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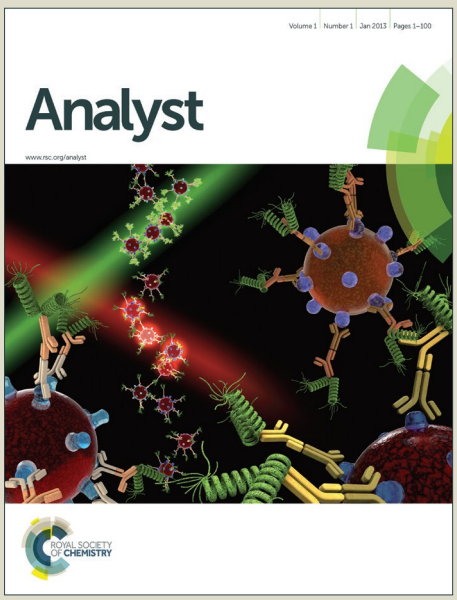

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ARTICLE TYPE

Label-Free Cyclic Assembly of G-quadruplex Nanowires for Cascade Amplification Detection of T4 Polynucleotide Kinase Activity and Inhibition

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Several fluorescence methods have been developed for sensitive detection of PNK activity based on signal amplification techniques, but they need fluorescently labeled DNA probe and superabundant assistant enzyme. We have addressed these limitations and report here a label-free and enzyme-free amplification strategy for sensitively and specifically studying PNK activity and inhibition via hybridization chain reaction (HCR). First, the phosphorylation of hairpin DNA H1 by T4 PNK make it be specifically digested by lambda exonuclease (λ exo) from 5′ to 3′ direction to generate a single-stranded initiator which can successively open hairpin H2 and H3 to trigger autonomous assembly of long DNA nanowires. Meanwhile, intermolecular G-quadruplex formed between H2 and H3, thereby providing a fluorescence enhancement of N-Methyl mesoporphyrin IX (NMM) which is a highly quadruplex-selective fluorophore. So, the PNK activity can be facilely and sensitively detected by using NMM as a signal probe which provide a low background signal to improve overall sensitivity, resulting in detection limit of 3.37 $\times 10^{-4}$ U/mL. More importantly, the successful application for detecting PNK activity in complex biological matrix and studying the inhibition effects of PNK inhibitors demonstrated it provides a promising platform for screening PNK inhibitors as well as detecting PNK activity. Therefore, it is a highly sensitive, specific, reliable and cost-effective strategy which shows great potential for biological process research, drug discovery, and clinic diagnostics.

Introduction

The 5′-polynucleotide kinase (PNK), as a crucial class of enzyme that catalyzes the phosphorylation of nucleic acids with 5′-hydroxyl termini, has been well-known for playing a vital role in a majority of normal cellular events, including DNA recombination, DNA replication, and DNA repair.[1-5] Aberrant PNK behaviors and blocked DNA phosphorylation states may be closely related to some serious human disorders, for example Bloom's syndrome, Werner syndrome, and Rothmund–Thomson syndrome.[6] On the other side, inducing DNA lesion and interfering with DNA repair pathways of tumor cells leading to the necrosis and apoptosis of tumor cells could thereby sensitize cells to DNA-targeting therapies.[7] So, study the inhibitors for PNK is instructive to the effective therapeutic protocols and kinase-targeted drug discovery. On account of the significance of PNK, the development of assays for PNK activities and its potential inhibitors is therefore of fundamental importance.

Traditionally, autoradiography, radical isotope ^{32}P-labling, and polyacrylamide gel electrophoresis (PAGE) are used to detect the PNK activity.[5,8-13] However, these methods have the shortcomings of expensive instruments, sophisticated operating procedures, or even potential radioactive hazards which prohibit their wide applications. To overcome these drawbacks, alternative methods have been developed, such as fluorescence assays, colorimetric assays, chemiluminescence methods, and electrochemical methods, in which fluorescence strategies attract much attention due to their high sensitivity and more flexible sensing modes. For example, Song and co-workers had utilized a singly-labeled DNA-hairpin probe coupled with exonuclease cleavage to realtime monitor the activity and kinetics of T4 PNK.[14] Lin et al.[15] and Zhang et al.[16], respectively, used functional materials such as graphene oxide and copper nanoparticle as signal conversion element for PNK detection. He et al. designed a hairpin sequence which could form G-quadruplex induced by K^+ ions and chose a Ir (III) complex as a G-quadruplex-selective probe for detecting the polynucleotide kinase activity.[17] Although these fluorescence methods have made great improvements toward PNK detection, it should be noted that further improvement of the analytical performances, particularly sensitivity, is still in high demand. As well known, studying the activity of PNK and its potential inhibitors is of great important not only for investigating the phosphorylated biochemical process during nucleic acid metabolism but also valuable for developing effective molecularly targeted therapies and kinase-targeted drug discovery. Furthermore, these processes often have a relatively thirsty demand on sensitivity. Currently, several signal amplification strategies were developed to improve sensitivity of PNK activity assay. Wang et al. successfully screened DNA phosphorylation process by coupling split DNAzyme and ligation-triggered DNAzyme cascade amplification.[18] Hou et al. combined exonuclease enzyme

