Talanta 143 (2015) 101–106

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Hairpin assembly circuit-based fluorescence cooperative amplification strategy for enzyme-free and label-free detection of small molecule

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ARTICLE INFO

Article history:
Received 10 March 2015
Received in revised form
16 May 2015
Accepted 25 May 2015
Available online 27 May 2015

Keywords:
Hairpin assembly circuit
Fluorescence cooperative amplification
Enzyme-free
Label-free
Small molecule

ABSTRACT

Here, we developed an enzyme-free, label-free, and sensitive fluorescence cooperative amplification strategy based on a hairpin assembly circuit which coupled catalytic hairpin assembly (CHA) with hybridization chain reaction (HCR) for small molecule adenosine. A double-strand DNA probe with aptamer-catalysis strand (Apt-C) and inhibit strand (Inh) was designed for adenosine recognition and signal transduction which was named as Apt-C/Inh. Hairpins H1 and H2 were employed for constructing the CHA, and hairpins H3 and H4 for the HCR. Through the binding of adenosine and the Apt-C, the Inh was released from the Apt-C/Inh. Then the free Apt-C initiated the CHA through successively opening H1 and H2, generating H1/H2 complex and recyclable Apt-C. Next, the released Apt-C entered another CHA cycle, and the H1/H2 complex further initiated the HCR of H3 and H4 which induced the formation of the concatemers of H3/H4 complex. Such a process brought the two ends of hairpins H3 into close proximity, yielding numerous integrated G-quadruplexes which were initially sequestered in the stem and two terminals of H3. Finally, N-methyl mesoporphyrin IX (NMM) was added to generate an enhanced fluorescence signal. In the proposed strategy, driven only by the energy from hybridization, one target could trigger multiple HCR events via CHA-based target-cycle, leading to a remarkable enzyme-free amplification for adenosine. The detection limit could achieve as low as 9.7×10^{-7} mol L^{-1}. Furthermore, G-quadruplexes were applied to construct label-free hairpin assembly circuit, which made it more simple and cost-effective. The satisfactory recoveries were obtained when detecting adenosine in spiked human serum and urine samples, demonstrating the feasibility of this detection strategy in biological samples.

1. Introduction

Detection and quantification of small molecules such as bisphnol A, mycotoxin, adenosine and the corresponding derivatives have played essential roles in environmental analysis, food safety and clinical diagnosis [1–3]. Research progress in the detection of small molecules have been achieved by colligating several transducing techniques, including electrochemical, optical, and mass-sensitive transducer [4–7]. Among them, fluorescence methods have been widely adopted because of high sensitivity, rapid analysis with spatial resolution, and little proclivity to sample or cell damage [8]. Several fluorescence sensing systems for small molecules in which aptamers act as recognition elements have been successfully implemented due to the unique natures of aptamers, the high affinity and specificity [9,10]. Despite the simple detection achieved, a poor detection limit is often obtained owing to the ratio 1:1 between the target input and the signal output [11]. For this reason, it is essential to develop a target recognition scheme capable of amplifying signal readout for small molecules detection.

Several enzyme-mediated amplification strategies have been introduced to amplify the aptamer/target interaction including exonuclease III digestion [12], rolling circle amplification (RCA) [13] and nicking endonuclease assisted signal amplification [14]. Although these enzyme-mediated assays have made significant progress towards the improvement of sensitivity, the protein enzyme is sensitive towards circumstance and easy to be inactive, which may disturb the signal amplification capability resulting in the problem of the poor repeatability. Enzyme-free DNA circuits [15,16] that rely on toehold-mediated DNA strand displacement to

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http://dx.doi.org/10.1016/j.talanta.2015.05.072
0039-9140/

achieve signal amplification have been increasingly used as sensing elements in bioanalysis. Owing to the simple hairpin design algorithms and high amplification efficiency, the catalytic hairpin assembly (CHA) [17] and the hybridization chain reaction (HCR) [18,19] have enabled enzyme-free amplified strategies to detect small molecules [20,21]. However, most of these sensing systems always require labels to obtain detectable signals and the labeling process requires stringent purification steps or leads to the reduced bioaffinity [22]. As an excellent label-free signal output, G-quadruplex structure has drawn an ever-growing interest in various bioanalysis [23,24]. Specially, G-quadruplex structure has been used for HCR to construct label-free signal amplified biosensors [25,26]. However, one copy of the initiators can only trigger an HCR event to yield nicked double helices, thus limit the detection sensitivity of biomolecules.

Herein, a hairpin assembly circuit-based label-free fluorescence strategy was designed by integrating CHA and HCR, in which one target triggered multiple HCR events by CHA-based target-cycle, thus effectively improving the detection sensitivity of target molecules. We take adenosine as the model analyte which is an endogenous nucleoside and a powerful potent vasodilator. It is able to control the contraction of vascular smooth muscle in brain and eye [27–29], modulate the release of neurotransmitters [30], and be used as the treatment and diagnosis agent of tachyarrhthmias and ischemia. Hence, selective and sensitive detection of adenosine is valuable for medicinal development and clinical diagnosis. In this work, the binding event of aptamer-catalysis strand and adenosine directly catalyzes the self-assembly of the hairpins H1 and H2, realizing initial target-cycle amplification. And the heteroduplex of H1 and H2 further promotes the self-assembly of H3 and H4, ultimately leading to an enzyme-free cooperative amplification owing to the integration of CHA and HCR. Functional G-quadruplexes are designed to form at the end of H3/H4 complexes after self-assembly. N-methyl mesoporphyrin IX (NMM), which has a pronounced structural selectivity for G-quadruplex, is added to obtain label-free fluorescence signal [31]. It is demonstrated that hairpin assembly circuit is a reliable and effective signal amplifier and the detection limit for adenosine is achieved as low as 9.7×10^{-7} mol L^{-1}. Notably, the label-free strategy eliminates the requirement for fluorescent labeling, making the system more simple and cost-effective.

