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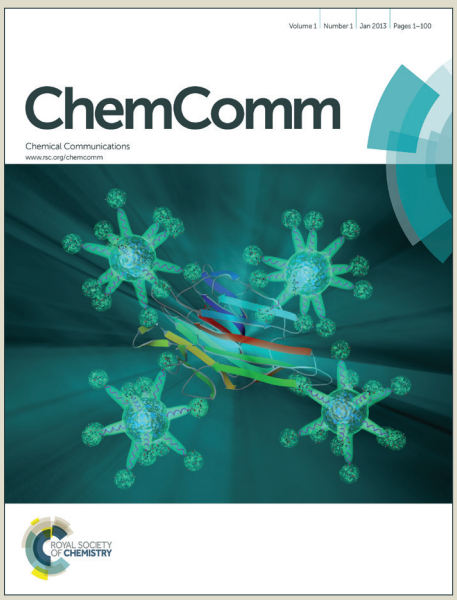

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Sensitive detection of T4 polynucleotide kinase activity based on β-cyclodextrin polymer enhanced fluorescence combined with exonuclease reaction

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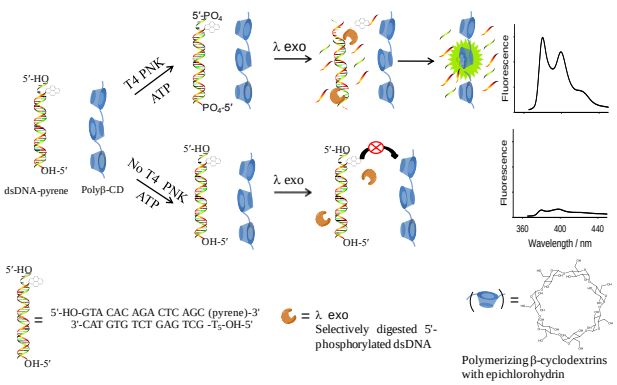
A strategy for T4 polynucleotide kinase activity detection was proposed based on β-cyclodextrin polymer (polyβ-CD) and exonuclease reaction. The fluorescence of pyrene enhanced more than 10 times in the presence of polyβ-CD, and a simple detection of T4 PNK was achieved with a detection limit of 0.02 unit/mL.

Phosphorylation of the 5′-hydroxyl termini of DNA plays a critical role in a majority of normal cellular events, including DNA recombination, DNA replication, and repair of DNA during strand interruption.[1, 2] Polynucleotide kinase (PNK) can catalyze the transfer of the terminal phosphate of adenosine triphosphate (ATP) to the 5′-hydroxyl termini of nucleic acids, and it has been widely used in DNA adducts[3] / oligonucleotides[4] detection and DNA lesions repair. [5]

Traditionally, the activity of PNK was detected by radical isotope ^{32}P-labeling, polyacrylamide gel electrophoresis (PAGE) and autoradiography.[2, 6] However, these methods were time-consuming, laborious, complex, costly or radio labeling. To overcome these drawbacks, sensitive, robust, and convenient enzymes assays, including fluorescence assays[1, 7-14], colorimetric assays[15], electrochemical assays[16] and luminescence assays[17-20] have been developed. Among them, fluorescent methods were particularly attractive due to their high sensitivity, easy readout, low sample volume, simple operation and feasibility of quantification. For example, Hou et al[7], Chen et al[8] and our group[1] developed fluorescence assays by using molecular beacon to investigate the phosphorylation process of nucleic acids by PNK. Huang et al[9] and Wu et al[10] developed PNK fluorescence assays by using single-labeled DNA probes and nanomaterials. Although great advances have been made by these fluorescence quenching or restoring methods towards PNK assays, some of them require complex and expensive double label of molecular beacon, and the quenching or restoring efficiency of fluorescence dyes is limited. Much of the motivation behind improving PNK assays is to develop simple and sensitive fluorescence methods with minimal DNA modification and enhanced signal amplification.

Cyclodextrin polymers have showed efficient fluorescence emission enhancement for fluorophors.[21, 22] The presence of multiple exterior interaction sites on cyclodextrin polymer brings about enhanced recognition ability and forms high affinity host-guest complex with fluorophors, associated with excellent and stable fluorescence enhancement.[21, 22] What is more, cyclodextrin polymers exhibited unique characteristics in terms of good stability and solubility. However, despite their excellent properties, cyclodextrin polymers have rarely been applied in fluorescence assays.

