcription are integrated for the activation of the *trans*-cleavage ability of Cas12a. The integrated triplex signal amplification endows the strategy with low background and high sensitivity. This design expands the scope of CRISPR-Cas-based sensing toolbox and shows great potential in the biological and biomedical studies.

The different expression levels of microRNAs (miRNAs) in normal tissues, benign tumors and malignant tumors make them important biomarkers for cancer diagnosis and prognosis evaluation,[1, 2] which highlights the urgency and importance of developing highly sensitive and specific miRNA assays.

The CRISPR-Cas system (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein) as an adaptive immune system of prokaryote has been widely used as a revolutionary tool for gene editing in eukaryotic organisms.[3-5] The mature CRISPR RNA (crRNA) produced by precursor crRNA (pre-crRNA) processing is essential to form an active CRISPR effector complex.[6] The commonly employed Cas9 gene editing system utilizes additional host-expressed RNA endonuclease for crRNA maturation,[7] while differently, Cas12a is capable of shearing crRNAs by itself due to its inherent RNase activity.[8]

Furthermore, superior to Cas9, after assembly with crRNAs, Cas12a displays a unique collateral *trans*-cleavage property.[9, 10] Namely, upon specific binding to the target sequences, the *trans*-cleavage ability of the Cas12a-crRNA complex will be activated to non-specifically cleave single-stranded DNA (ssDNA) in the surrounding. Taking benefits of this extraordinary property, many Cas nuclease-based methods have been developed for nucleic acids detection.[11-23] However, all of these methods need the preparation of Cas12a-crRNA complex in advance and the use of pre-amplification steps such as RPA,[12] PCR[15] etc. to amplify nucleic acid targets, which limits their application in detecting quite short sequences or discriminating single base variations in the target sequence. Besides, the requirement of protospacer-adjacent motif (PAM) recognizing sequence also restricts the targeting capabilities of Cas12a-crRNA in double-strand targets.

Recently, our group has proposed a new CRISPR sensing mechanism based on Cas12a self-powered crRNA processing from the rolling circle transcription (RCT) products, which broadens the scalability of CRISPR-Cas system for nucleic acids analysis.[24] Herein, we wish to further advance the new mechanism of Cas12a self-powered processing/recruiting of crRNA for ultrasensitive miRNA detection by integrating triplex cascading amplification including hyperbranched rolling circle amplification (HRCA), T7 transcription amplification and Cas12a-based amplification reaction (denoted as Cas-TCA).

This new Cas-TCA strategy is schematically illustrated in Fig. 1, where miR-21 is selected as a model target. The padlock probe is composed of three regions, a crRNA-direct repeat sequence (the Cas binding motif, denoted as DR, yellow), a T7 promoter sequence (blue), and a target-complementary sequence (red). When binding with miR-21 (Fig. 1a), the 5′-end and 3′-end sequences of the padlock probe become adjacent and are ligated under the action of SplintR ligase. In the presence of phi29 DNA polymerase, the RCA reaction is initiated by using circularized padlock probe as the template and miRNA as the primer, producing a long tandem DNA sequence. The 21* primers, the complementary sequence of miR-21 sequence, can hybridize to the complementary sequences in different regions of the RCA products and reversely extend along them. Subsequently, the extending of backward primer can displace the forward extended primers

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with the assistance of phi29 DNA polymerase. As a result, a lot of single-stranded DNA (ssDNA) and double-stranded (dsDNA) with various lengths will be produced. In the meantime, the newly released ssDNA in turn serve as the template for T7* primer binding to trigger a new cycle of multiple-site hybridization, primer-extension, and strand-displacement events. In this way, a larger number of dsDNA/ssDNA mixture molecules are further produced. As such, each ssDNA product will serve as the template to initiate the next round of extension and displacement reaction until they are completely consumed, forming the first amplification step of the highly efficient hyperbranched RCA. Afterward, as illustrated in Fig. 1b, the abundant dsDNA products all contain numerous repeat T7 promoter sequences, which can independently initiate the transcription amplification by virtue of T7 RNA polymerase, producing numerous repeated RNA transcripts (the pre-crRNAs) that contain alternating spacer and direct repeat sequences, forming the second step of transcription amplification. Then, as illustrated in Fig. 1c, the Cas12a will recognize the direct repeat sequences of the pre-crRNA and trims its 5′-end, generating mature crRNA which can assemble with Cas12a to recognize the dsDNA activators and activate the collateral cleavage activity of Cas12a. As a result, the fluorophore/quencher-labeled ssDNA reporter will be cleaved with a high efficiency (turnover rate ~1250 per second),[11] leading to the third round of signal amplification accompanying with remarkable fluorescence enhancement. In contrast, if miR-21 is absent, the ligation reaction of padlock probe cannot take place, thus, no Cas-TCA as well as the fluorescence enhancement will be triggered at all.