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reaction and bimolecular beacons (biMBs)-based signal amplification proposed a novel strategy for the sensitive determination of T4 PNK activity.[19] But these methods ofen need fluorescence-labeled DNA probes or superabundant assistant enzyme. Consequently, the establishment of a label-free and enzyme-free amplification approach for the sensitive detection of T4 PNK activity is greatly desirable.

As a promising amplification technique for ultrasensitive bioanalysis, hybridization chain reaction (HCR) has aroused considerable attention owing to its enzyme-free and isothermal operation. HCR is based on the chain reaction of recognition and hybridization events between two hairpin DNA probes, in which a long nicked dsDNA molecule is formed by a cascade of toehold mediated DNA strand displacement events triggered by an initiator DNA and can be used to design enzyme-free and powerful amplification cascades to achieve polynomial or exponential amplification of input signals.[20,21] Conventional HCR usually needs two labeled hairpin DNA probes. Herein, making the best of the enzyme-free amplification ability of HCR technique, a label-free amplification strategy has been proposed for studying protein kinase activity and inhibition. A quadruplex-selective fluorescence probe, NMM, was used as signal probe. Three hairpin DNAs without any labeling were designed as molecular recognition probes. As well known, λ exo can selectively remove the 5′-phosphorylated strand of dsDNA.[22] So, the stem part of phosphorylated H1 can be digested by λ exo to generate a initiator DNA that can trigger HCR between H2 and H3 DNA probe to autonomous assembly of dsDNA polymer accompanied with interchain G-quadruplex. The G-quadruplex formation could cause a significant increase in fluorescence intensity, which is utilized for detecting PNK activity and studying inhibition of PNK activity. To the best of our knowledge, it is the first time that realizes label-free fluorescence amplification for studying T4 PNK activity and inhibition by HCR, which shows great potential for studying biological process based on HCR technique and also is of great theoretical and practical importance in bioanalysis, drug screening and clinic diagnostics.

Experimental Section

Chemicals and Reagents

Lambda exonuclease (λ exo, 5 U/μL) was purchased from New England Biolabs (NEB, U.K.). T4 polynucleotide kinase (PNK, 10 U/μL), EcoRI, BamHI endonucleases, adenosine triphosphate (ATP), adenosine diphosphate (ADP) were obtained from Sangon Biotech Co. (Shanghai, China). Lysozyme (Lys) and Immunoglobin G (IgG) were purchased from Beijing Dingguo Biotechnology Co., Ltd (Beijing, China). Protein kinase A (PKA, catalytic subunit from recombinant E.coli strain) was obtained from New England Biolabs (NEB, U.K.). N-Methyl mesoporphyrin IX (NMM) was purchased from Frontier Scientific, Inc (Logan, Utah, USA). The sequences of oligonucleotides used in the assay were listed as follows: hpDNA1 (5′-GTACTTAAACACCTTCTTCTATACATTTGAC AGAAGAAGGTGTTTAAGTAC-3′, H1), hpDNA2 (5′-AGGG CGGGTGGGTGTTTAAGTTGGAGAATTGTACTTAAACAC CTTCTTCTTGGGT-3′, H2), hpDNA3 (5′-TGGGTCAATTCTC CAACTTAAACTAGAA GAAGGTGTTTAAGTTGGGTAGG GCGGG-3′, H3). They were synthesized by Shanghai Sangon Biotechnology Co. (Shanghai, China). The H1 oligonucleotide stock solutions were prepared with PNK buffer (70 mM Tris-HCl solution pH 8.0, 10 mM $MgCl_2$, 5 mM DTT). H2 and H3 DNA stock solutions were prepared in phosphate buffer (0.4 M NaCl, 50 mM Na_2HPO_4, pH 7.4). Each hairpin probe was heated to 95 ºC for 2 min and then allowed to cool to room temperature for 1 h before use. Millipore MilliQ (18 MΩ cm) water was used in all experiments.