2. Materials and methods

2.1. Materials

All of the DNA samples used in the study were synthesized and purified by Invitrogen Biotechnology Co. Ltd. (Beijing, China), and their sequences were given in Table S1 and S2. N-Methyl Mesoporphyrin IX (NMM) was obtained from Frontier Scientific Inc. (Logan, Utah, USA). Adenosine was obtained from Sigma-Aldrich (St. Louis, MO, USA). Cytidine, uridine and Guanosine were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). All the other chemicals were of analytical grade and used without further purification. Human serum and urine samples were provided by Qilu Hospital of Shandong University. Unless otherwise stated, all solutions were prepared in ultrapure water. NMM stock solution was prepared with dimethyl sulfoxide (DMSO) and stored at −20 °C. All experiments were performed in TNaK buffer (20 mM Tris, pH 7.5, 600 mM NaCl, 5 mM KCl).

2.2. The fluorescence measurements procedure

All fluorescence measurements were carried out on a Hitachi F-2500 spectrometer (Japan). The excitation wavelength was set at 399 nm and the emission spectrum was collected from 550 to 680 nm, and the PMT detector voltage was set at 700 V. Before experiment, all hairpins and the mixture of Apt-C and Inh were heated at 90 °C for 5 min and then gradually cooled down to room temperature in order to form the stable hairpin configuration and Apt-C/Inh duplex. In a typical experiment, 5 μL Apt-C/Inh and 5 μL adenosine were first incubated at 37 °C for 1 h. Then 5 μL H1 and 5 μL H2 were added. After 30 min at 37 °C, 5 μL H3, 5 μL H4 and 10 μL TNaK buffer were added into the mixture and incubated at 37 °C for 45 min. At last, 2 μL NMM and 8 μL KCl was added, and kept at 37 °C for 30 min before the fluorescence detection.

2.3. The nondenaturing polyacrylamide gel electrophoresis procedure

Firstly, adenosine with Apt-C/Inh was incubated at 37 °C for 1 h. Then H1 and H2 were added, and incubated at 37 °C for 24 h. Meanwhile, H1, H2, H1/H2 and H1/H2/Apt-C/Inh were also incubated for 24 h. 10 μL of each sample and 2 μL 6 × loading buffer were mixed together. The gels were prepared with 1 × TAE /Mg buffer (40 mM Tris, pH 8.0, 2 mM EDTA · Na_2, 20 mM acetic acid, 12.5 mM $(CH_3COO)_2Mg \cdot 4H_2O$). After stained with ethidium bromide for 5 min, the gel was imaged with UV imaging system (Bio-RAD Laboratories Inc. USA).

3. Results and discussion

3.1. The principle of the hairpin assembly circuits-based fluorescence cooperative amplification strategy

In this study, based on the hairpin assembly circuit, a novel enzyme-free and sensitive fluorescence strategy for adenosine was developed which combined the CHA with HCR. The principle is depicted in Scheme 1. In this strategy, it consists of aptamer-catalysis strand (Apt-C), inhibit strand (Inh), and the hairpins (H1 and H2 for CHA, H3 and H4 for HCR). G-quadruplex segments were sequestrated in the stem and two terminals of H3 (in red). When H3 is in its hairpin configuration, intact G-quadruplex can not form due to two reasons: the block of stem and the steric hindrance from the two terminals. Without adenosine, Apt-C is inactivated by hybridizing with Inh and thus the metastable hairpins can coexist in solution. The binding of adenosine and its aptamer makes Apt-C/Inh less stable and release Inh strand. Then the free Apt-C as a trigger strand opens H1 by toehold-mediated strand displacement, forming an Apt-C/H1 intermediate. In the Apt-C/H1, the trigger domain is no longer occluded and can bind with the H2, again initiating a branch migration process to form a H1/H2/Apt-C complex. This complex is inherently unstable, and Apt-C dissociates from the H1/H2 complex, completing the CHA reaction and allowing Apt-C to act as a catalyst to trigger the hybridization of additional pairs of H1 and H2 hairpins. Simultaneously, the H1/H2 complex with the overhung 5' terminal can further trigger the cross opening of H3 and H4, resulting in the close proximity of two ends of adjacent hairpins H3. Thus, the G-quadruplex subunits self-assemble into numerous integrated G-quadruplex structures on the formed nicked double helices by HCR. Finally, NMM is added for the label-free fluorescence signal. In summary, a single adenosine can generate several H1/H2 complexes which further trigger assembly of H3 and H4, leading to the cooperative amplification of the signal output.

3.2. Verification of the feasibility by fluorescence spectra characteristics

To verify the feasibility of the present strategy, we investigated the fluorescence emission spectra under different conditions, as