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Cyclic up-regulation fluorescence of pyrene excimer for studying polynucleotide kinase activity based on dual amplification

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ABSTRACT

Due to its important biological and clinical roles of polynucleotide kinase (PNK), accurate monitoring of PNK activity and inhibition is highly desirable. Herein, a homogeneous and sensitive fluorescence assay has been proposed for the detection of PNK activity by integrating target recycling signal amplification of DNA toehold strand displacement reaction (TSDR) with gamma-cyclodextrin (γ-CD) enhancement of pyrene excimer. A label-free hairpin DNA1 (H1) and two singly pyrene-labelled DNA, H2 and H3, are designed. Accompanying the occurrence of the efficient enzyme reactions, namely phosphorylation-actuated λ exonuclease reaction, a single-stranded DNA as a trigger DNA (tDNA) of TSDR can be released from H1. Then, tDNA drives circulatory interactions between H2 and H3 to continuously form H2/H3 duplex, resulting in formation of pyrene excimer and a "turn on" fluorescence signal of pyrene excimer. Furthermore, the fluorescence of pyrene excimer is further amplified by introducing gamma-cyclodextrin (γ-CD), which can regulate the space proximity of two pyrene molecules. Thus, TSDR-induced cyclic formation of pyrene excimer and γ-CD enhancement can specifically up-regulate the fluorescence of pyrene excimer for detection of PNK activity, the detection limit is 9.3×10^{-5} U mL^{-1}, which is superior to those of most existing approaches. Moreover, the proposed strategy can also be successfully utilized to study inhibition efficiency of different PNK inhibitors as well. Therefore, a dual amplification approach is provided for nucleic acid phosphorylation related researches.

1. Introduction

DNA phosphorylation plays a significant role in regulation of majority vital cellular events, such as nucleic acid metabolism and DNA damage repair (Wang et al., 2002; Whitehouse et al., 2001; Ma and Yeung, 2010; Tang et al., 2005). Polynucleotide kinase (PNK), as a prominent member of the 5′-kinase family, possesses the capacity to phosphorylate DNA via catalyzing the transfer of the γ-phosphate residue from nucleoside triphosphate (ATP) to 5′-hydroxyl termini of nucleic acids (Becker and Hurwitz, 1967; Cameron and Uhlenbeck, 1977). The normal function of polynucleotide kinases is a prerequisite for ligation-based healing of DNA lesions to maintain gene integrity (Wang et al., 2002), while the aberrant PNK behaviors or lockage of DNA phosphorylation would perturb the cellular responses to DNA damage or lesions and closely associated with some serious human disorders such as Bloom's syndrome, Rothmund–Thomson syndrome and Werner syndrome (Sharma et al., 2006). Freschauf's group has reported that PNK inhibition could potentially sensitize human tumors to γ-radiation, meaning that PNK inhibitors might be promising drugs for effective cancer treatment (Freschauf et al., 2009; 2010). Similarly, PNK has also been widely applied in the detection of DNA adducts (Phillips and Arlt, 2007) or oligonucleotides (Frauendorf et al., 2003) and the repair of DNA lesions (Galburt et al., 2002). On account of these important biological and clinical roles of PNK, accurate monitoring of PNK activity and inhibition is highly desirable.

Up to now, various approaches for the determination of DNA phosphorylation have been developed by using T4 PNK as the model target. The traditional T4 PNK assays are radioactive ^{32}P-labeling, polyacrylamide gel electrophoresis (PAGE) and autoradiography (Wang et al., 2002; Whitehouse et al., 2001). However, several inherent drawbacks, such as sophisticated operating procedures, high cost, limited sensitivity and the potential radioactive hazards to human health, greatly limit their wide application. Therefore, alternative methods circumventing the aforementioned problems need to be explored for the investigation of the phosphorylation of nucleic acids. Recently, various methods toward convenient detection of PNK activity were developed, such

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as fluorescence assays (Hou et al., 2014; Tao et al., 2014; Shi et al., 2015), colorimetric assays (Jiang et al., 2013), surface plasmon resonance methods (Luan et al., 2010), nanochannel biosensors (Lin et al., 2013), bioluminescent sensors (Du et al., 2014), flow cytometric bead assays (Zhang et al., 2015) and electrochemical methods (Wang et al., 2012; 2013). There into, fluorescence-based PNK assays have aroused special attentions for evaluating DNA phosphorylation due to their high sensitivity, easy readout, low sample volume, flexible design and feasibility of quantification. Song proposed a β-cyclodextrin polymer-based fluorescence enhancement assay for PNK activity and inhibition (Song et al., 2015). Lin reported a graphene oxide (GO) based strategy for PNK activity and inhibition detection (Lin et al., 2011). Zhang developed a CuNPs-based PNK detection method (Zhang et al., 2013). Tao proposed a photo-induced electron transfer (PIET) based PNK detection assay (Tao et al., 2015). Despite great advances toward the DNA phosphorylation assay in each method, sensitivities of these methods are greatly limited due to the lack of an amplification mechanism. Consequently, the establishment of amplified fluorescence strategies for sensitive detection of DNA phosphorylation is highly desirable. Zhu developed an amplified fluorescence assay for PNK via an exonuclease III-aided trigger DNA recycling (Zhu et al., 2014). Liu reported sensitive detection of DNA phosphorylation by ligation-triggered DNAzyme cascade amplification (Liu et al., 2014). Cheng proposed the detection of PNK activity by coupling DNA strand displacement reaction with enzymatic-aided amplification (Cheng et al., 2015). Although all these methods have an obviously amplified fluorescence response in DNA phosphorylation determination than the traditional ones, they are time-consuming and need superabundant assistant enzyme to realize signal amplification. Further advances towards time-saving, enzyme free amplification strategy for sensitive and specific PNK detection are still urgently needed.