Apparatus

All fluorescence measurements were performed on Hitachi F-7000 fluorescence spectrophotometer (Kyoto, Japan). The vertical electrophoresis system was purchased from Bio-Rad Laboratories, Inc. The Molecular Imager system was purchased from Shanghai Peiqing science & Technology. Co., Ltd (Shanghai, China). Circular Dichroism spectras were measured on a Chirascan Circular Dichroism Spectrometer (Applied Photophysics Ltd, England, UK). Fluorescence microscopy images were recorded using TH4-200 (Olympus, Japan).

Assay of Kinase Activity and Inhibition

H1 (100 nM) was mixed with certain amounts of T4 polynucleotide kinase in 25 μL buffer (70 mM Tris-HCl solution pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP) and 0.022 U/μL λ exo at 37 ℃ for 30 min. After that, the above mixture was heated at 75 ºC for 10 min to inactivate the T4 PNK and λ exo. Then, the above solution was added into 300 nM H2 and 300 nM H3 in 75 μL of HCR reaction buffer (0.4 M NaCl, 50 mM Na_2HPO_4, pH 7.4). The total volume of reaction solution is 100 μL. To investigate the effects of inhibitors on the phosphorylation process of T4 PNK, ADP (0.25-5 mM), $(NH_4)_2SO_4$ (5-30 mM) and Na_2HPO_4 (5-30 mM) were selected as inhibitors of PNK in the inhibition experiments. The inhibitors were contained in the phosphorylation reaction buffer, respectively. After addition of 100 nM H1, 1 mM ATP, 0.022 U/μL of λ exo, 5 U/mL of PNK, the reaction was kept at 37 °C and the procedures were similar to that mentioned above.

Fluorescence microscopy image

First, the glass slides were washed with distilled water, followed by an ethanol and acetone wash and then were UV/ozone cleaned. Then, the DNA polymer containing interchain G-quadruplex solution was dropped on the surface of the clean glass slides.

Gel Electrophoresis

A 12.5 % native polyacrylamide gel was prepared by using 1 × TBE buffer (100 mM Tris-HCl, 83 mM boric acid, 1 mM EDTA, pH 8.0) as running buffer. 5.0 μL of each DNA sample was loaded into the lanes and performed at a constant potential of 150 V for 30 min. After stained for 30 min in ethidium bromide solution, and then photographed with Molecular Imager with Gel Doc system.

Circular Dichroism Spectroscopy

The CD spectra of haripin and double-stranded polymer were measured by using a Chirascan Circular Dichroism Spectrometer. CD spectra were recorded using a quartz cell of 1 mm optical path length and an instrument scanning speed of 100 nm/min

with a response time of 2s at room temperature. CD spectra were obtained by taking the average of three scans made from 210 to 320 nm. All the DNA samples were dissolved in the buffer (0.4 M NaCl, 50 mM Na_2HPO_4, pH 7.4) and heated to 90 °C for 5 min, gradually cooled to room temperature, and incubated at 4 °C overnight. The background of buffer was subtracted from CD data.

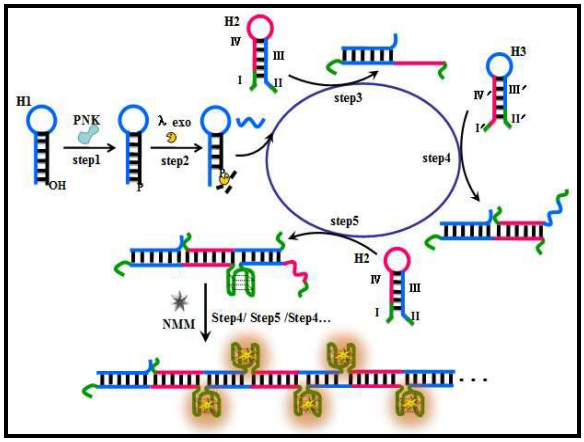
PNK activity detection in diluted cell extracts

Hela cells were cultured in DMEM medium supplemented with 10 % fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin at 37 ℃ under 5 % humidified CO_2. Cell extracts were prepared according to the previous reports.[23] The harvested cells were resuspended in 20 μL 10 mM Tris-HCl (pH 7.8) containing 150 mM NaCl. Equal volume of lysis buffer was added into the suspension containing 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 2 mM EDTA, 2 mM DTT, 40 % glycerol, 0.2 % NP40, and 0.4 mM phenylmethylsulfonyl fluoride. The mixture were incubated for 1.5 h at 4 ℃ with occasional shake. Cell debris was removed by centrifugation at 16000 g for 10 min, and the supernatant was recovered. Diluted cell extracts were added to the assay solution (1%). The detection procedure was the same as those described in the aforementioned experiment for PNK detection in pure reaction buffer.