The Cas-TCA strategy possesses a series of advantages. First, superior to the conventional RCA, HRCA is of a continuously expending process. It accumulates a large number of dsDNA until the ssDNA products are completely consumed, which greatly increases the amplification efficiency. Second, the newly formed dsDNA structures all contain multiple T7 promoter sites for T7 RNA polymerase amplification. The integration of multi-targeted transcription mechanism and HRCA allows for the successive formation of pre-crRNA in abundance. Third, as to the traditional Cas12a-based methods, the pre-synthesis and purification steps have to be performed to obtain crRNA. However, the incomplete purification of crRNA may increase the risk of the nonspecific background. In this research, crRNA is generated by the target-responsive amplification and further processed/recruited by Cas12a, without the involvement of any pre-synthesis and purification steps. Therefore, the triplex cascading signal amplification constituted by the high *trans*-cleavage activity Cas12a-crRNA and the successive HRCA/T7 transcription process ensures the high sensitivity of Cas-TCA, enabling sensitive detection of as low as 10 zmol miRNA.

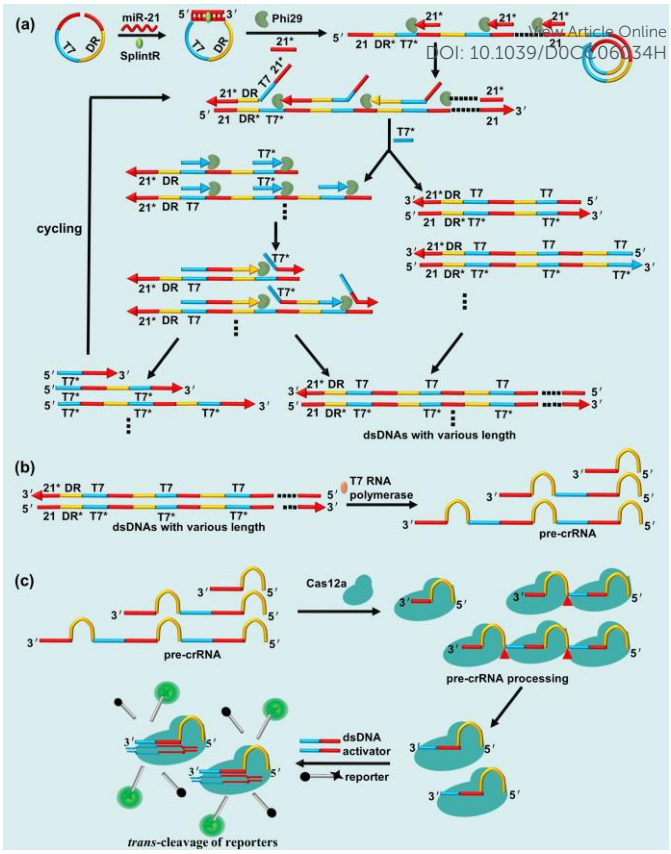


Fig. 1 Schematic illustration of the Cas-TCA strategy for miRNA detection.

Whether the Cas-TCA design is specifically responsive to miR-21 was first investigated to verify its feasibility. The results (Fig. 2a) showed that only in the presence of miR-21 and all of the involved enzymes (SplintR ligase, phi29 DNA polymerase, T7 RNA polymerase and Cas12a) could the significantly enhanced fluorescence signal be observed. However, even in the existence of miRNA target but without any of these enzymes, no fluorescent signal could be aroused, suggesting that all these materials were necessary in this work. It is also noteworthy that only trivial fluorescence signal was recorded in the negative control group (without target miR-21).

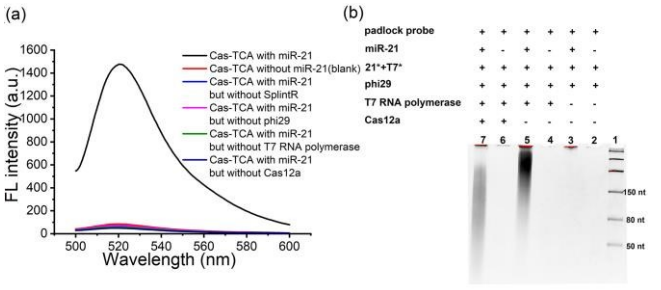


Fig. 2 Evaluating the feasibility of the Cas-TCA assay for miRNA detection. (a) Fluorescence spectra of the Cas-TCA system obtained with different reaction components for miR-21 analysis. The concentration of miR-21 is 300 zmol. (b) dPAGE analysis of the amplification products under different conditions. Lane 1: ssRNA ladder; Lane 2: HRCA products without miR-21; Lane 3: HRCA products with miR-21 target (8 fmol); Lane 4: T7 transcription products without miR-21; Lane 5: T7 transcription products with miR-21 target (8 fmol); Lane 6: Cas12a-based crRNA processing products without miR-21. Lane 7: Cas12a-based crRNA processing products with miR-21 target (8 fmol).

