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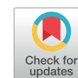
Ultrasensitive, label-free detection of T4 ligase and T4 polynucleotide kinase based on target-triggered hyper-branched rolling circle amplification



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ABSTRACT

Ligase and polynucleotide kinase (PNK) play important roles in DNA repair process. In this work, a simple, convenient and ultrasensitive fluorescence biosensor for label-free detection of ligase and PNK was proposed on the basis of target-triggered hyper-branched rolling circle amplification (HRCA). In the presence of PNK and ligase, the 5′-OH end of a linear Padlock DNA strand could be phosphorylated by PNK and then be converted by ligase into a circular template to trigger subsequent exponential HRCA reaction, producing amounts of double-stranded DNA (dsDNA) fragments, accompanied by the significant fluorescence increase of dsDNA-intercalating dye–SYBR Green I (SG I). In the absence of ligase or PNK, however, HRCA primer and uncyclized Padlock strands were completely digested by exonucleases, thus providing a low background for the sensing system. Therefore, by recording the fluorescence change of SG I, ligase and PNK activity could be facilely determined. The proposed biosensor exhibited excellent detection sensitivity for ligase and PNK activity with the detection limits of 3.4×10^{-4} U/mL and 3.8×10^{-4} U/mL, respectively. Such a sensing strategy could be easily extended to studies on inhibitors of these two enzymes, thus might offer a promising tool in clinical molecular diagnostics and cancer therapy.

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1. Introduction

DNA damage, which can be induced by various factors, is a very common phenomenon in biology, and is highly relevant with biological dysfunction and instability of the genome [1]. Effective repair of DNA damage is extremely important for maintaining the integrity and stability of genome [2]. During DNA repair, an essential step is the ligation reaction between 5′-phosphoryl (5′-P) and hydroxyl (3′-OH) termini at the break points of genomic DNA [3]. DNA ligase and polynucleotide kinase (PNK) are two kinds of repair enzymes used in the ligation reaction. DNA ligase can catalyze the formation of phosphodiester bonds between adjacent 3′-OH and 5′-P termini at single break in dsDNA [4,5]. In many cases, however, the broken DNA strands contain 5′-OH but not 5′-P termini [6]. Therefore, phosphorylation of 5′-OH termini is required prior to ligase-mediated DNA repair. PNK is a functional enzyme with 5′-kinase activity, it can generate 5′-P at damaged 5′-OH terminal [7], thus permitting subsequent ligase-mediated DNA repair. So both DNA ligase and PNK enzymes are indispensable in the DNA repair process. In addition, the activity levels of DNA ligase and PNK are closely related to the pathogenesis of various diseases, such as Bloom's syndrome, Werner syndrome and so on [8,9]. Recent clinical studies have shown that inhibiting DNA ligase and PNK can potentially reduce the risk of cancer cell invasion and metastasis [10] and increase the sensitivity of human tumors to γ-radiation therapy [11,12]. Collectively, developing a simple, convenient, and sensitive detection method for DNA ligase and PNK activity assay is significant for early diagnosis and prognostic evaluation of many diseases.

Conventionally, radioactive isotope ^{32}P-labeling, polyacrylamide gel electrophoresis and autoradiography are commonly used for the activity analysis of these enzymes [13–15]. However,

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these methods suffer from several intrinsic drawbacks, such as complicated detection procedures and potential radioactive health hazards, which greatly limited their broad applications. Up to now, a variety of new approaches have been explored for the detection of DNA ligase and PNK activity, including fluorescence [16–23], electrochemistry [24–28], colorimetry [29,30], nucleic acid amplification [31–35] and nanomaterials-based strategies [36–39]. Among them, the fluorescent ones have attracted considerable attention due to their distinct advantages of high sensitivity, simple operation and fast response. Although some of fluorescent assays work well for DNA ligase and PNK detection, the utilization of fluorescence-labeled probes [40], nanoparticles [36–38] or DNAzyme [41] might increase the complexity of probe design and synthesis, thus limiting their widespread applications. Based on above considerations, label-free fluorescent strategy is a good choice to realize facile, reliable and cost-effective assay. To further improve the detection sensitivity, it is very necessary to incorporate highly efficient amplification strategies in biosensor design.

As an isothermal nucleic acid amplification technique, rolling circle amplification (RCA) shows many unique advantages in biosensing applications [42]. Based on RCA reaction, our group has reported two elegant design strategy for determining DNA ligase and PNK activity, and obtained satisfactory results [7,43]. However, since conventional RCA is linear amplification process, its limited amplification efficiency can't meet the demand of high sensitivity in many assays. Recently, by introducing of endonuclease, we developed nicking endonuclease mediated RCA strategy [44,45], which allowed RCA reaction to be performed in an exponential amplification mode and significantly improved the sensitivity. Nevertheless, the use of nicking endonuclease and G-quadruplex structure will certainly increase the detection cost and the complexity of probe design. Overall, the development of a simple, cost-effective, reliable, and sensitive RCA method for enzyme detection is still urgently needed.

