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Phosphorothioated and phosphate-terminal dumbbell (PP-TD) probe-based rapid detection of polynucleotide kinase activity†

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Polynucleotide kinase (PNK), a bifunctional enzyme with 5′-kinase and 3′-phosphatase activities, plays an important role in DNA repair and is associated with various diseases. Here, we developed a primer-free, sensitive, and isothermal quantitative assay to detect PNK activity. In the presence of PNK, the 3′-phosphate group of the substrate was digested with 3′-OH, initiating the amplification reaction. Elongated dsDNA binds to the dsDNA-specific fluorescent dye EvaGreen, leading to a significant enhancement in fluorescence intensity. The limit of detection (LOD) of this method was 7.7×10^{-7} U μL^{-1}, which is comparable or even superior to that of previously reported methods. This approach also showed good quantitative ability in complex cell lysates, indicating potential for biological sample analysis. Additionally, this facile and sensitive assay can be used to screen for PNK inhibitors. The proposed method provides a promising platform for sensitive PNK activity monitoring and inhibition screening for drug discovery and clinical treatment.

Introduction

Polynucleotide kinase (PNK), a dual functional enzyme with 5′-terminal kinase and 3′-terminal phosphatase activities, plays an imperative role in DNA repair.[1] Mutations in genes encoding PNK have been proved to be associated with neurological diseases,[2,3] such as microcephaly with early onset seizures (MCSZ).[4–6] Given the significance of PNK, sensitive detection of its activity and screening of its potential inhibitors are crucial to identify biochemical interactions associated with DNA phosphorylation and to discover novel drugs.

Various methods have been developed to detect PNK activity, and conventional methods for accurate quantification, including radioisotope ^{32}P-labeling,[7] autoradiography, immunoassay,[8] and polyacrylamide gel electrophoresis (PAGE), have been widely used.[9] However, these methods have several intrinsic drawbacks, including radioactive hazards, laborious operation protocols, and time consumption, limiting their comprehensive application. To overcome these obstacles, alternative methods have been developed in recent years, including fluorescence,[10–14] electrochemical,[15–19] colorimetric analyses,[20–22] and some other methods.[23] Among these, the fluorescence method is widely used because of its ease of operation, increased sensitivity, and efficiency.[24,25] However, most fluorescence methods are hindered by intricate primer design and inevitable background interference. Therefore, a simpler and more sensitive detection platform for PNK activity is required.

In this work, we developed a small dumbbell probe for sensitive and rapid detection of PNK activity. This dumbbell probe has a phosphate group at the 3′-terminal and a phosphorothioate (PS) modification hairpin at the 5′-terminal and is called a phosphorothioated and phosphate-terminal dumbbell (PP-TD) probe. PS modification reduces the thermal stability of dsDNA and facilitates self-folding of terminal hairpins,[26] achieving higher amplification efficiency at a constant temperature.[27] The phosphate group at 3′-terminal of probe can be specifically hydrolyzed by PNK, which works as a "turn-on" switch to initiate self-folding and extension amplification utilizing DNA polymerase. Thus, without additional primers, the PP-TD probe could be specifically switched on by PNK and subsequently amplified into long concatemers isothermally and quickly. Finally, the amplification products were successfully detected by adding EvaGreen, a label-free dsDNA-specific fluorescent dye. The proposed assay could satisfactorily monitor PNK activity in cell lysates and screen inhibitors. This sensitive and selective PP-TD probe-based PNK detection assay could be a powerful tool for biomedical research and clinical diagnosis.

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Results and discussion

Design principle of the PP-TD probe for detecting PNK

Scheme 1 illustrates the working mechanism of the PP-TD probe for detecting PNK activity. The PP-TD probe was modified with a phosphate group at its 3′-terminal. This group can be hydrolyzed to a hydroxyl group in the presence of PNK, activating the PP-TD probe, thereby initiating the downstream reaction. Subsequently, Bst 2.0 DNA polymerase binds to the 3′ terminus of the active PP-TD probe, extending the DNA sequence to a stem-loop structure. As PS modification increased the instability of the extended dsDNA amplification products, the products could easily self-fold into two hairpin structures, as indicated in Scheme 1. A new round of extension can proceed in the presence of Bst 2.0 DNA polymerase with strand displacement and isothermal amplification activity. After several cycles, the PP-TD probe was amplified into a long concatemer. EvaGreen was added to the reaction system and embedded in extended repeat dsDNA to measure fluorescence. However, only weak fluorescence was detected in the absence of PNK, as the phosphate group at the 3′-terminal can block the amplification process and the formation of extended repeat dsDNA.