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The principle of the Cas-TCA design was also validated by the denaturing polyacrylamide gel electrophoresis (dPAGE) results. As illustrated in Fig. 2b, in the presence of the mixture of padlock probe, miR-21, 21*, T7* and phi29 DNA polymerase, a clear DNA band appeared at the top of the gel (lane 3), indicating the occurrence of HRCA amplification. With the addition of T7 RNA polymerase, a broad smearing band was observed (lane 5), clearly proved that HRCA products can act as the dsDNA transcription template to produce numerous pre-crRNAs with various lengths. Moreover, the pre-crRNAs were processed when Cas12a was further employed (lane 7). In contrast, in the negative control groups that without introducing miR-21 (lane 2, lane 4 and lane 6), no DNA bands can be observable. All of these results clearly confirm the feasibility of the Cas-TCA for specific miRNA sensing.

Towards the specific and sensitive detection of miR-21, the reaction conditions including the dosages of phi29 DNA polymerase, T7 RNA polymerase, Cas12a, and cleavage time of Cas12a were systematically optimized (ESI). Under the optimized conditions, the fluorescence responses of the Cas-TCA system against different concentrations of miR-21 were investigated. As shown in Fig. 3a, the fluorescence signal increased gradually with the increasing dosage of miR-21. Notably, as low as 10 zmol miR-21 can be accurately discriminated from the blank control. As displayed in Fig. 3b, the fluorescence intensities at 520 nm were linearly proportional to the concentrations of miR-21 in the range of 10 zmol to 1000 zmol. The correlation equation is $F = 4.52\ A_{\text{miR-21}}$ (zmol) + 28.15 with a correlation coefficient (R^2) of 0.9924, where F represents the fluorescence intensity (at 520 nm) and A is the amount of target miR-21 (zmol). As shown in Table S2, the sensitivity of the Cas-TCA is much higher than, or at least comparable to the previously reported RCA, T7 transcription or CRISPR-Cas-based miRNA sensing mechanisms. The significantly enhanced sensitivity may decide by: (1) the use of HRCA, which enables the production of numerous dsDNA for triggering multiple T7 transcription and subsequent Cas12a-based reaction, resulting in improved amplification efficiency; (2) the use of highly specific SplintR ligase,[25] which endows the method with a low background.

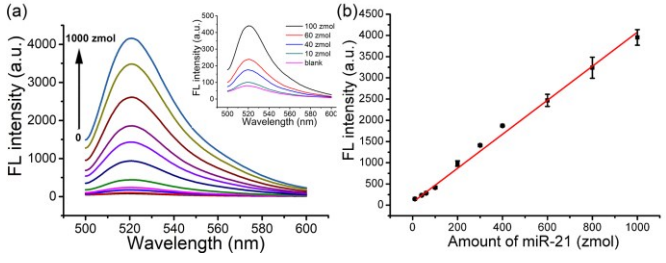


Fig. 3 (a) Fluorescence spectra of the Cas-TCA system for the detection of different concentrations of miR-21. From bottom to top, the concentrations of miR-21 are 0 (blank), 10, 40, 60, 100, 200, 300, 400, 600, 800 and 1000 zmol, respectively. (b) Correlation of the fluorescence intensities (520 nm) to the concentrations of miRNA. The error bars represent the standard deviation of three parallel measurements for each data point.

According to the principle of Cas-TCA illustrated in Fig. 1, HRCA is the basis of the subsequent efficient amplification reactions. To testify the crucial role of HRCA, Cas-TCA system was performed without adding T7* primer. In this way, only the reverse extension and the displacement of 21* primer can be conduct without the ssDNA product recycling, resulting in the low efficiency of yielding the dsDNA with T7 promoter. As can be seen from Fig. 4a, the standard Cas-TCA protocol with the 21*/T7* primers pair in HRCA can detect as low as 10 zmol miRNA. In sharp contrast, in the control group without adding T7* primer (Fig. 4b), only when the concentration of miR-21 was higher than 1 amol, can the fluorescence signal be distinguished from the blank control, showing a ~2 orders of magnitude decreased sensitivity. These results demonstrate the critical role of HRCA in the triplex signal amplification strategy.