Results and discussion

Sensing mechanism

Scheme 1 outlines the principle of an enzyme-free amplification strategy to monitor PNK activity by coupling exonuclease cleavage reaction with HCR. Three hairpin oligonucleotides were designed to keep their stable hairpin conformation in a kinetic trap, including H1, H2 and H3. The blue portion of H1 is designed containing HCR initiator strand which is complementary to the domain Ⅲ of H2. In the presence of PNK, the 5′-hydroxyl terminal of H1 was phosphorylated (step 1) and was further digested by λ exo from 5′ to 3′ direction to generate a single-stranded initiator strand



Scheme1 Schematic illustration of label-free signal amplification for detecting T4 polynucleotide activity and inhibition based on cyclic assembly of G-quadruplex nanowires induced by hybridization chain reaction.

(step 2).[22] The initiator strand can pair with the domain Ⅲ of H2 to release the domain Ⅳ and domain Ⅰ, step 3. Then, domain Ⅳ in the resulting structure hybridizes with the domain Ⅳ′ of H3, and opens the hairpin of H3 (step 4) to expose domain Ⅲ′ which is identical to the initiator strand of HCR in sequence and can start a subsequent reaction cycle. These make the G-quadruplex subunits (green portion of H2 and H3), domain Ⅰ, Ⅰ′, Ⅱ, Ⅱ′, to self-assembly into interchain G-quadruplex structure (step 5). So, each copy of initiator strand can give rise to an enzyme-free autonomous cross-opening of H2 and H3 via step 3, step 4 and step 5 to form long DNA nanowires which consist of multiple interchain G-quadruplex units. N-Methyl mesoporphyrin IX (NMM) is an anionic porphyrin which shows a pronounced structural selectivity for G-quadruplexes other than duplexes, triplexes, or single-stranded DNA forms.[24] So, it can selectively bind to interchain G-quadruplex units to produce a dramatically high fluorescence response, which can effectively and sensitively detect PNK activity.

Firstly, fluorescence analysis has been performed to verify the feasibility of this scheme**.** As shown in Figure 1A, NMM alone produces negligible fluorescence (curve a). Due to the weak interaction between DNA duplex and NMM, the mixture of NMM and H1 also showed low fluorescence (curve b). After addition of H2 and H3, the fluorescence intensity of NMM/H1/H2/H3 mixture increased modestly (curve c) since few toehold of H2 and H3 were frayed occasionally to form G-quadruplex complexes. However, a significant increase in NMM fluorescence was observed (curve d) when H1 incubated with both T4 PNK and λ exo in the mixture, indicating that a higher amount of G-quadruplex was continuously formed by HCR. Thus, the preliminary results demonstrated this label-free cascade amplification strategy is feasible to detect PNK activity.

Then, the non-specific interaction has been systematically investigated. From Figure 1B, the fluorescence of NMM/H1/H2/H3 remained unchanged upon addition of PNK alone (curve c, d). It suggested that PNK itself had no effect on the fluorescence intensity of NMM. Futhermore, the specificity of DNA phosphorylation was studied by replacing PNK with heat-inactivated PNK. The fluorescence of NMM/H1/H2/H3 was almost no change when co-incubated with heat-inactivated PNK and λ exo (curve e). So, H1 could release initiator strand of HCR only when H1 was phosphorylated and further digested by λ exo. Therefore, the fluorescence enhancement was due to the fact that the H1 was phosphorylated by PNK, demonstrating that this label-free amplification strategy is effective and reliable to study the activity of polynucleotide kinase.