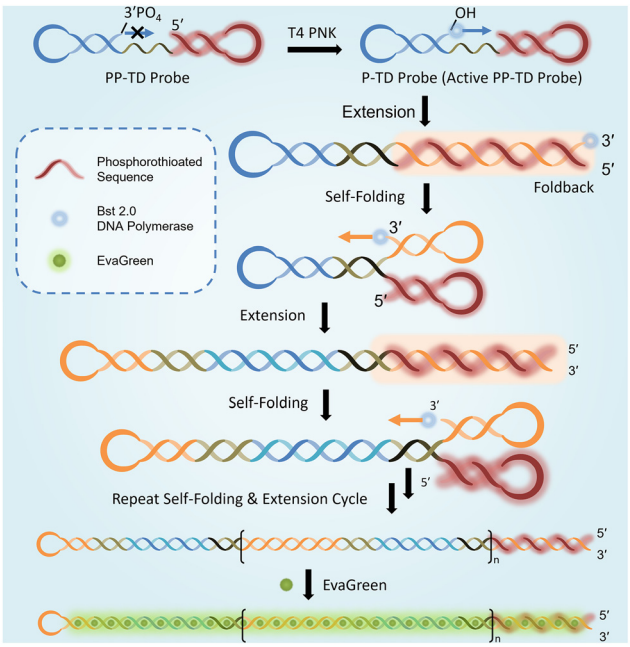
Feasibility research

In the developed detection method, PNK was used as a trigger to activate the PP-TD probe. An "active PP-TD probe", phosphorothioated-terminal dumbbell (P-TD) probe, whose phosphate group at the 3′-terminal was replaced by a hydroxyl group, was chosen to verify and test whether the ssDNA probe with PS-modified hairpin and palindromic structures at both ends can initiate its self-folding and extension reaction in the presence of Bst 2.0 DNA polymerase. As indicated in Fig. 1a, the fluorescence intensity was positively correlated with the concentration of the P-TD probe in an S-shaped curve, indicating that the dumbbell structure can realize self-folding and extension reactions.

The feasibility of the PNK-activation principle was also explored. The hydrolyzing reaction could not work in the absence of either active PNK or ATP. Hence, the fluorescence intensity under different conditions was compared to verify feasibility of this PNK system, considering the presence or absence of PNK and ATP and the addition of inactive PNK. Fig. 1b shows that in the presence of both active PNK and ATP, the PP-TD probe was activated due to the 3′-dephosphorylation of PNK. This activation mechanism was further illustrated by comparison with the P-TD probe, which indicated a similarly high amplification efficiency at the same time. All results indicate the need for specific 3′-dephosphorylation of PNK. Hence, it is feasible to develop a strategy for initiating self-sequence amplification by using the designed PP-TD probe.

The design of PP-TD probe

The structure of the PP-TD probe significantly affects its stability and amplification efficiency. Firstly, the length of the loop on the PP-TD probe was optimized (sequences are provided in Table S1†). The fluorescence signal reached a maximum when



Scheme 1 Schematic illustration for the PP-TD probe based PNK detection assay.

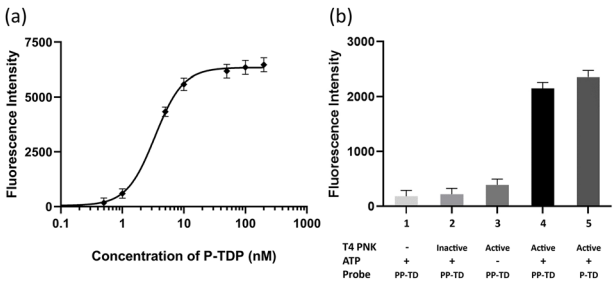


Fig. 1 Feasibility research of (a) self-folding and extension reaction and (b) PNK-activating principle.

