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Label-free monitoring of DNA polymerase activity based on a thrombin-binding aptamer G-quadruplex

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ABSTRACT

We have developed a label-free assay for the detection of DNA polymerase activity based on a thrombin-binding aptamer (TBA) G-quadruplex. In the presence of DNA polymerase, the 3′-OH termini of the hairpin substrate are immediately elongated to replace the TBA, which can be recognized quickly by the ThT dye and results in an increase of fluorescence. This method is highly sensitive with a detection limit of 0.1 U/mL. It is simple and cost-effective without any requirement of labeling with a fluorophore-quencher pair. Furthermore, the proposed method can also be applied to analyze the inhibition of DNA polymerase, which clearly indicates that the proposed method can be applied for screening of potential DNA polymerase inhibitors.

1. Introduction

DNA polymerase, which catalyzes the polymerization of four deoxyribonucleotides 5'-monophosphate (dNTP) residues into a DNA strand along the sequence of a template strand, plays a major role in DNA repair and DNA replication in bacterial and eukaryotic cells [1]. It has been demonstrated that numerous hyperproliferative diseases are closely related to DNA polymerase, such as autoimmune diseases and cancer [2,3]. Furthermore, DNA polymerase is crucial for numerous core biotechnology applications such as polymerase chain reaction (PCR), cDNA cloning and genome sequencing [4]. Therefore, the development of a simple, fast and cost effective approach for screening DNA polymerase activity and its inhibitors might be critical for future cancer therapy and drug discovery.

So far, several common methods have been developed for assaying DNA polymerase activity, mainly including gel electrophoresis methods and incorporation of radioactive labeling of pseudo-thymidine [5,6]. However, these traditional techniques are usually laborious and insensitive. To circumvent the obstacles of traditional methods, fluorometric methods have attracted much attention due to avoided radiation exposure, easy operation and reduce time. In the past decades, numerous fluorescent methods have been developed for DNA polymerase in vitro assays, such as the dsDNA quantitation dye, the dsDNA-specific fluorescent copper nanoparticles (CuNPs), the FRET-based DNA probes, and the molecular beacons (MB) [7–10]. Despite avoiding the use of radioactivity, some of these methods are limited by expensive labels and reagents. Graphere oxide, a super quencher for DNA polymerase activity detection, has been employed by Xu and collaborators [11]. However, they require an expensive labeling probe, and synthesis of graphene oxide is usually time-consuming. Song et al. [12] have reported a simple and rapid fluorometric method for real-time monitoring of DNA polymerase activity using singly labeled smart probe. Nevertheless, they still require the design of a fluorescence labeling probe, which often suffers from problems like high cost.

Thioflavin T (ThT), a benzothiazole dye, can induce the G-rich oligonucleotide sequence to form the G-quadruplex structures and the G-quadruplex structures enhancements in ThT fluorescence [13–16]. Recently, ThT-based fluorescent probes have been used in different fields [17,18]. Zhou et al. have reported that ThT can be used as a fluorescence probe to detect T4 polynucleotide kinase activity [19]. Sugimoto and co-works have reported an approach for monitoring RNA metabolism based on ThT-based fluorescent probes [20]. Due to its inherent advantages such as low background fluorescence, simplicity and low cost, ThT-based fluorescent probes have a great potential in biomedical applications. In this work, a simple and label-free ThT-based fluorescent probe is described for real-time monitoring of the activity of DNA polymerase.

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2. Materials and methods

2.1. Reagents and materials

Klenow Fragment (KF-) which lacks both the 3′-5′ exonuclease and the 5′-3′ exonuclease activities of Polymerase I and 10 × NEB buffer 2 (100 mM Tris-HCl, 500 mM NaCl, 100 mM $MgCl_2$, 10 mM DTT, pH = 7.9) were obtained from New England Biolabs (Beverly, MA, USA). The dNTP mixture was obtained from Takara Biotechnology Co., Ltd (Dalian, China). Thioflavin T (ThT) and chloroquine were purchased from Sigma-Aldrich. All other reagents were of analytical-reagent grade. Ultra-pure water (18.2 MΩ cm^{-1}) was obtained from a Milli-Q water purification system (Millipore Corp, Bedford, MA, USA) and was used throughout the experiments. The oligonucleotides were synthesized and purified by Sangon Biotechnology Co., Ltd (Shanghai, China). The self-annealing hairpin substrate was 5′-CCAACCACACCAACCAGT CGCACC-TAAAGGGTGCG-3′, and the thrombin-binding aptamer (TBA) sequence was: GGTTGGT GTGGTTGG. All the DNA oligonucleotides were dissolved in TE buffer and were stored at −20 °C before use.