Additionally, the feasibility and reliability of this strategy has been verified by fluorescence imaging and colorimetric analysis. As shown in Figure 1C-a, long bright wires were observed when H1 was phosphorylated by PNK. But, the same phenomenon was not observed when H1/H2/H3 incubated with heat-inactivated PNK and λ exo (Figure 1C-b). Thus, the luminescent nanowires implied that the self-assembly of G-quadruplex nanowires induced by HCR were formed and this phenomenon was consistant with the previously reported result.[25] Moreover, it can get the similar result by naked eyes with the aid of a UV transilluminator. As shown in Figure 1D,

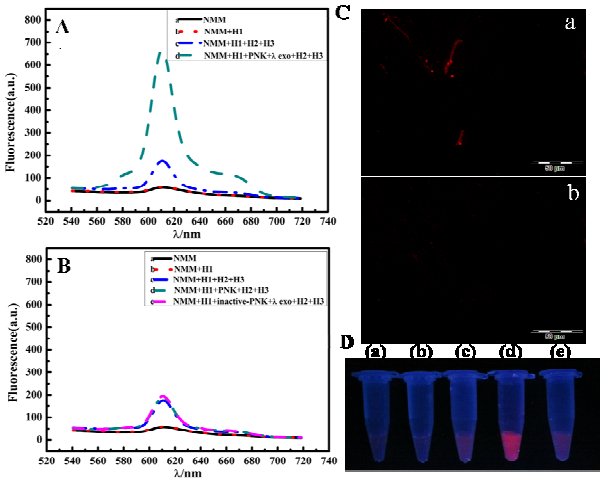


Figure 1. (A) Fluorescence response of NMM under different conditions to verify the principle. (B) Fluorescence response of NMM under different conditions to exclude nonspecific influences. The concentrations of NMM, PNK and λ exo are 250 nM, 5 U/mL, and 0.022 U/μL respectively. The concentrations of H1, H2, and H3 are 100 nM, 300 nM, 300 nM respectively. (C) Fluorescence microscopy image of the DNA nanowires. The exciting mode is GWA channel and the bar is 50 μM scale. (D) Photographs of solutions containg (a) NMM, (b) H1 + NMM, (c) H1 + H2 + H3 + NMM, (d) H1 + PNK + λ exo + H2 + H3 + NMM and (e) H1 + inactive-PNK + λ exo + H2 + H3 + NMM, which were taken using a UV transilluminator. The concentrations of NMM, H1, H2, and H3 are 5 μM, 300 nM, 1.5 μM, 1.5 μM respectively.

red nanowires formed when H1 was phosphorylated by PNK. Hairpin probes alone or heat-inactivated PNK could not trigger autonomous assembly of DNA to form interchain G-quadruplex. All results discussed above revealed that PNK activity can be reliably and specifically detected by the proposed strategy.

Assay of gel electrophoresis

To verify DNA phosphorylation and hybridization chain reaction, gel electrophoresis was utilized. First, the DNA phosphorylation induced cleavage of H1 by λ exo was studied. In Figure 2A, the DNA band in lane 1 was corresponding to DNA H1. The same band was observed from lane 2 and 3 when H1 was incubated with PNK or λ exo alone. However, the

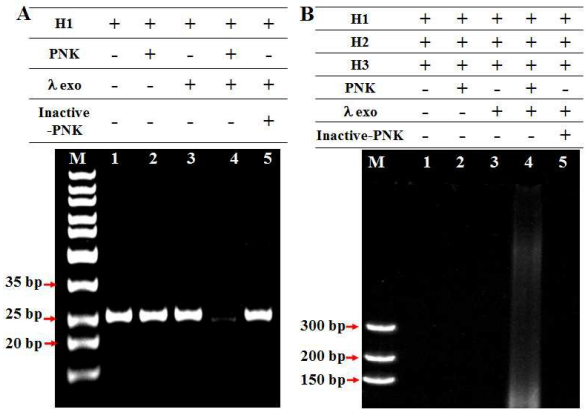


Figure 2. (A) Electrophoresis analysis of specific cleavage of H1 by λ exo. (B) Electrophoresis assay to verify T4 PNK-triggered hybridization chain reaction.

band of H1 became shallow when H1 incubated with PNK and λ exo together (lane 4), implying that the DNA phosphorylation and λ exo cleavage reaction effectively achieved. Then, DNA phosphorylation triggered HCR was also examined. As seen in Figure 2B, long DNA polymers did not form when H1 mixed with H2 and H3. The same results were observed when H1/H2/H3 mixture incubated with PNK or λ exo alone (lane 2 and 3). As a comparison, new long DNA bands appeared in the lane 4 when H1/H2/H3 mixture incubated with PNK and λ exo together, suggesting that PNK induced HCR occurred as we had expected. On the contrary, the same band corresponding to long DNA polymers was not observed when PNK was replaced by heat-inactivated PNK. The results of gel electrophoresis strongly support the principle of HCR-based amplification strategy.