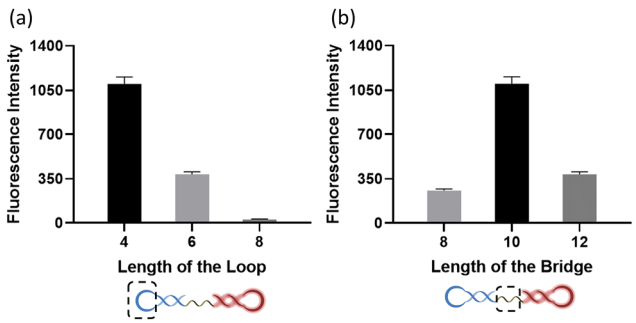


Fig. 2 Optimization of PP-TD probe sequence. (a) Fluorescence intensity *vs.* length of the loop. (b) Fluorescence intensity *vs.* length of the unpaired stem.

the length of the loop was 4 nt (Fig. 2a). A longer loop may have created increasing instability to the structure, leading to failed self-folding. We changed the length of the unpaired fragment on the stem (sequences with different bridge lengths are provided in Table S1†). As shown in Fig. 2b, the maximum fluorescence intensity reached a moderate bridge length of 10 nt. A bridge that is too short is likely to cause steric hindrance during the enzyme degradation process, and a bridge that is too long may not be conducive for maintaining the stability of the palindromic structure.

Optimization of the reaction conditions

Since appropriate reaction conditions are critical for enzyme degradation and amplification processes, the reaction conditions were optimized to improve the performance of our strategy. The concentration of the PP-TD probe was first examined, because it plays an essential role in dephosphorylation and amplification. As shown in Fig. S1a,† an improved fluorescence response was achieved at a concentration of 50 nM. Thus, 50 nM was chosen as the optimal PP-TD probe concentration. As an energy donor, ATP plays a key role in phosphorylation. With increasing ATP concentrations from 0 to 10 mM, the fluorescence intensity reached a maximum at 1 mM (Fig. S1b†). Therefore, 1 mM was chosen as the optimal working concentration of ATP for phosphorylation. Low concentration of ATP cannot provide efficient energy for the phosphorylation reaction, whereas further addition of ATP inhibits the reaction through competitive binding to PNK. DNA polymerase also plays a vital role in the system, considering that the enzyme-catalyzed reaction rate is correlated with the enzyme concentration. Amplification process of the proposed method is also catalyzed by DNA polymerase. Therefore, we screened the concentration of Bst 2.0 DNA polymerase in the range of 2.5 to 40 U mL^{-1}. As shown in Fig. S1c,† Bst 2.0 DNA polymerase concentrations greater than 15 U mL^{-1} had insignificant improvement in the fluorescence intensity; therefore, 15 U mL^{-1} was determined as the optimal concentration.

Selectivity of the PNK assay

Selectivity of the detection assays is critical in practical biological detection systems; therefore, we verified the selectivity of the proposed assay. Four enzymes which enzymes which worked in DNA replication and repair were chosen as control substitutes: T4 DNA ligase, Klenow fragment, Nb·BbvCl, and RecA. As shown in Fig. 3, only PNK significantly enhanced the fluorescence signal. These results suggest that this method possesses satisfactory specificity for detecting PNK activity.

Quantitative detection of PNK

We estimated the sensitivity and quantitative ability of the proposed approach under optimized conditions. Fig. 4 illustrates the linear relationship between the fluorescence intensity at 525 nm and the logarithm of the PNK concentration. Linear regression was calculated as FI = 149.4 lg C + 986.4 (R = 0.9935) where FI is the fluorescence intensity and C is the concentration of PNK. PNK can be quantified in the range of 1.0 × 10^{-6} to 5.0 × 10^{-5} U μL^{-1}. The LOD of PNK was estimated to be 7.7 × 10^{-7} U μL^{-1}, which is 3.3 times the standard deviation of the background. The LOD is comparable to or advanced to fluorescence methods reported in recent years and the proposed method requires a relatively short detection time (Table S2†).

