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Enzyme-free dual-amplification strategy for the rapid, single-step detection of nucleic acids based on hybridization chain reaction initiated entropy-driven circuit reaction

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ABSTRACT

Developing robust, isothermal and simple nucleic acids detection strategies is of great significance to clinical diagnosis and bioanalysis. Recently, hybridization chain reaction has attracted intense interest owing to its isothermal reaction conditions, enzyme-free nature, and simple sequence design. However, strategies based on HCR alone may not offer satisfactory sensitivity when trace targets are required to be analyzed. Here, we developed an enzyme-free and isothermal dual-amplification sensing strategy for the rapid and one-step detection of nucleic acids based on hybridization chain reaction and entropy-driven circuit reaction (HCR-EDCR). The target DNA initiates upstream HCR to assemble polymeric double stranded DNA (dsDNA) nanowires composed of many tandem trigger units that motivate downstream EDCR to continuously liberate report strand, resulting in the generation of an amplified fluorescence readout signal. This isothermal and homogenous strategy exhibits high sensitivity and good selectivity with a limit of detection of 87 fM, without the involvement of any enzymes. The whole dual-amplification can be completed with one-step in 45 min. Moreover, the developed HCR-EDCR approach can be easily adapted for detecting different analytes just by substituting the target-specific sequence. Therefore, this cascaded strategy may offer a new promising model for various diagnostic and biological analysis.

1. Introduction

Detection of nucleic acids is of great significance to clinical diagnosis and bioanalysis. However, the abundance of nucleic acids is always low in clinical sample. Thus, exploration of the multiplexing amplification strategy is still highly desirable for sensitive detection of nucleic acids. In recent years, the isothermal amplified biosensing strategies, including strand displacement amplification (SDA) [1], rolling circle amplification (RCA) [2], duplex-specific nuclease (DSN) amplification [3], nicking endonuclease (NEase) assisted amplification [4], and loop-mediated isothermal amplification (LAMP) [5,6], have attracted substantial research interests owing to its fast response, high amplification efficiency, and low cost [7]. These strategies are conducted at constant temperature without thermocycling required in polymerase chain reaction (PCR). However, these enzyme-based amplification techniques may be unsatisfactory because of the harsh experimental conditions and unstable enzyme activities.

To avoid the above deficiencies, many non-enzymatic nucleic acid circuits have been used for isothermal signal amplification. For example, the catalyzed hairpin assembly (CHA) [8], entropy-driven circuit reaction (EDCR) [9], and hybridization chain reaction (HCR) [10,11] have been adapted to bioassay. HCR is a nucleic acid self-assembly process, where the initiator triggers a cascade of hybridization events between two species of DNA hairpins to generate long-nicked double-stranded DNA (dsDNA) nanowires. Given the advantages of enzyme-free nature, efficient isothermal amplification, mild experimental conditions, and structural flexibility, HCR has been extensively used for the sensitive and specific detection of various analytes, including nucleic acids [12], proteins [13], and tumor cells etc [14]. However, HCR amplification strategies alone are usually not sensitive enough when analytes only exist at extremely low concentrations [15,16]. For highly sensitive detection of nucleic acids, various enzyme-

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based amplification strategies are advisably integrated to HCR, for instance, SDA [17], RCA [18], DSN [19], and NEase [20]. Despite the improved sensitivity, these cascaded amplified methods require multi type of enzymes and separating steps with initially enzymatic amplification followed by HCR reaction. In addition, enzyme-based strategies often suffer from false positives during amplification process [21,22]. Recently, the cascaded enzyme-free circuits, including HCR − HCR, DNAzyme − HCR, and CHA − HCR, have been successfully constructed for the amplified detection of analytes [23–27]. However, these methods suffer from either long reaction time, or circuit leakage that may results in relatively high background signals. Therefore, integration of HCR with enzyme-free isothermal strategies to construct rapid and robust assay platforms is still highly desirable.

The EDCR introduced by Zhang and co-workers is one of the most promising strategies owing to the exclusive entropy-driven force [28]. This amplification strategy employs a series of single-stranded linear DNA molecules to play multiple roles in the catalytic circuit via toehold-mediated strand displacement reaction. Based on fluorescent monitoring or electrochemical measurement, the enzyme-free EDCR strategy is programmed to detect nucleic acids, showing excellent thermo-stability, minimized background noise, low detection limit, and robust resistance to complex environment [29–31].

In this work, a novel enzyme-free isothermal signal amplification method integrating HCR-EDCR is constructed for highly sensitive and selective detection of nucleic acids. The target DNA initiates the successive cross-opening of two DNA hairpins (H1 and H2), generating polymeric dsDNA nanowires consisting of the numerous tandem reconstituted triggers (STs). Then, the STs bind to downstream the terminal of the three-stranded DNA complex QPR. With the help of fuel strand, the ST is cyclically reused, and many reporter strands are released, leading to a significantly amplified fluorescence readout signal. The enzyme-free HCR-EDCR circuit with synergistic amplification performance is proved to be a simple, rapid and robust sensing method for detection of nucleic acids. Moreover, it is a versatile sensing platform for highly sensitive and selective determination of other important biomarkers, through a simple and convenient 'plug-and-play' fashion.