2.2. Fluorescence measurements

All fluorescence measurements were carried out on an F2700 fluorescence spectrophotometer (Hitachi, Japan) with an excitation wavelength of 425 nm and an emission wavelength of 490 nm for ThT. Each experiment was carried out with a final volume of 100 μL and the mixtures were incubated at 25 °C for about 5 min to reach equilibrium. Next, a defined amount of DNA polymerase was introduced into the solution and the fluorescence intensity of the sample was recorded.

2.3. Real-time monitoring of DNA polymerase activity

Two samples were prepared: Hairpin substrate, TBA, dNTP, and ThT were added into sample A and sample B, respectively. Assays were carried out in 1 × NEB buffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9). In the experiment involving real-time monitoring of the DNA polymerase, samples A and B were incubated at 25 °C for about 5 min, and 20 U/mL of KF- was only added to sample B thereafter.

2.4. Optimization of analysis conditions

In order to obtain the best possible results, a variety of crucial reaction parameters were optimized, including the concentration of hairpin substrate, the ratio of hairpin substrate to TBA, the concentration of dNTP, and ThT. The hairpin substrate concentration range was 50–500 nM, the ratio hairpin substrate to TBA range was 2:1 to 1:4, the dNTP concentration range was 10–400 μM, and the ThT concentration range was 10–40 μM.

2.5. Activity assay of DNA polymerase

In this assay, 300 nM hairpin substrate, 600 nM TBA, 100 μM dNTP, and 20 μM ThT were placed into 100 μL of 1 × NEB buffer 2, incubated at 25 °C for about 5 min, and a defined amount of KF- was added to the solution with stirring for 2 s. The fluorescence intensity of sample was recorded thereafter. To determine the DNA polymerase activity, DNA polymerase reactions were carried out with KF- concentrations varying from 0.1 to 20 U/mL (0.1, 0.25, 0.5, 2.5, 5, 10, 15, and 20 U/mL). The initial velocity of the fluorescence intensity (V) could be defined as the signal in this experiment and the initial velocity could be calculated with the following equation:

$$V = \frac{F_{200s} - F_0}{200}$$

Here, F_0 represents the fluorescence intensity before adding DNA polymerase and F_{200s} is the fluorescence intensity after addition of DNA polymerase 200 s.

2.6. Influence of inhibitors on DNA polymerase activity

In order to further evaluate the inhibitor screening ability of the proposed assay, a known inhibitor of KF-, namely chloroquine at different concentrations was added to the reaction buffer, respectively. The detection processes were recorded using the aforementioned procedure.

3. Results and discussion

3.1. Strategy rationale

The general principle of our approach can be found highlighted in Fig. 1. The substrate template, namely hairpin substrate, features a self-hairpin structure with a long tail. The tail of the hairpin substrate can hybridize with TBA to prevent TBA from forming a G-quadruplex structure. In the presence of DNA polymerase (KF-) and dNTPs, the 3′-OH terminus of the hairpin substrate is immediately elongated to replace TBA, which can be recognized quickly by the ThT dye ultimately resulting in an increase of fluorescence [15]. Thus, the DNA polymerase activity can be monitored in real time based on this principle.

3.2. Real-time monitoring of DNA polymerase activity

In order to confirm the feasibility of the strategy, two separate experiments were carried out. Fig. 2 shows the relative time courses in the presence and absence of DNA polymerase. In the absence of DNA polymerase, no fluorescence intensity change could be seen in curve A due to the hairpin substrate hybridizing with the TBA sequence, preventing TBA from forming a G-quadruplex structure. As shown in curve B, in the presence of DNA polymerase, an increase in fluorescence intensity could be observed, further confirming that this assay may reflect the activity of DNA polymerase. Therefore, the activity of DNA polymerase can be monitored in real time using this approach.

3.3. Optimization of experimental conditions

In order to achieve a good detection performance, four main influencing factors were optimized through a series of experiments as shown in Fig. 3. Firstly, the concentration of hairpin substrate was optimized. The results obtained showed that the reaction velocity reached a plateau at 300 nM (Fig. 3A). Thus, 300 nM was used as the concentration of hairpin substrate for the following experiments. Moreover, the molar ratio of hairpin substrate to TBA was optimized with a fixed concentration of hairpin substrate (300 nM) and different concentrations of TBA (150, 300, 600, 900 and 1200 nM). As shown in Fig. 3B, the reaction velocity increased gradually as the molar ratio of hairpin substrate to TBA increased and reached a maximum value at a molar ratio of 1:2. Therefore, 1:2 was chosen as the optimum molar ratio of hairpin substrate to TBA in the following experiments. Fig. 3C highlights the changes of the reaction velocity with the concentration of dNTPs. As can be seen from inspection of Fig. 3C, the reaction velocity increased with an increasing dNTPs concentration and reached a maximum at 100 μM. Thus, 100 μM was used throughout subsequent