Herein, a strategy for sensitive detection of PNK has been developed by combining β-cyclodextrin polymer-based fluorescence enhancement of pyrene with exonuclease reaction. As a proof-of-concept of our approach, T4 PNK was chosen as the model, and single pyrene-labeled dsDNA (dsDNA-pyrene) acted as the signal report probe (Scheme 1).



Scheme 1 Schematic representation of combining β-cyclodextrin polymer-based fluorescence enhancement of pyrene with exonuclease reaction for sensitive T4 polynucleotide kinase activity detection.

In the absence of T4 PNK, the dsDNA-pyrene with 5′-hydroxyl terminal could not be digested by λ exonuclease (λ exo), and pyrene attached on the dsDNA was difficult to enter the cavity of polyβ-CD because of steric hindrance. So the fluorescence of pyrene kept weak. In the presence of T4 PNK, the dsDNA-pyrene was phosphorylated at 5′-terminal by T4 PNK, and the resulting 5′-phosphoryl termini product was then digested by λ exo, yielding pyrene-labeled mononucleotides. Because of weak steric hindrance of mononucleotides, the pyrene could easily enter the hydrophobic cavity of polyβ-CD, accompanied with significant fluorescence enhancement. Thus, the quantitative detection was achieved through the relation between the activity of T4 PNK and fluorescence signal.

The phosphorylation-induced digestion of dsDNA-pyrene by λ exo was primary in this strategy, and was examined by gel electrophoresis. As shown in Fig. 1A, in the absence of T4 PNK or λ exo, a bright band was obtained, indicating no dsDNA-pyrene digestion. When T4 PNK and λ exo were both present, no obvious band was observed, indicating no dsDNA-pyrene exist and a high efficiency of phosphorylation-induced digestion of dsDNA-pyrene by λ exo in the presence of T4 PNK.

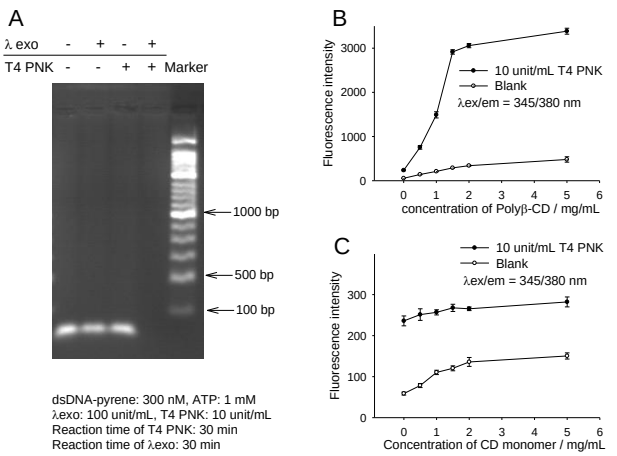


Fig. 1 (A) Agarose gel (2%) electrophoresis image. (B) Fluorescence enhancement of various concentration of polyβ-CD to the system with 10 unit/mL T4 PNK. (C) Fluorescence enhancement of various concentration of CD monomer to the system with 10 unit/mL T4 PNK. Error bars indicated the standard deviations of three experiments.

The efficient fluorescence enhancement of pyrene was the basis of our strategy. As shown in Fig. 1B, in the presence of T4 PNK, the fluorescence of pyrene enhanced greatly with the increasing concentration of polyβ-CD and more than 10 times fluorescence intensity was detected when the polyβ-CD was 1.5 mg/mL, which proved the efficient fluorescence enhancement of pyrene by polyβ-CD. In the absence of T4 PNK, the fluorescence intensity kept low with various concentration of polyβ-CD because of steric hindrance of dsDNA. Hence the feasibility of our proposed strategy was achieved. As the control, the fluorescence intensity of pyrene with CD monomer was also investigated. As shown in Fig. 1C, the fluorescence intensity of the system in the presence/absence of T4 PNK kept low and showed little change at various CD monomer concentrations. It was reported by Markus Hollas [21] that compared to monomer β-cyclodextrin, the overall complexation constant of the β-cyclodextrin polymers and pyrene increased by more than 2 orders of magnitude, which may be the reason for excellent fluorescence enhancement of pyrene by β-cyclodextrin polymer.

