Talanta 179 (2018) 51–56

Contents lists available at ScienceDirect

Talanta

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

Integrating DNA structure switch with branched hairpins for the detection of uracil-DNA glycosylase activity and inhibitor screening

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ARTICLE INFO

Keywords:
DNA structure switch
Fluorescent amplification
Uracil-DNA glycosylase
Quick response
High sensitivity

ABSTRACT

The detection of uracil-DNA glycosylase (UDG) activity is pivotal for its biochemical studies and the development of drugs for UDG-related diseases. Here, we explored an integrated DNA structure switch for high sensitive detection of UDG activity. The DNA structure switch containing two branched hairpins was employed to recognize UDG enzyme and generate fluorescent signal. Under the action of UDG, one branched hairpin was impelled folding into a close conformation after the excision of the single uracil. This reconfigured hairpin could immediately initiate the polymerization/nicking amplification reaction of another branched hairpin accompanying with the release of numerous G-quadruplexes (G4s). In the absence of UDG, the DNA structure switch kept its original configuration, and thus the subsequent polymerization/nicking reaction was inhibited, resulting in the release of few G4 strands. In this work, Thioflavin T was used as signal reporter to target G4s. By integrating the DNA structure switch, the quick response and high sensitivity for UDG determination was achieved and a low detection limit of 0.0001 U/mL was obtained, which was superior to the most fluorescent methods for UDG assay. The repeatability of the as-proposed strategy was demonstrated under the concentration of 0.02 U/mL and 0.002 U/mL, the relative standard deviation obtained from 5 successive samples were 1.7% and 2.8%, respectively. The integrated DNA structure switch strategy proposed here has the potential application for the study of mechanism and function of UDG enzyme and the screening the inhibitors as potential drugs and biochemical tools.

1. Introduction

Repair of DNA lesions are essential for organisms to block mutagenesis of the genome and maintain the integrity of their genetic information [1,2]. The base excision repair (BER) pathway acts throughout the cell cycle to surmount the deleterious effects of DNA damage. DNA glycosylases are responsible for initiating the BER process by removing the damaged or mismatched bases and then generating apurinic/apyrimidinic (AP) sites in DNA for the downstream BER repair processes by other enzymes [1–3]. Uracil-DNA glycosylase (UDG) is one of the most important BER enzymes which can prevent uracil lesions of the genome by catalyzing the hydrolysis of the N-glycosylic bond between the deoxyribose and uracil base. Abnormal UDG activity would suppress the cellular responses to uracil lesions, which is closely associated with the etiology of various diseases including human immunodeficiency [4], lymphoma [5] and Bloom syndrome [6]. Consequently, it is highly desirable to exploit efficient methods for the detection of UDG activity for its biochemical studies and the development of drugs for UDG-related diseases.

The commonly-used analytical methods for UDG activity detection include gel-based assays [7], radioisotopic labeling [8], streptavidin paramagnetic bead capture techniques [9], electrochemical method [10] and colorimetric method [11], suffer from potential radioactive dangers, complicated electrode modification or limited sensitivity, respectively. As an alternative, fluorescence-based methods are attractive due to the advantages of safety, simplicity and robust assays for UDG activity. Among them, the methods were mainly based on the independent DNA probes as the target recognition and signal output elements, which achieved collaborative detection of UDG activity [12,13]. However, the sensitivities of the above fluorescent assays were entirely moderate, and thus signal amplification strategies have been introduced into the fluorescent UDG activity assays. Among them, polymerization/nicking amplification reaction was applied because of the high amplification ability and operational ease [14,15]. Wang et al. developed an enzyme-assisted bicyclic cascade signal amplification strategy for assaying UDG activity [16]. Additionally, Wu et al.

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http://dx.doi.org/10.1016/j.talanta.2017.10.052
Received 7 August 2017; Received in revised form 19 October 2017; Accepted 25 October 2017
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demonstrated a toehold-mediated polymerization/nicking reaction for sensitively detection of UDG enzyme [17]. In these methods, the primer for the amplification process was obtained through the polymerization/nicking process of the recognition probe of UDG, leading to the increase of design complexity and response time. Furthermore, recognition probe and the signal output probe were independently designed, which could form DNA dimers similar to "template-dimer" type nonspecific background amplification [18].

To develop a polymerization/nicking amplification method for UDG activity which is characteristic of simple probe design, quick response and low-background for UDG activity, we designed an integrating DNA structure switch (DSS) with two branched hairpins and used it to develop a fluorescent amplification strategy for UDG activity. The uracil removal in DSS converted the overhung primer into a complete hybrid to initiate the polymerization/nicking amplification reaction immediately, generating label-free fluorescent signal. Due to the integration of the recognition element and signal element, the quick response and the high sensitivity for UDG determination was achieved and a low detection limit of 0.0001 U/mL was obtained which was superior to the most reported fluorescent methods for UDG assays. Furthermore, the method was also used to screen the inhibitor of UDG enzyme. The integrated DSS strategy developed here has the potential application for the study of mechanism and function of UDG enzyme and the screening the inhibitors as potential drugs and biochemical tools.