Identification of Double-stranded Polymer and G-quadruplex Formation

To further identify changes in DNA conformation, circular dichroism (CD) spectra of DNA probes under various conditions were measured. From Figure 3, It was clearly found that the CD spectra of hairpin DNAs mixture (H1/H2/H3) showed a positive and a negative peak around 275 nm and 248 nm, respectively.[26,27] A blue shift of positive peak from 275 to 265 nm and 248 to 245 nm for negative peak was observed when H1, H2 and H3 incubated with PNK and λ exo. An increase in the amplitude was also obtained as well. According to the reports in the literature, a dominant positive peak around 260 nm and a negative peak around 240 nm are characteristic of parallel quadruplexes.[28,29] So, changes in CD spectra suggested the formation of double-stranded polymer containing parallel G-quadruplex structure.[30,31] On the contrary, the conformation of H1/H2/H3 mixture was unchanged when they incubated with λ exo. The same result was discovered when PNK was replaced by heat-inactivated PNK. The CD result is in accordance with fluorescence and electrophoresis, demonstrating that it is a reliable molecular amplification strategy for studying PNK activity.

Optimization of assay conditions

To achieve the high accuracy and sensitivity, influences of

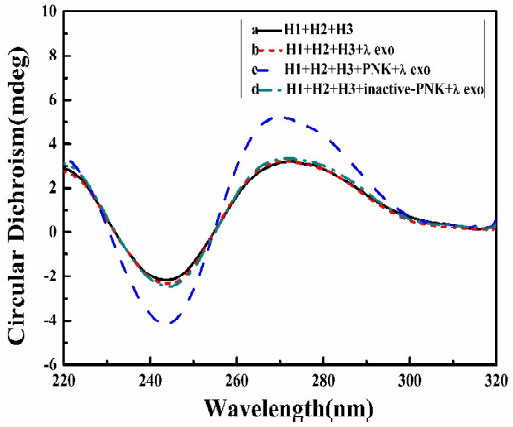


Figure 3. Circular dichroism spectra of H1/H1/H3 under different condition. The concentrations of PNK, λ exo, H1, H2 and H3 are 50 U

mL^{-1}, 0.22 U μL^{-1}, 1 μM, 3 μM, 3 μM, respectively.

some main factors should be strictly controlled. Firstly, the factors related to phosphorylation process were studied. As can be seen from Figure S1A, the fluorescence intensity increased with the increase of ATP concentration and reached its maximum at the ATP concentration of 1 mM. While, a slight decrease in the rate was observed when the concentration of ATP was higher than 1.0 mM, which is probably due to a competitive binding reaction between ATP and DNA substances to T4 PNK. The enzymatic reaction time and λ exo amount also should be carefully considered. As shown in Figure S1B and Figure S1C, the best performance was obtained when phosphorylated time was 30 min and λ exo concentration was 0.022 U/μL.

Next, factors related to HCR had been investigated. NMM was used as a signal probe whose concentration would affect the sensitivity. As presented in Figure S2A, the fluorescence efficiency reached its maximum when the concentration of NMM was 0.25 μM. Thus, 0.25 μM NMM was used as a signal probe throughout the experiment. The hairpin probes (H2 and H3) as energy sources materials for the HCR reaction were closely related to sensitivity of PNK assay. From Figure S2B, the optimum concentration of H2 and H3 was 300 nM. Finally, the optimal reaction time was also studied. As shown in Figure S2C, the highest HCR efficiency was achieved after 4 h. To consider timesaving as well as hybridization efficiency, the best reaction time of HCR was chose as 4 h.

Assay of PNK Activity

Under the optimized experimental conditions, this cascade amplification method was applied to investigate sensitivity toward PNK activity. As shown in Figure 4, the fluorescence gradually increased with the increasing of PNK concentration, indicating that this strategy had an effective and calculable response for detection of PNK activity. From the inset of Figure 4, it can be discovered that the proposed method presented a good linear response (R^2=0.9923) of fluorescence intensity against the logarithm of PNK concentration over the range from 0.001 to 5 U/mL. A detection limit of 3.37×10^{-4} U/mL was obtained,

