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Detection of T4 polynucleotide kinase activity based on cationic conjugated polymer-mediated fluorescence resonance energy transfer

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ABSTRACT

A simple but robust strategy for sensitive detection of T4 polynucleotide kinase (T4 PNK) activity is developed by means of a DNA phosphorylation-accelerated λ exonuclease cleavage reaction coupled with cationic conjugated polymer (CCP)-mediated fluorescence resonance energy transfer (FRET). Firstly, a label-free hairpin DNA with a 5′-hydroxyl end is designed as the substrate of T4 PNK. SYBR Green I (SGI), a double-stranded DNA (dsDNA)-specific fluorescent dye, can fluoresce only when intercalated to the stem region of the hairpin DNA. When mixed with CCP, the SGI-binding hairpin DNA will be brought in close proximity with the CCP due to strong electrostatic interaction, leading to efficient FRET from CCP to SGI. However, in the presence of T4 PNK, the hairpin DNA would be phosphorylated at its 5′-terminus and thus can be immediately recognized as the initial cleavage site of λ exonuclease. The phosphorylation-actuated λ exonuclease reaction will cleave the stem of the hairpin to yield a single-stranded DNA, which is unable to combine with SGI and as a result, the FRET signal would decrease gradually in correlation to the T4 PNK activity. Therefore, by recording the change of FRET ratio, T4 PNK activity can be facilely determined in a mix-and-read manner. Due to the light harvesting and fluorescence amplification properties of CCP, high sensitivity is achieved for this homogeneous assay. This new strategy provides a simple detection procedure, easy readout and cost-effective manner for T4 PNK analysis, which shows great potential in the study of polynucleotide kinase-related biological processes.

1. Introduction

DNA breaks induced by either ionizing radiation (Henner et al., 1983) or nucleases (Torriglia et al., 1998) have serious impacts on the biofunction of the genome. Therefore, efficient repair of such DNA lesions is extremely important for maintaining gene integrity. Generally, DNA repair is achieved through the ligation between the 5′-phosphate and 3′-hydroxyl terminus at the break points by DNA ligases (Lindahl and Wood, 1999; Zhang et al., 2013). Unfortunately, numerous DNA breakages are associated with the generation of 5′-OH termini (Chen et al., 2013), which should be pre-phosphorylated by kinases before the ligase-mediated DNA repair. In this regard, T4 polynucleotide kinase (T4 PNK), which can catalyze the transfer of γ-phosphate group of ATP to the 5′-OH group of nucleic acid molecules (Phillips and Arlt, 2007), plays a critical role in the processes of DNA damage repair, replication and recombination. It has been clearly verified that aberrant T4 PNK activity will result in abnormal DNA phosphorylation states, which are closely associated with the etiology of various human diseases such as Bloom's syndrome, Werner syndrome and Rothmund–Thomson syndrome (Sharma et al., 2006). Therefore, due to its clinical and biological importance, the precise determination of T4 PNK activity is essential for further understanding the biological processes of nucleic acid metabolism and PNK-targeted drug development.

Conventionally, polyacrylamide gel electrophoresis (PAGE) and radioactive methods by use of γ-^{32}P-ATP during the phosphorylation reaction (Bernstein et al., 2009; Karimi-Busheri et al., 1998; Wang and Shuman, 2001) used to be considered as the standard protocols for assaying T4 PNK activity. However, several inherent drawbacks, such as complicated procedures and the potential radioactive hazards to human health, greatly limit their wide application. Therefore, significant efforts have been paid to develop nonradioactive PNK assays. Up to now, many approaches for the detection of T4 PNK activity have been reported by use of various analytical techniques such as electrochemistry, colorimetric methods, nucleic acid amplification and nanomaterials-based strategies (Huang et al., 2013; Jiang et al., 2013; Tang et al., 2014; Wang et al., 2012). Notably, fluorescence-based PNK assays are

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extremely attractive and have become the most popular tools for evaluating DNA phosphorylation due to their flexible design and easy readout (Hou et al., 2014; Liu et al., 2014a, 2014b; Ma et al., 2013). Although these fluorescent assays have made great advances for evaluating T4 PNK activity, they usually rely heavily on the use of functional nucleic acid probes such as molecular beacon or DNAzyme, which need skilled and sophisticated sequence design. Moreover, these methods also require specific dye-labeled or even dye/quencher double-labeled DNA probes, which are costly and difficult to synthesize. Therefore, it still remains a great challenge to develop a label-free, low-cost, fast response, convenient and sensitive approach for evaluating T4 PNK activity.

In recent years, water-soluble fluorescent conjugated polymers have gained much attention in the field of biosensing. These fluorescent polymers contain a large number of absorbing units with a delocalized electronic structure, which results in rather high absorption coefficients. Along the whole backbone of the conjugated polymer, the excitation energy can transfer to suitable energy acceptor sites over long distance, leading to a greatly amplified fluorescence resonance energy transfer (FRET) signal. Due to their unique light harvesting and fluorescence amplification properties, fluorescent conjugated polymers have been widely used as the energy donor in fabricating FRET-based sensing platform for various biomolecules (Bai et al., 2013; Cheng et al., 2012; Duan et al., 2010; Feng et al., 2010; Ren and Xu, 2009; Zhu et al., 2012). Notably, based on the FRET between conjugated polymer and DNA intercalating dyes, Pu and coworkers have proposed a label-free strategy for the facile detection of nuclease activity (Pu et al., 2010), which greatly simplify the FRET design because expensive fluorophore-labeled DNA probes are no longer required.