Here, a dual-amplification strategy based on new signal conversion mechanism was developed for PNK activity and inhibition evaluation. It is based on the fluorescence up-regulation of pyrene by formation pyrene excimer via toehold-mediated DNA strand displacement (TSDR) which provides a fast, efficient, specific, simple reaction process and can realize signal amplification by programming biomolecular self-assembly pathways without the help of functional enzymes (Yurke et al., 2000). As a spatially sensitive fluorescent dye, pyrene can form an excimer when an excited-state molecule is brought into close proximity with a pyrene moiety in the ground state (Huang et al., 2011). More importantly, the fluorescence of pyrene excimer is apparently different from the fluorescence of pyrene monomer, which offers a good choice for using pyrene as signal probe to realize reliable signal transfer in the complex matrix. Meanwhile, the fluorescence of pyrene excimer is sensitive to the change in microenvironment. So, to further improve sensitivity, γ-cyclodextrin (γ-CD) was introduced to offer protection for the pyrene excimer's emission and further enhance the fluorescence of pyrene excimer. In the presence of T4 PNK, DNA phosphorylation triggers TSDR to continuously form pyrene excimer, leading to "turn-on" fluorescence signal of pyrene excimer. In this strategy, time-saving and enzyme free signal amplification detection of DNA phosphorylation can be readily achieved by integrating toehold strand displacement reaction based target recycling signal amplification with γ-cyclodextrin enhancement signal amplification, which ensures high specificity as well as high sensitivity.

2. Experimental section

2.1. Chemicals

Adenosine triphosphate (ATP), Ethidium bromide (EB), adenosine diphosphate (ADP), dithiothreitol (DTT), γ-CD, EcoRI, T4 polynucleotide kinase (PNK, 10 U μL^{-1}) were bought from Shanghai Sangon Biotech Co. (Shanghai, China). Immunoglobin G (IgG) and lysozyme (Lys) were purchased from Beijing Dingguo Biotechnology Co., Ltd (Beijing, China). Lambda exonuclease (λ exo, 5 U μL^{-1}) and cAMP-dependent protein kinase A (PKA) were obtained from New England Biolabs (NEB, U.K.). The sequences of the DNA oligonucleotides were listed as follows: hairpin DNA1 (5′-CTTCTCAGTTAGGGTTAGACAAAAACACACACACACTTT TGTCTAACCCTAACTGAGAAG-3′, H1), hairpin DNA2 (5′-pyrene-TTCTCAGT TAGGGTTAGACATCAACTTCATCACTTCTAACCCTAAC-3′, H2), hairpin DNA3 (5′- TTCATCATTCTCAGTTAGGGTTA-GAAGTGATGAAGTTGATGTCT AACCCTAACTGAGAA-pyrene-3′, H3). Oligonucleotides used in this work were obtained from Takara Bio, Inc. (Dalian, China). Ultrapure water obtained from a Millipore filtration system was prepared throughout the assays.

2.2. Instruments

All fluorescence measurements were carried out on Hitachi F-7000 fluorescence spectrophotometer (Kyoto, Japan). Circular dichroism (CD) spectra were obtained on a Chirascan Circular Dichroism Spectrometer (Applied Photophysics Ltd, England, UK). The electrophoresis was conducted with a vertical electrophoresis system bought from Bio-Rad Laboratories, Inc and analyzed by the Molecular Imager system purchased from Shanghai Peiqing science & Technology. Co., Ltd (Shanghai, China).

2.3. Amplification detection of PNK activity and inhibiton

Firstly, 100 nM H1, certain concentration of T4 PNK and 0.01 U μL^{-1} λ exo were mixed in 50 μL buffer (70 mM Tris–HCl, pH 8.0, 1 mM ATP, 5 mM DTT, 10 mM $MgCl_2$). After incubation at 37 °C for the phosphorylation and λ exo cleavage reaction, the above mixture was heated at 75 °C for 10 min to terminate the reaction and cooled to room temperature. Then, the above solution was transferred into 50 μL buffer (70 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$) containing H2 (300 nM) and H3 (300 nM). After incubation of 30 min, 1.5 mM γ-CD was introduced to the resulting solution and incubated for another 5 min. Finally, the resulted solutions were characterized by fluorescence spectrophotometer and the excimer fluorescence was recorded. To investigate the effects of inhibitors on the phosphorylation process of PNK, ADP, $(NH_4)_2SO_4$ and Na_2HPO_4 were used as the model inhibitors in the inhibition assays. Various concentrations inhibitors were contained in the phosphorylation reaction buffer, respectively. After addition of 100 nM H1, 1 mM ATP, 0.01 U μL^{-1} of λ exo, 1 U mL^{-1} of PNK, the following procedures were similar to that mentioned above.

2.4. Kinase selectivity assay

To investigate the specificity of this proposed method for T4 PNK, 1 U mL^{-1} of EcoIR/PKA /heat-inactivated T4 PNK and 1.0 μM of IgG/Lysozyme/Bovine serum albumin (BSA) were added into the buffer respectively. The phosphorylation and enzymatic free amplification reaction were the same as those described in the aforementioned experiments for T4 PNK activity detection in reaction buffer.