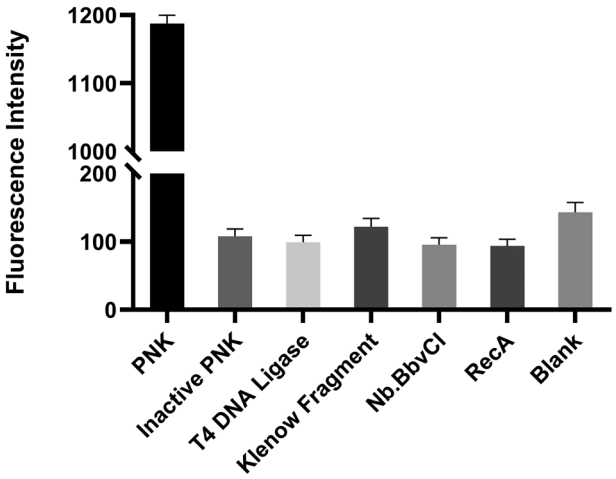


Fig. 3 Selectivity of the proposed PNK detection assay.

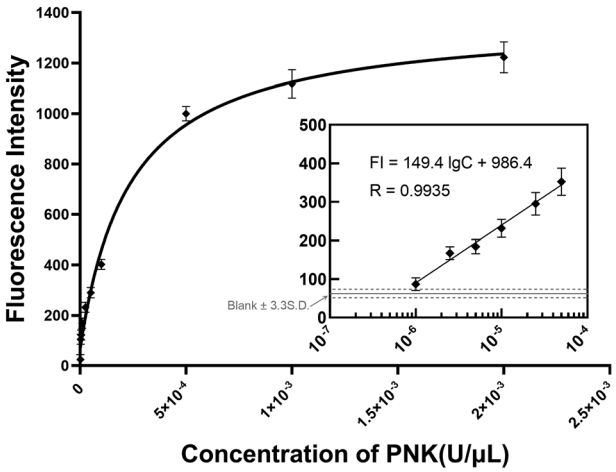


Fig. 4 Concentration-dependent fluorescence response for detection of PNK activity.

PNK activity detection in diluted cell extracts

To further examine the capability of the proposed assay in real sample analysis, cell lysates (A549) were used to simulate the intracellular environment. Different concentrations of PNK were spiked into 100-fold diluted cell lysates. Fluorescence intensity changed linearly with the logarithm of PNK concentration in the range of 2.5 × 10^{-5} to 1.0 × 10^{-3} U μL^{-1}, and the values were close to those in the reaction buffer (Fig. S2†).

Linear regression was calculated as FI = 415.1 lg C − 2840.7 (R = 0.9904) and the results indicate that this assay has potential for use in biological sample detection.

PNK inhibitor screening assay

Since abnormal activity of PNK can induce serious neurological diseases, screening for PNK inhibitors is of great significance in drug discovery and clinical treatment. $(NH_4)_2SO_4$ and NaH_2PO_4 were used in inhibition assays. These inhibitors influenced the phosphorylation by salt effects that possibly changed the conformation of PNK into an inactive state,[28] without interfering with the Bst 2.0 DNA polymerase activity. As shown in Fig. 5, the fluorescence intensity decreased gradually as the concentration of the inhibitor increased. This is because the depressed activity of PNK resulted in a decrease in the probes that could be amplified by the system. The half-maximal inhibition values (IC_{50}) for $(NH_4)_2SO_4$ and NaH_2PO_4 were 16.7 mM and 8.2 mM, respectively, which is consistent with previous reports.[29–32] These results demonstrated that the proposed PP-TD probe is promising for PNK inhibitor selection in drug discovery.

Experimental

Apparatus

The fluorescence measurements were conducted with a Synergy™ H1 full-function microplate reader (Berton Biotek).