2. Experiment section

2.1. Materials and apparatus

Oligonucleotides used in this work were synthesized and purified by HPLC at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China), and their sequences and modifications were listed in Table S1. Thermodynamic parameters and secondary structures of all oligonucleotides were calculated using bioinformatics software (http://www.bioinfo.rpi.edu-/applications/). (In the secondary structure of DSS, the 3′ terminal was protruding, and thus could inhibit the occurrence of the polymerization/nicking reaction, and then the 3′ terminal of the DSS' secondary structure was blunt which could initiate the polymerization/nicking reaction.) Tris (hydroxymethyl) aminomethane hydrochloride (purity > 99%) was purchased from Aladdin (Shanghai, China). Ethylenediamine tetra acetic acid disodium (purity > 99%) was purchased from Sinopharm Chemical Reagent Beijing Co., Ltd (Beijing, China).

Uracil-DNA glycosylase (UDG), uracil glycosylase inhibitor (UGI), human 8-oxoG DNA glycosylase 1 (hOGG1), human alkyl adenine DNA glycosylase (hAAG), Exonuclease III (Exo III), Klenow fragment (3′-5′ exo-) polymerase and Nb.BbvCI nicking enzyme were obtained from New England Biolabs Ltd. (Beverly, MA, USA). Thioflavin T (purity > 97%) was purchased from Abcam (Cambridge, UK). The other reagents were of analytical grade and used as received. The ultrapure water which was obtained from a Millipore Milli-Q water purification system (> 18.25 MΩ) was used to prepare all of the solutions.

2.2. Fluorescent amplification strategy for UDG activity assay and inhibitor screening

For assaying UDG activity, 100 nM DSS and various amounts of UDG were added to the reaction buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 10 mM NaCl, 2 mM $MgCl_2$, pH 8.0) to give a total volume of 10 μL. The mixture was incubated at 37 °C for 45 min to allow the base excision reaction to take place. After the base excision reaction, dNTPs (10 mM), Nb.BbvCI nicking enzyme (0.1 U μL^{-1}), KF polymerase (0.05 U μL^{-1}), and 1 × NEBuffer 2 (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) were added. Then the DNA amplification reaction in a volume of 40 μL was carried out at 37 °C for 2 h.

The fluorescence intensity of the mixture solution was measured on a Hitachi F-7000 spectrofluorophotometer (Hitachi, Japan). The instrument was operated under the following parameters: λ_{ex} = 425 nm (bandpass 5 nm), λ_{em} = 492 nm (bandpass 10 nm), PMT detector voltage = 700 V. The control experiment was carried out under the same condition without UDG. For assaying the inhibition of UDG, the different concentrations of UGI and UDG were firstly added to the enzyme reaction buffer containing 100 nM DSS, and the mixture solution was incubated at 37 °C for 45 min. Then the polymerization/nicking reaction and the fluorescence measurement were accordant with the operations in UDG assay.

2.3. Preparation of cells lysates

The HeLa and MCF-7cells samples were pelleted by centrifugation (5 min, 3000 rpm, 4 °C) and resuspended in 20 mL of lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na_3VO_4 and leupeptin, pH 7.5) on ice using a sonicator (four pulses at 200 W for 30 s with a tapered microtip). The mixture solution was then centrifuged at 12,000 rpm for 30 min at 4 °C to remove insoluble material. The resulting supernatant was collected and filtered through a 0.45 μm filter membranes, yielding crude lysate [19,20].

3. Results and discussions

3.1. Design of DNA structure switch and rationale of fluorescent amplification strategy for UDG activity

Our strategy for UDG activity assay comprises a UDG-responsive DNA structure-switch process followed with an autonomous replication-scission-displacement reaction that is able to produce a great abundance of G4 sequences for signal readout, as illustrated in Scheme 1. One integrating DSS is designed with two regions: region I includes the UDG recognition sequence, an inactivated overhung trigger sequence and an auxiliary complementary sequence (red loop). Region II includes a half recognition sequence of (5′-CCTCAGC-3′) for Nb.BbvCI nicking enzyme in the loop (in purple) and the complementary sequence of G4 DNA at the stem and 5′ overhung domain (in green). The action of the UDG could remove uracil base from the deoxyriboses phosphate backbone of DSS, leading to the generation of AP site and a lower melting temperature of the region I which could result in its hairpin reconfiguration and folding into a close conformation. This converts the overhung trigger into a complete hybrid to enable an extension reaction in the presence of KF polymerase and dNTPs. The replication of the region II finally yields the duplex strand with a full recognition site for nicking enzyme. Hence, a cycle of nicking enzyme cleavage, polymerase extension, and subsequent G4 strands release is created. In contrast, the region I of the DSS keeps its original configuration in the absence of UDG enzyme, and thus the subsequent replication-scission-displacement reaction is inhibited. Hence, there are few G4 strands released. In this work, we use ThT as a signal reporter to target G4 which has long been used to detect amyloid fibrils [21–23]. Recently, it has been reported that ThT possesses the prominent structural selectivity for G4 such as the human telomeric motif [24] and can be used to discriminate this structure from duplexes or single strands. It exhibits weak fluorescence in free form, but significantly enhance fluorescence signal via binding to G4 DNA. Through this character, we can realize quantitative measurements of the UDG activity successfully.

3.2. Verification of conformational change of DSS and fluorescent amplification strategy

The key to the success of our strategy was the conformational change of DSS and the subsequent DNA amplification process. Firstly, we verified that the action of UDG on DSS induced the hairpin

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