2. Experimental section

2.1. Materials and reagents

The oligonucleotides (Table S1) were obtained from Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). GoldView was purchased from SBS Genetech (Beijing, China). Salmon sperm DNA was purchased from Sigma-Aldrich (St. Louis, MO, USA). 20-bp DNA ladders were purchased from TaKaRa (Dalian, China). All oligonucleotides were dissolved in TE buffer (pH 8.0, 10 mM Tris-Acetate, 1 mM EDTA) to give the stock solutions, which were diluted in appropriate buffer prior to use. The reaction buffer contained 10 mM Tris (pH 7.0), 480 mM NaCl and 5 mM $MgCl_2$. All the chemicals were of analytical grade and used without further purification. All solutions were prepared with ultrapure water (Millipore, Milford, MA).

2.2. Probe preparation

Hairpin H1 and H2 were heated to 95 °C for 5 min, and slowly cooled down to room temperature. The three-strand DNA complex probes were prepared by annealing the mixture of the quencher strand (Q, 2.5 μM), byproduct strand (2.5 μM) and report strand (2.5 μM) in 1 × TAE/Mg^{2+} buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0, with 12.5 mM $MgCl_2$ added) at 90 °C for 5 min, then cooled down to room temperature. The obtained DNA solutions were stored at 4 °C for further use.

2.3. HCR-EDCR cascade reaction

The HCR-EDCR cascade reaction was carried out by mixing 25 μL H1, 25 μL H2, 25 μL three-strand complex, 25 μL fuel strand and varying concentrations of target DNA in reaction buffer to give a total volume of 100 μL, and the final concentrations of H1, H2, three-strand complex, fuel strand were 0.25 μM. The reaction mixtures were incubated at 37 °C for 45 min.

2.4. Fluorescence measurement

A Cary Eclipse Fluorescence Spectrophotometer (Agilent, California) and quartz fluorescence cell with an optical path length of 1.0 cm were used to conduct fluorescence measurement. The emission spectra were recorded from 490 to 600 nm under excitation wavelength of 480 nm in steps of 1 nm at room temperature. The excitation and emission slit widths were set at 5 nm. The maximum fluorescence emission intensity was obtained at 518 nm. Prior to each measurement, the cuvette was rinsed 5 times in distilled water, 3 times in 70% ethanol, another 6 times in distilled water and finally dried with nitrogen.

2.5. Nondenaturing polyacrylamide gel electrophoresis (PAGE)

The native PAGE was performed on DYY-6C electrophoresis analyzer (Liuyi Instrument Company, China) with 6% native polyacrylamide gel in 1 × TBE buffer (pH 8.3, 89 mM Tris − boric acid, 2 mM EDTA) at 110 V for 40 min. The gel was then imaged on a Biorad ChemDoc XRS (Bio-Rad, U.S.A.) after GoldView staining for 30 min.

3. Result and discussion

3.1. Principle of HCR-EDCR operation

The principle of the enzyme-free HCR-EDCR strategy for highly sensitive detection of nucleic acids is illustrated in Scheme 1. The dual-amplification sensing strategy is composed of two different DNA hairpin structures H1 and H2, a fuel strand F, and a three-strand DNA complex QPR. The three-strand DNA complex is formed through hybridization of a reporter strand R with a FAM modification at the 5′-end, a quencher strand Q with a BHQ1 modification at the 3′-end, and a byproduct strand P, resulting in the quenching of FAM fluorescence. H1 includes the sequence a–b complementary to the target DNA. H2 is further elongated with two isolated DNA segments c and d at its 5′- and 3′-ends, respectively. In the presence of target DNA, H1 is opened through the toehold-mediated strand displacement reaction. The newly exposed sticky sequences in H1 hybridize with the e segment in H2 to unfold H2. Then, the newly released sticky sequences in H2, in turn, hybridize with domain b in another H1. An autonomous cross-assembling of hairpins H1 and H2 can bring the separated segments c and d into close proximity, leading to the assembly of dsDNA nanowires and the formation of tandem adjacent regions c and d namely reconstituted trigger (ST). The ST binds to three-strand complex QPR and releases a byproduct P while simultaneously exposing a single stranded toehold in the intermediate I. The single stranded toehold provides an initiation site for reaction between the intermediate I and the fuel strand F. Then, the fuel strand F binds to the intermediate I and displaces both the ST and a report stranded R, leading to recovery of fluorescent. Subsequently, the released ST can react with other molecules of three-strand complex QPR and engages in multiple turnover events, resulting in a significantly amplified fluorescent signal for highly sensitive detection of target DNA.

3.2. Validation of the dual-amplification strategy in each module

To ensure proper operation of the HCR-EDCR strategy, the circuit in