Kinase activity detection in real-time was of great scientific and biological interest.[1] To confirm the ability of this strategy in real-time monitoring, the fluorescence emission intensity of system in the presence/absence of T4 PNK was investigated. As shown in Fig. 2, the emission maximum in the absence of T4 PNK at 380 nm kept very low, indicating that there was no significant change of dsDNA-pyrene. The emission maximum in the presence of T4 PNK was gradually increased with the addition of λ exo, with the slope of the curve decreasing gradually as time lapsed. This indicated the phosphorylation-induced digestion of dsDNA-pyrene by λ exo occurred and pyrene entered the hydrophobic cavity of polyβ-CD for fluorescence enhancement.

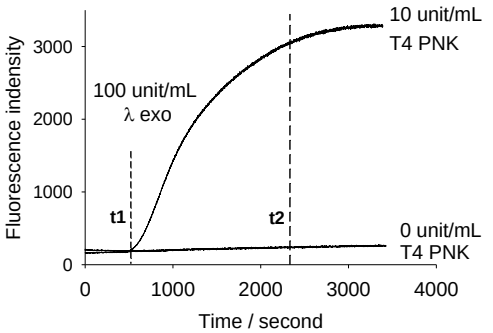


Fig. 2 Real-time monitoring the activity of T4 PNK. λ exo and T4 PNK were added at time t1. The fluorescence intensity of the system reached 90% of the maximum at t2 (1800 seconds from t1 to t2).

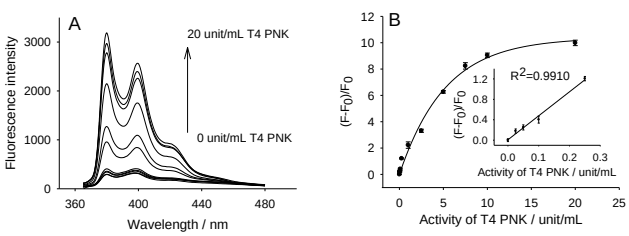


Fig. 3 (A) Fluorescence spectra of detection system in the presence of various activity of T4 PNK (from bottom to top): 0, 0.025, 0.05, 0.1, 0.25, 1, 2.5, 5, 7.5, 10 and 20 unit/mL. (B) Plot of T4 PNK *vs* $(F-F_0)/F_0$. Inset is the calibration curve for concentration of T4 PNK from 0 to 0.25 unit/mL. The concentration of polyβ-CD, dsDNA-pyrene, ATP and activity of λ exo were 1.5 mg/mL, 300 nM, 1 mM and 100 unit/mL, respectively. Error bars indicated the standard deviations of three experiments.

To characterize the detection range of T4 PNK activity, the fluorescence emission spectra of the system containing various amount of T4 PNK were measured under the optimized condition (Fig. S2 in ESI). As shown in Fig 3A, the fluorescence intensity of the system enhanced with the increase of T4 PNK activity from 0 to 20 unit/mL. The fluorescence increasing factor was $(F-F_0)/F_0$, where F and F_0 were the fluorescence intensities of the detection system

with and without T4 PNK respectively. A linear dependence of fluorescence increasing factor on T4 PNK activity in the range of 0-0.25 unit/mL was found (Fig. 3B, insert). The detection limit was 0.02 unit/mL (S/N = 3), which was lower than that of the radical isotope ^{32}P-labeling methods and comparable to the results obtained from previous fluorescence assays (Table S1 in ESI).

The influence of proteins and enzymes for T4 PNK activity assay was evaluated by investigating the response of the system to T4 PNK in the presence of proteins and enzymes, such as BSA, avidin, ssDNA binding protein (SSB), lysozyme, thrombin and Bst DNA polymerase (Bst pol). As shown in Fig. S3 (in ESI), at various conditions, the fluorescence intensity of the system kept weak in absence of T4 PNK and, in the presence of T4 PNK, the fluorescence intensity of the system enhanced greatly and kept similar at various conditions as in buffer. These results indicated that these proteins and enzymes did not influence the detection of T4 PNK obviously, which presumably originated from the specific phosphorylation reaction of T4 PNK and digestion reaction of λ exo.

We used 50% cell extract to investigate the feasibility of this strategy in biological samples. T4 PNK-containing cell extract samples were prepared by adding T4 PNK in 50% cell extract contained reaction buffer and then the target was detected. The calibration curve was obtained using 50% cell lysate with various activity of T4 PNK added. As shown in Fig 4, a plot of the fluorescence intensity at 380 nm versus the activity of T4 PNK revealed a dynamic correlation between the fluorescence intensity and activity of T4 PNK in the range from 1 to 8 units/mL, The result suggested that the signal change of the method is dependent on the activity of T4 PNK in complicated biological sample, such like cell lysate.

