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ARTICLE *in* ANALYTICAL CHEMISTRY · DECEMBER 2013

Impact Factor: 5.64 · DOI: 10.1021/ac403458b · Source: PubMed

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Amplified Detection of T4 Polynucleotide Kinase Activity by the Coupled λ Exonuclease Cleavage Reaction and Catalytic Assembly of Bimolecular Beacons

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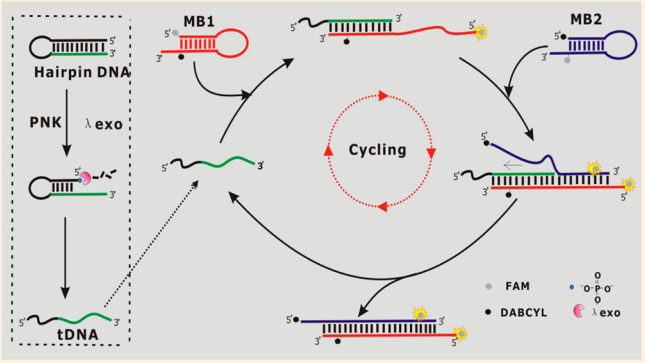
Supporting Information

ABSTRACT: The phosphorylation of nucleic acid catalyzed by polynucleotide kinase is an indispensible procedure involved in many vital cellular activities such as DNA recombination and DNA repair. Herein, a novel strategy for the sensitive determination of T4 polynucleotide kinase (PNK) activity and inhibition was proposed, which combined exonuclease enzyme reaction and bimolecular beacons (bi-MBs)-based signal amplification. A hairpin probe (HP) with 5′-hydroxyl termini and two different types of molecular beacons (MBs), MB1 and MB2, is designed. Taking advantage of the efficient enzyme reactions, namely the phosphorylation of HP by PNK and the λ exonuclease cleavage reaction, the trigger DNA fragment can be released from HP and is used to trigger the catalytic assembly of bimolecular beacons, resulting in a remarkably amplified fluorescence signal toward PNK activity detection. The detection limit of this method toward PNK was obtained as 1 mU/mL, which was superior or comparable with the reported methods. Furthermore, the facile and sensitive method can also be used to screen the inhibition effects toward several common inhibitors. It provides a promising platform for sensitive determination of nucleotide kinase activity and inhibition, and also shows great potential for biological process research, drug discovery, and clinic diagnostics.



The phosphorylation of nucleic acids with 5′-hydroxyl termini, catalyzed by 5′-polynucleotide kinase, has been well-known to play a crucial role in many vital cellular activities, including DNA recombination, DNA replication, and DNA repair during strand damage and interruption.[1−4] Generally, several exogenous and endogenous agents, for instance, chemical substances,[5] ionizing radiation,[6] as well as nucleases,[7] can induce strand breaks accompanied by the generation of hydroxyl at the 5′-end of DNA. Thus, normal function of 5′-polynucleotide kinase is indispensable for the 5′-phosphate termini dependent DNA healing process and the maintenance of gene integrity. T4 polynucleotide kinase (PNK), as a founding member of 5′-kinase family, was first discovered in 1965.[8] It can catalyze the transfer of the γ-phosphate residue of nucleoside triphosphate (ATP) to 5′-hydroxyl termini of nucleic acids and has been widely used in assay of DNA adducts,[9−12] and repair of DNA lesions.[13−16] Furthermore, abnormal PNK behaviors would suppress the cellular responses to DNA damage or lesions and directly relates to some vital human diseases, for example loom's syndrome, Werner syndrome, and Rothmund−Thomson syndrome.[17]

Traditionally, the DNA phosphorylation kinase activity was analyzed by autoradiography, radical isotope ^{32}P-labling, and polyacrylamide gel electrophoresis (PAGE).[18−25] However, their intrinsic limitations, such as complexity, laboriousness, and potential radioactive hazards, prohibit their wide applications. To overcome these drawbacks, sensitive, robust, and convenient PNK assays, including fluorescence assays,[26−32] colorimetric assay,[33] nanochannel biosensor,[34] and electrochemical methods,[35,36] have been recently developed to detect PNK activity and inhibition, in which fluorescence-based strategies attract much attention due to their high sensitivity. For example, Song and Zhao designed a singly labeled hairpin DNA probe for real-time monitoring of the activity and kinetics of PNK.[26] Jiao et al. developed a novel label-free perylene probe for the detection of polynucleotide kinase activity.[27] Chen et al. proposed a novel amplified fluorescence sensing platform for PNK assay.[28] Wu et al.[29] and Lin et al.,[30] respectively, realized the detection of PNK activity by using

Received: October 24, 2013
Accepted: December 13, 2013
Published: December 13, 2013