In view of the distinct features of fluorescent conjugated polymers, we wish to report herein a label-free, simple and sensitive method for assaying T4 PNK activity by coupling a phosphorylation-accelerated λ exonuclease (λ exo) cleavage reaction with a cationic conjugated polymer (CCP)-amplified FRET strategy. In this protocol, the cationic poly[(9,9-bis(6′-N,N,N-triethylammonium)hexyl) fluorenylene phenylene dibromide] (PFP) serves as the FRET donor while SYBR Green I (SGI), which can intensively fluoresce only when bound to the stem region of a label-free hairpin DNA, acts as the FRET acceptor. The SGI-incorporated hairpin DNA will be brought in close proximity with the PFP due to strong electrostatic interaction, leading to efficient FRET from PFP to SGI and an amplified fluorescence signal. However, T4 PNK-induced phosphorylation of the 5′-terminus of the hairpin DNA will actuate λ exo-catalyzed cleavage of the stem region of the hairpin structure to yield a single-stranded DNA (ssDNA), which is unable to incorporate SGI and as a result, the FRET signal would decrease gradually as a function of T4 PNK activity. Due to the light harvesting and fluorescence amplification properties of CCP, high sensitivity is achieved for this homogeneous assay, which shows great potential in the study of polynucleotide kinase-related biological processes as well as in screening of potential inhibitor drugs.

2. Experimental

2.1. Materials and reagents

T4 PNK (10,000 U/mL) and λ exo (5 U/μL) were purchased from New England Biolabs (NEB, UK). Adenosine triphosphate (ATP) and adenosine diphosphate (ADP) were obtained from Sangon Biotech (Shanghai, China). SYBR green I (20 ×) was purchased from Zeesan Biotech co., Ltd. (Xiamen, China). Poly[(9,9-bis(6′-N,N,N-triethylammonium)hexyl) fluorenylene phenylene dibromide] (PFP) was kindly provided by Prof. Shu Wang of Institute of Chemistry, Chinese Academy of Sciences. The stem-loop hairpin DNA (HPDNA), 5′-**CTGGCGCTTGATGGTA***TTTT***TACCATCAAGCGCCAG**-3′) was synthesized by Takara (Dalian China). All other chemicals were of analytical grade and ultrapure water was used throughout this work.

2.2. Standard assay procedures for the detection of T4 PNK activity

Firstly, the HPDNA (1 μM in 70 mM Tris–HCl buffer containing 10 mM $MgCl_2$, pH 7.6) was heated at 95 °C for 5 min and then allowed to cool to room temperature for 1 h before use. Then 90 nM HPDNA, 1 mM ATP, 3 units (U) of λ exo and series dilutions of T4 PNK were mixed in a total 50 μL of enzyme reaction buffer (70 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.6). After incubation at 37 °C for 1 h to simultaneously perform the phosphorylation and λ exo-based DNA cleavage, this reaction system was heated to 80 °C for 5 min to deactivate the enzymes, and then gradually cooled to 37 °C and kept at this temperature for 1 h. Afterwards, this reaction system was further transferred into another 150 μL of Tris–HCl buffer containing SGI (final concentration of 0.2 ×) and PFP (final concentration of 1 μM), and the fluorescence emission spectra of such mixture was recorded immediately. The fluorescence emission spectra (400–650 nm) were recorded on a PerkinElmer LS-55 fluorescence spectrophotometer with the excitation wavelength of 380 nm.

2.3. Kinase inhibition study

The inhibition study was performed by using $(NH_4)_2SO_4$, Na_2HPO_4 or ADP as the model inhibitors of T4 PNK. Typically, the experiments were carried out via similar procedures as those for T4 PNK assay stated above, except for the pre-incubation of a fixed T4 PNK concentration (5 U/mL) with varied concentrations of inhibitor.

2.4. Gel electrophoresis

The structure of HPDNA before and after the treatment of T4 PNK/λ exo was characterized by polyacrylamide gel electrophoresis (PAGE), which was carried out in a 16% (w/v) polyacrylamide gel with TBE buffer (pH 8.0). After 5 μL of sample was loaded on the gel, electrophoresis was performed at a constant voltage of 110 V for 90 min. The gel was stained by 1 × SYBR Gold and visualized on a Gel Doc EZ Imager (Bio-Rad).

3. Results and discussion

3.1. Design principle of the PFP-based T4 PNK assay

Fig. 1 illustrates the design principle of the proposed T4 PNK assay by combining a phosphorylation-initiated λ exo cleavage reaction with the CCP-based FRET technology. As demonstrated here, PFP serves as the sensing elements in this design. Besides, λ exo is another essential component of this strategy. λ exo is a highly processive 5′→3′ exodeoxyribonuclease that selectively digests the 5′-PO_4 end of dsDNA at a high speed, which is over 300-times more quickly than digesting dsDNA with the 5′-OH end (Perkins et al., 2003; Subramanian et al., 2003; Zhao et al., 2012). Herein, a label-free stem-loop HPDNA with a 5′-OH terminus is used as the substrate of T4 PNK. When T4 PNK is absent, the 5′-OH end of the hairpin will not be recognized by λ exo to conduct the cleavage reaction, leaving the stem-loop structure intact. SYBR Green I (SGI), which cannot stain ssDNA but strongly fluoresce when bound to the small groove of dsDNA, will intercalate into the stem region of the hairpin to generate strong fluorescence. The