Recently, Li et al. reported a background-eliminated fluorescence assay for sensitive detection of PNK activity by using multifunctional magnetic probes and polymerization nicking reactions mediated hyperbranched rolling circle amplification (HRCA) [35]. The work gives extraordinarily high sensitivity with a detection limit of 4.36×10^{-5} U/mL. However, relatively complicated operations, including PNK-triggered polymerization extension and nicking endonuclease cleavage reactions, magnetic separation, and subsequent HRCA processes, might hinder its wide use to some extent. To achieve simple but sensitive detection of ligase and PNK activity, a target enzyme-triggered HRCA-based fluorescence assay platform was proposed in this work. Such a platform was demonstrated to work well for label-free, cost-effective, highly sensitive and specific analysis DNA ligase and PNK activity, and could be easily extended to studies on inhibitors of these two enzymes.

2. Experimental section

2.1. Materials and reagents

All of the DNA oligonucleotides (Padlock, ligation template (LT), Primer and Reverse Primer. Table S1) used in this work were synthesized and purified by Sangon Biotech. Co. Ltd. (Shanghai, China). The concentrations of the oligonucleotides were represented as single-stranded concentrations, which were determined by measuring the absorbance at 260 nm. The molar extinction coefficient was determined using a nearest neighbor approximation (http://www.idtdan.com/analyzer/Applications/OligoAnalyzer). Adenosine diphosphate (ADP) and deoxyadenosine triphosphate (dATP) were obtained from Sigma-Aldrich. T4 ligase, T4 PNK, Phi29 DNA polymerase, exonuclease I (Exo I), exonuclease III (Exo III), restriction endonuclease EcoR I, restriction endonuclease Nb.BbvCl, Taq polymerase, Bst polymerase and deoxyribonucleoside 5′-triphosphate mixture (dNTPs), were obtained from New England Biolabs (Beijing, China). SYBR Green I (SG I, 20×), agarose, ethidium bromide (EB), 6× loading buffer, and DNA Marker IV were obtained from Tiangen Biotech. Co. Ltd. (Beijing, China). All chemical reagents were analytical grade and used without further purification. Deionized and sterilized water (resistance >18 ΩM cm^{-1}) was used throughout the experiments.

2.2. Detection of T4 ligase activity

The process of T4 ligase activity detection can be divided into two steps: circular template preparation and subsequent HRCA. In circular template preparation step, 100 nM Padlock was mixed with 10 U/mL T4 PNK in 1 × T4 ligase buffer (50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM adenosine triphosphate (ATP)). The mixture was incubated at 37 °C for 1 h to achieve the phosphorylation of 5′-OH end of Padlock. Then, the mixture was heated at 65 °C for 10 min to inactivate T4 PNK. 300 nM LT was added to this mixture. To ensure that Padlock could fully hybridize with LT, the mixture was incubated at 37 °C for 0.5 h. Then, different concentrations of T4 ligase were added. The mixture was allowed to incubate at 25 °C for 1 h to ensure that the 5′-P and 3′-OH ends of Padlock were ligated to form a circular template. The ligation reaction was terminated by a thermal treatment at 65 °C for 10 min. Next, 20 U Exo I and 20 U Exo III were added and the mixture was incubated at 37 °C for 1 h to digest the leftover ssDNA and dsDNA. The Exo I and Exo III enzymes were then denatured by incubating at 90 °C for 10 min. Finally, as-prepared circular template was used immediately or stored at −20 °C until use.

HRCA reaction mixture was prepared by combining above-prepared circular template with SYBR Green I (2×), 50 nM Primer, 50 nM Reverse Primer, 4 mM dNTPs, 1 × Phi29 buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, and 4 mM dithiothreitol (DTT), pH 7.5), and 5 U Phi29 DNA polymerase. The final volume was 100 μL. The reaction mixture was incubated at 30 °C for 3 h and terminated by heating at 65 °C for 10 min. The fluorescence signal of the reaction mixture was recorded on a Shimadzu RF-5301 fluorescence spectrometer (Shimadzu Ltd., Japan) in the range of 505–650 nm by setting the excitation wavelength at 497 nm. The fluorescence signal intensity at 525 nm was used for the quantitative analysis of T4 ligase activity.

2.3. Detection of T4 PNK activity

Padlock (100 nM) and different concentrations of T4 PNK were incubated in 1 × T4 ligase buffer (50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM ATP) at 37 °C for 1 h and then at 65 °C for 10 min. Then, 300 nM LT was added to this solution, and the mixture was incubated at 37 °C for 0.5 h. After addition of 5 U/mL T4 ligase, the mixture was allowed to incubate at 25 °C for 1 h to ensure the formation of the circular template. The following experimental steps were same as those used in T4 ligase detection.

2.4. Enzyme inhibitor assay

As for inhibition assay of T4 ligase, dATP was selected as the model inhibitor. Typically, the experiment was carried out similar to above stated T4 ligase assay, except the pre-incubation of 0.5 U/mL T4 PNK with varied concentrations of dATP.

In the inhibition assay for T4 PNK, ADP was used as the model inhibitor. Similar experimental procedures to PNK assay were employed except that 0.5 U/mL PNK was pre-mixed with different concentrations of ADP.

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