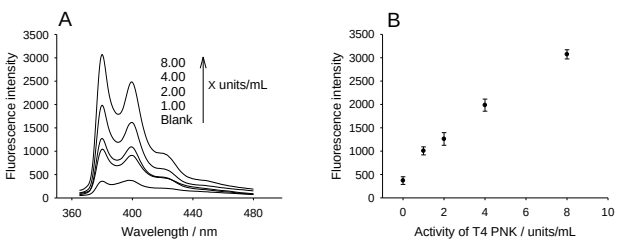


Fig. 4 (A) Fluorescence spectra of the system for various activity of T4 PNK in 50% cell lysate. (B) Fluorescence intensity at 380 nm of the system as a function of the various activity of T4 PNK in 50% cell lysate. The concentration of polyβ-CD, dsDNA-pyrene, ATP and activity of λ exo were 1.5 mg/mL, 300 nM, 1 mM and 100 units/mL, respectively. Error bars indicated the standard deviations of three experiments.

The application of our proposed strategy for T4 inhibitors screening was also investigated by using three known T4 PNK inhibitors (ADP, NaH_2PO_4, and $(NH_4)_2SO_4$), which were considered to have no inhibition effect on the activity of λ exo.[7] The inhibition assays were carried out with T4 PNK at a fixed activity of 10 unit/mL. As seen in Fig. 5, the fluorescence intensity decreased gradually with increasing concentration of inhibitor, which indicated that the activity of T4 PNK became weaker. 1 mM ADP, 20 mM NaH_2PO_4 and 10 mM $(NH_4)_2SO_4$ effectively suppressed about 50% decrease of T4 PNK activity. The half inhibition concentrations of these three inhibitors were approximate to those previously reported.[1, 7, 12]

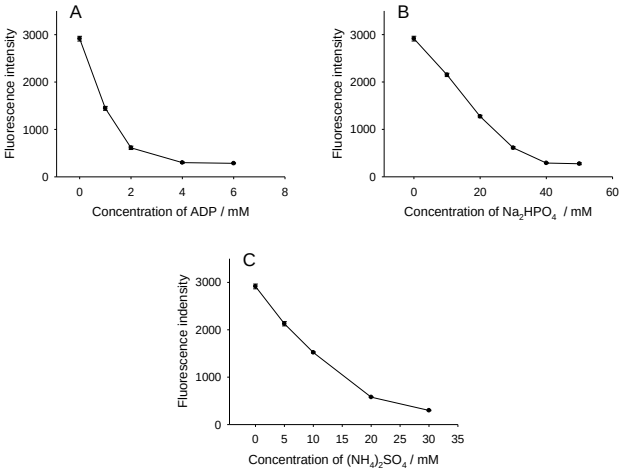


Fig. 5 The capability of the detection system for inhibitors screening. Fluorescence intensity of the detection system in the presence of different concentration of ADP, Na_2HPO_4 and $(NH_4)_2SO_4$ was shown in Fig. 4A, Fig. 4B and Fig. 4C respectively. The concentration of polyβ-CD, dsDNA-pyrene and ATP was 1.5 mg/mL, 300 nM and 1 mM. The activity of λ exo and T4 PNK was 100 unit/mL and 10 unit/mL. The excitation/emission wavelength was set at 345/380 nm. The error bars indicated the standard deviations of three experiments.

In conclusion, we have successfully developed a simple and sensitive T4 PNK activity detection strategy that combines β-cyclodextrin polymer-based fluorescence enhancement of pyrene with exonuclease reaction. This strategy offered several advantages: 1) Owning to the excellent fluorescence enhancement of pyrene by polyβ-CD (more than 10 times), sensitive detection of T4 PNK activity was achieved with a limit detection of 0.02 unit/mL; 2) It avoided double-labeling and complex design of DNA probe; 3) It worked well in complex samples; 4) It also has the potential for screening of T4 PNK inhibitors. Altogether, the developed strategy provides a new opportunity for T4 PNK assay and holds a great potential to apply in the researches of DNA phosphorylation-relevant process, drug developments, and clinical diagnostic.

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Notes and references

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Electronic Supplementary Information (ESI) available: chemicals and materials, experimental details, synthesis of β-cyclodextrin polymer, conditions optimization, investigation of the influence of proteins and enzymes, detection in cell extracts, and inhibitors screening as noted in the text. See DOI: 10.1039/c000000x/

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