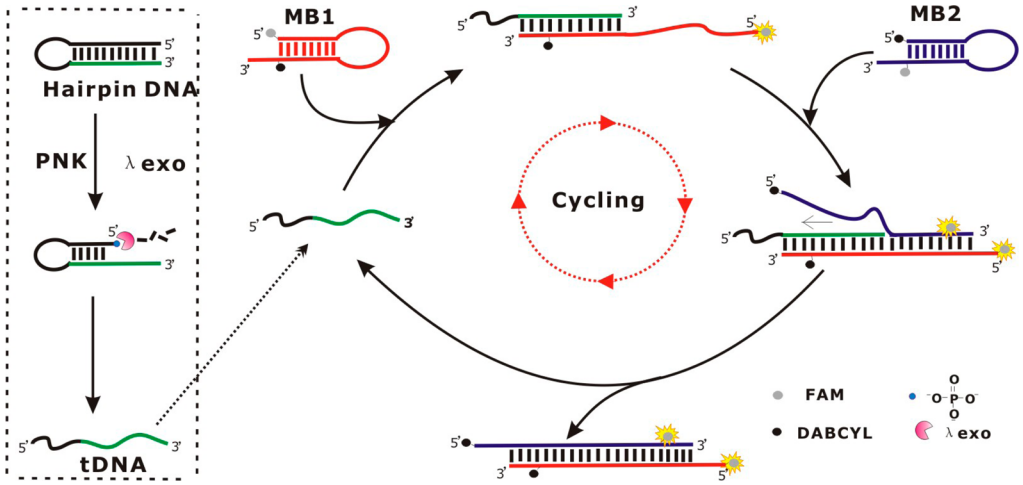
   dx.doi.org/10.1021/ac403458b | Anal. Chem. 2014, 86, 884−890

Table 1. Sequences of Oligonucleotides Used in the Experiments[a]

name	sequence (from 5′ to 3′)
MB1	5′-(FAM)-**CTAACTCGCACTACTTAC**GCTCAACTTCATCAC***GTAAGTAGTGCGAGTTAG****-(DABCYL)-TGATGAA-* 3′
MB2	5′-(DABCYL)-**ACGCTCAACTTCATCACT**AACTCGCACTACTTAC**AGTGATGAAGTTGAGCGT**-(FAM)-AAGTAGT-3′
HP	5′-**CTGTAAGTAGTGCGAGTTAGTGATGAA**ACACACACACAC***TTCATCACTAACTCGCACTACTTACAG****-3′**
phosphorylated-HP	5′-(PO_4)-**CTGTAAGTAGTGCGAGTTAGTGATGAA**ACACACACACAC***TTCATCACTAACTCGCACTACTTACAG****-3′**
mimicking DNA	5′-ACACACACACACTTCATCACTAACTCG CACTACTTACAG-3′

[a]The boldface letters indicate the stem sequences of the MBs and HP. The italicized letters in HP and MB1 represent the sequences complementary to each other.

Scheme 1. Schematic Illustration for the Detection of PNK Activity Based on the Coupled λ Exo Cleavage Reaction and Catalytic Assembly of Bimolecular Beacons



graphene oxide as a super quencher. Huang et al. demonstrated a novel sensitive fluorescence polarization biosensor for amplified detection of PNK activity.[31] Although these previous methods have made great advances toward the DNA phosphorylation assay, the further improvement of the analytical performances, particularly sensitivity, is still in high demand to satisfy the development needs of biological research, clinic diagnostics, and drug discovery.

Very recently, enzyme-free signal amplification strategy has attracted considerable attention for the development of bioassays owing to its enzyme-free and isothermal operation. For example, enzyme-free and amplified biomolecule detection has been achieved by the analyte-induced autonomous cross-opening of hairpin combined with different signal reporter system.[37−39] The target catalyzed hairpin assembly has also been developed for enzyme-free DNA detection, in which the signal amplification is achieved by the cycling use of the target.[40−43] These developments showed that the enzyme-free signal amplification strategy has great potential in bioanalysis and can be extended to develop assays for other targets.

Herein, a simple and sensitive strategy for evaluating PNK activity is proposed, which combines λ exonuclease (λ exo) cleavage reaction and bimolecular beacons (bi-MBs)-based signal amplification. λ exo is a highly efficient and processive 5′ to 3′ cleavage enzyme, catalyzing the removal of 5′-mononucleotides one at a time from one strand of 5′-phosphate double-strand DNA (dsDNA) to generate single-strand DNA (ssDNA) and mononucleotides.[44−46] Taking advantage of the efficient enzyme reactions, namely the phosphorylation of hairpin probe (HP) by PNK and the λ exo cleavage reaction, the trigger DNA fragment (tDNA) can be released from the HP with the amounts of tDNA positively related to PNK activity. The tDNA can then trigger the catalytic assembly of bimolecular beacons, resulting in an amplified fluorescence signal toward PNK activity detection. To the best of our knowledge, it is the first time fluorescence signal amplification was realized by coupling the λ exo cleavage reaction and catalytic assembly of bimolecular beacons for the detection of PNK activity and inhibition. It shows huge potential for further research on the disease-related biochemical process and is also valuable to the molecular-target therapies and the nucleotide kinase-target drug discovery