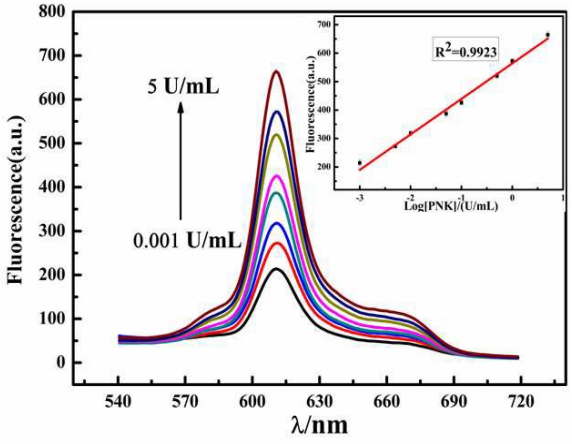


Figure. 4 PNK concentration-dependent fluorescence response. (Inset) Linear correlation of the fluorescence change vs. logarithmic concentrations of PNK in the range of 0.001 to 5 U mL^{-1}. Error bar were calculated from four replicate measurements.

which was superior or comparable with the most previous reports (Table S1). The results explained that sensitive and accurate kinase activity measurement can be achieved in a wide concentration range through this label-free amplification platform.

PNK activity detection in diluted cell extracts

The application of the proposed strategy was also tested by detecting T4 PNK in complex biological samples. In order to examine the possibility of detecting cellular T4 PNK activity profiling, HeLa cell extracts were added to the reaction buffer to mimic the intracellular environment. As can be seen from Fig S3, the similar results were obtained in the diluted cell extracts. The fluorescence intensity increased when the concentration of PNK ranged from 0.005 to 5 U/mL. The result demonstrated that the proposed approach could work well in complex biological samples, which holds great value for further using in real sample analysis.

Specificity of the PNK Assay

To further verify the specificity of PNK detection, the influence of other proteins on the PNK assay has been investigated. Five common proteins, including BamHI and EcoRI, IgG, Lys and PKA, were chosen to evaluate the specificity of PNK assay. It clearly illustrated in Figure 5A that only PNK caused a significant increase in fluorescence intensity, whereas the other proteins and inactive PNK failed to cause obvious changes in fluorescence intensity. Furthermore, the fluorescence difference was obviously distinguishable by naked

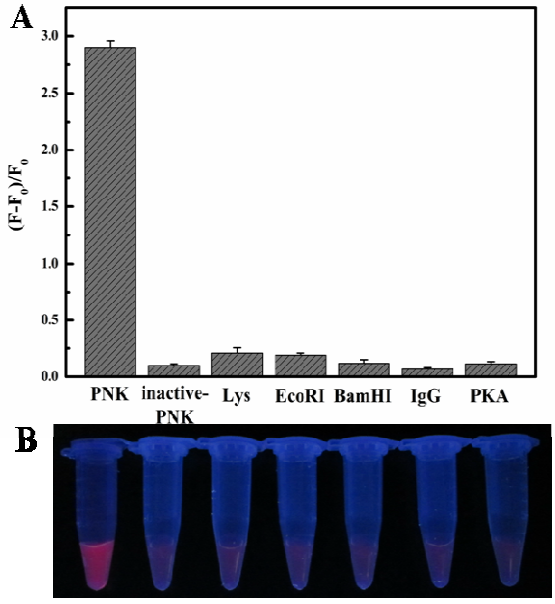


Figure 5. Selectivity of the proposed amplification strategy for T4 PNK assay. (A) Bar chart of fluorescence enhancement efficiency in the presence of different proteins. The concentrations of PNK, inactive PNK, EcoR I, BamHI and PKA are 5 U·mL^{-1}. The concentrations of IgG and Lys are 1 μM; (B) Photographs of solutions corresponding with (A) taken using a UV transilluminator, [NMM] = 5×10^{-6} M, [H1] = 300 nM, [H2] = 1.5 μM, [H3] = 1.5 μM. Error bars were calculated from four replicate measurements.

eyes under UV illumination. As shown in Figure 5B, only the solution having PNK exhibited a strong red light. So, it is a highly specific and reliable method for studying DNA phosphoryation.