Materials and reagents

The oligonucleotides were synthesized by Sangon Biotech Co. (Shanghai, China) and Table S1† shows the sequences. T4 PNK, Bst 2.0 DNA polymerase, T4 DNA ligase, Nb.BbvCl, and RecA were purchased from New England Biolabs (NEB, U.K.). Adenosine triphosphate (ATP) was purchased from Sangon Biotech (Shanghai, China). Klenow Fragment and deoxynucleotide (dNTP) mixture were obtained from Takara Bio Inc. (Dalian, China). EvaGreen® Dye, 20× in water was purchased from Biotium (California, USA). Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), and trypsin were from GIBCO (Waltham, MA). A549 cell line was obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

Detection of PNK

Certain concentration of PNK were incubated with PP-TD probe in reaction buffer for 30 min at 37 °C, hydrolyzing the phosphate group at the 3′-end to a hydroxyl group, followed by inactivation of PNK *via* incubation at 95 °C for 5 min. Then, a 20 μL reaction mixture was set up, containing dNTP (10 mM), Isothermal buffer (1×), Bst 2.0 DNA polymerase (0.4 U) together with the reaction solution from the first step (diluted 5 times) and incubated at 60 °C for 1 h. Finally, 1× EvaGreen were added into the reaction buffer as the indicator. After 10 min incubation at 25 °C, the fluorescence signal was measured with excitation of 485 nm and emission of 525 nm by spectro fluorophotometer.

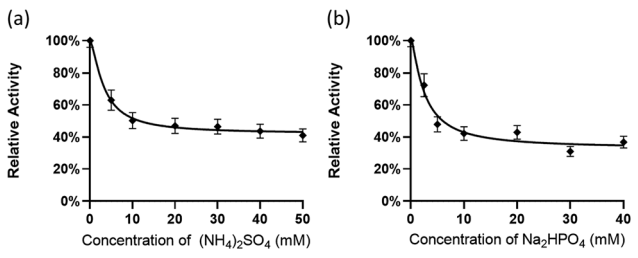


Fig. 5 Inhibition effects of (a) $(NH_4)_2SO_4$ and (b) Na_2HPO_4 on phosphorylation.

Cell culture and activity detection of PNK in cell lysates

Lysates of Human lung adenocarcinoma cell lines (A549) was chosen to simulate the complicated environment of real samples.

Cell lines were cultured in DMEM with 10% FBS and were incubated at 37 °C with 5% CO_2. Then, trypsin was used for cell digestion and cells were resuspended in DMEM. After centrifuged at 1000 rpm for 3 min, the supernatant was discarded and the cells were washed with phosphate buffer, resuspending in reaction buffer. After ultrasonication and centrifugation (14 000 rpm, 30 min, 4 °C), the supernatant was used as cell lysates in following experiments. PNK at a series of concentrations were spiked into the cell lysates and then were detected using the same method as mentioned in Part "Detection of PNK".

Inhibition detection

In inhibition detection, two known inhibitors of PNK ($(NH_4)_2SO_4$ and NaH_2PO_4) were used. Inhibitors at a series of concentrations were introduced into the hydrolyze process. The subsequent processes were consistent with mentioned in Part "Detection of PNK".

Conclusions

A novel assay was developed for PNK detection based on PP-TD probes with high sensitivity and selectivity. This detection system requires no additional primers to initiate amplification of DNA palindrome sequences, thus avoiding interference of complex by-products from primer interactions. This also simplifies the process because the amplified product itself works as a template for the next round of amplification. Furthermore, this assay enabled detection of PNK activity in cell lysis dilutions and has great potential for quick analysis and on-site screening. Finally, the proposed assay can be applied to screen for PNK inhibitors, providing a basis for drug discovery and clinical treatment.

Author contributions

Xianfeng Jiang: conceptualization, data curation, formal analysis. Xudan Shen: writing original draft, methodology. Jadera Talap: visualization. Dan Yang: data curation. Su Zeng:

funding acquisition, supervision. Hui Liu: validation, supervision. Sheng Cai: methodology, funding acquisition, supervision, project administration.

Conflicts of interest

There are no conflicts to declare.

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