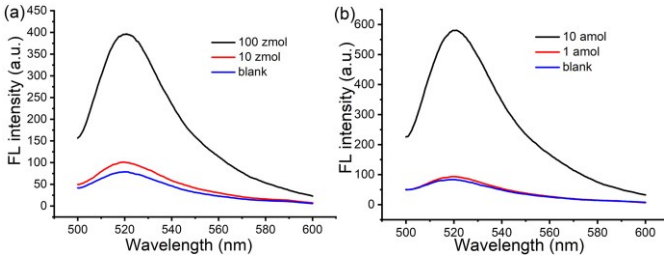


Fig. 4 Evaluation the effect of the HRCA on the amplification efficiency of Cas-TCA. (a) The fluorescence spectra produced by different concentration of miR-21 according to the standard Cas-TCA protocol. (b) The florescence spectra produced by different concentration of miR-21 according to the Cas-TCA but without T7* primer. Other conditions are the same as described in the experimental section.

For the detection of target miRNA in complex biological samples, the specificity of the assay is also crucial. Here, the specificity of the proposed assay for miR-21 was interrogated by detecting different kinds of miRNA species and a synthetic sequence with only a single base difference to miR-21 (named miR-21-1). As displayed in Fig. 5, a strong fluorescence signal could be aroused only by miR-21, whereas other miRNA sequences including miR-21-1 all generated negligible responses which were almost identical to the background signal. The reason why miR-21-1 produces little interference signals is that its mismatched base is located at the exact ligation site. All of these results suggest that the proposed Cas-TCA assay is of high specificity.

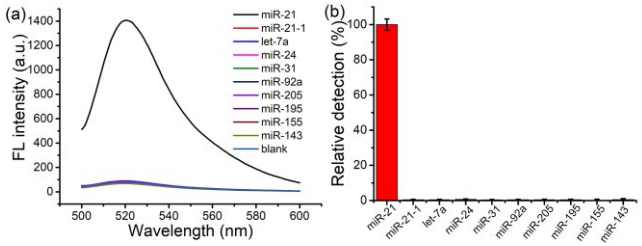


Fig. 5 Specificity evaluation of the Cas-TCA for miRNA analysis. (a) Fluorescence responses aroused by different kinds of miRNA species. (b) Relative fluorescence responses of other miRNA species compared with that of miR-21. The relative detection refers to the ratio of the fluorescence response induced by the other miRNA species to that induced by miR-21 (normalized to be 100%). The concentration of all different miRNA is 300 zmol. The error bars represent the standard deviation of three parallel measurements for each data point.

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The application of the Cas-TCA for miRNA analysis in complex cell extracts was then investigated. As displayed in Fig. S2, the amount of miR-21 in 50 pg total small RNA extracted from MCF-7 cells was determined to be 895 zmol. The reliability of the Cas-TCA assay was further validated by using the conventional stem-loop RT-PCR. According to the RT-PCR protocol, the miR-21 level in 50 pg total small RNA was calculated to be 995 zmol, which is in line with the Cas-TCA assay.

In summary, we have established a Cas12a-based triplex cascading amplification assay for the sensitive detection of miRNA. This Cas-TCA is rationally fabricated by integrating HRCA/T7 transcription-based amplification for the target-responsive pre-crRNA generation and Cas12a for self-powered crRNA processing/recruiting and the activated *trans*-cleavage amplification. This elegant cascading amplification design rationally transduces the target recognition events into remarkably enhanced fluorescence signals, endowing the assay ultrahigh sensitivity that has push the detection limit of miRNA down to 10 zmol. This Cas-TCA expands the scope of CRISPR-Cas-based sensing toolbox, showing great potential in designing new Cas-based sensing system as well as in the biological and biomedical studies.

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Conflicts of interest

There are no conflicts of interest to declare.

Notes and references

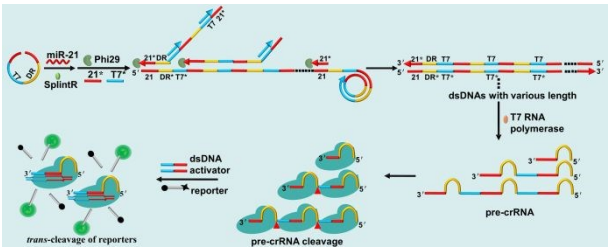
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