Evaluation of Inhibition of PNK Activity

As we known the inhibition of nucleotide kinase has a crucial effect on cellular nucleic acid regulation and metabolism. The capacity of the developed method for studying the inhibition of T4 PNK was evaluated by using several known inhibitors, such as ADP, ammonium sulfate and sodium hydrogen phosphate. As depicted in Figure S4A, the fluorescence intensity decreased gradually with the increasing of ADP concentration. The inhibition effect is possibly due to the reversible phosphorylation reaction between ADP and 5′-phosphate DNA, resulting in the transfer of phosphate from DNA to ADP.[14] In addition, as shown in Figure S4B and S4C, ammonium sulfate and sodium hydrogen phosphate were further used to evaluate the salt effects on PNK. The fluorescence intensity gradually decreased with increasing concentrations of ammonium sulfate and sodium hydrogen phosphate, and 13.8 mM ammonium sulfate and 15.9 mM sodium hydrogen phosphate caused a 50 % decrease in fluorescence intensity, respectively. Generally, the reason for the salt effects on PNK activity is attributed to the facts that high salt concentration leads to more stable structure of H1 with the inhibition of the reactivity of 5′-hydroxyl group, and also leads to the change in conformation of PNK with reduced activity.[32,33]

Under the same concentration, it was clearly see adenosine diphosphate (ADP) led to a more effective inhibition of PNK activity (Figure 6A). Gel electrophoresis was also utilized to study the influence of ADP, ammonium sulfate and sodium hydrogen phosphate on PNK activity. From Figure 6B, the band of H1 was nearly unchanged when it was incubated with PNK or λ exo only. But it obviously shallowed when H1 incubated with both PNK and λ exo, indicating that it was phosphorylated and then specifically effectively cleaved. Compared lane 5 with lane 6 and 7, ADP showed better inhibition efficiency than ammonium sulfate and sodium hydrogen phosphate. GE result was in accordance with fluorescence result. All these results suggested that this label-free amplification strategy can reliably study T4 PNK inhibition. It reveals great potential for screening PNK inhibitors, which is of great importance in discovery of kinase-targeted drugs.

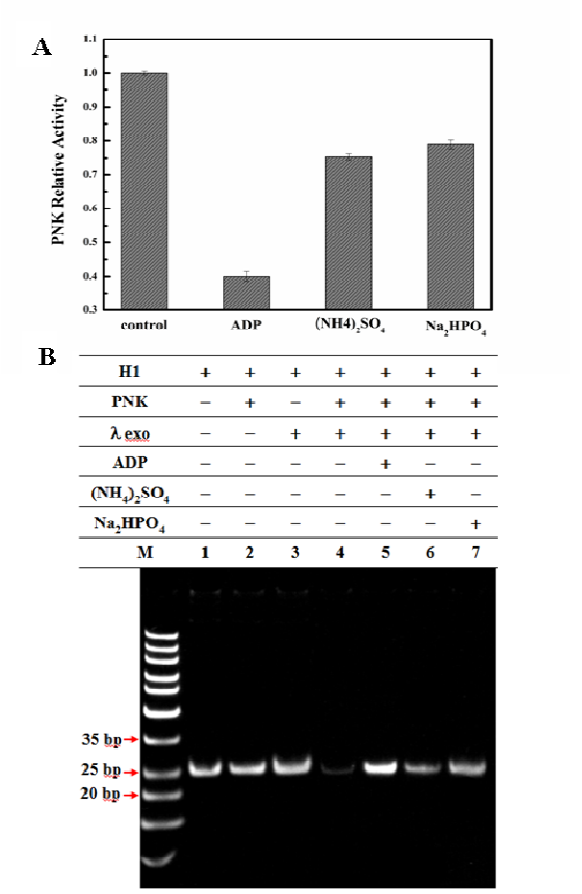


Figure 6. Inhibition effects of ADP, $(NH_4)_2SO_4$, and Na_2HPO_4 on phosphorylation. (A) Bar chart of inhibition effects toward PNK relative activity. (B) Electrophoresis analysis of inhibition effects toward PNK relative activity. Error bars were calculated from four replicate measurements.

Conclusions

In conclusion, taking advantage of enzyme-free amplification of HCR, an enzyme-free and label-free amplification strategy has been developed for cascade amplification detection of PNK activity and inhibition. By making the best of interchain G-quadruplex formation, all DNA probes did not fluorescent-labeled, achieving label-free and cost-effective signal amplification. High specificity ensured by taking full advantages of hairpin DNA and specific cleavage of λ exo. The detection limit is down to 3.37×10^{-4} U/mL which is lower than most existing works. In addition, the activity of T4 PNK in complex biological samples can be successfully evaluated. More importantly, this strategy also exhibits excellent performance in evaluating the inhibition effects of different inhibitors on phosphorylation. Therefore, this label-free strategy not only provides a new, accurate and convenient platform for monitoring the activity and inhibition of nucleotide kinase but also shows great potential in disease diagnostics, drug discovery and biological process research.

Acknowledgements

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