EXPERIMENTAL SECTION

Reagents. T4 polynucleotide kinase (10 units/μL), λ exonuclease (5 units/μL), adenosine triphosphate (ATP), and adenosine diphosphate (ADP) were purchased from MBI Co. Ltd. Dithiothreitol (DTT) was purchased from Shanghai Generay Biotech Co., Ltd. Tris(hydroxymethyl)aminomethane (Tris) was obtained from Sigma-Aldrich. The DNA sequences were obtained from Takara Bio Inc. (Dalian, China). Other reagents were of analytical grade and used without further purification. The sequences of oligonucleotide probes used in this work are listed in Table 1.

Preparation of DNA Stock Solution. All DNA samples were prepared in 70 mM Tris-HCl buffer (pH 8, 10 mM $MgCl_2$). The concentration of the HP stock solution was 0.5 μM, and the concentrations of the two different MB stock solutions were 3 μM. Before PNK enzyme reaction, all DNA samples were pretreated with the following procedure: heated to 90 °C and incubated for 5 min, and then cooled to 37 °C and incubated for 1 h. The obtained DNA solutions were stored at 4 °C for further use.

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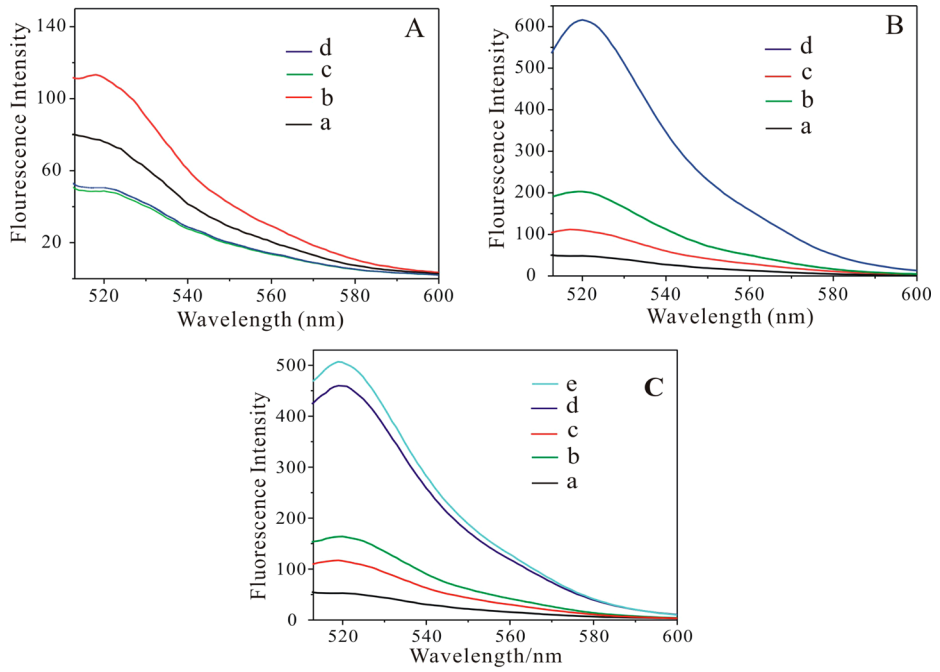

Figure 1. (A) Fluorescence response for the system containing MB1 + MB2 (a, b) or MB 1 only (c, d) recorded at incubation time of 0 (a, c) and 2 h (b, d). (B) Fluorescence response of the solution containing (a) MB1, (b) MB1 + mimicking DNA, (c) MB1 + MB2, and (d) MB1 + MB2 + mimicking DNA after an incubation time of 2 h. The concentrations of MB1, MB2, and mimicking DNA were 100, 100, 10 nM, respectively. (C) Fluorescence response of the solution containing (a) MB1 + HP + λ exo, (b) MB1 + HP + λ exo + PNK, (c) MB1 + MB2 + HP + λ exo, (d) MB1 + MB2 + HP + λ exo + PNK, (e) MB1 + MB2 + phosphorylated-HP + λ exo. The phosphorylation and exonuclease reaction was carried out in the buffer containing 50 nM HP or phosphorylated-HP, 1 mM ATP, 10 units λ exo, and 5 U/mL PNK. The concentrations of both MB1 and MB2 were 100 nM.

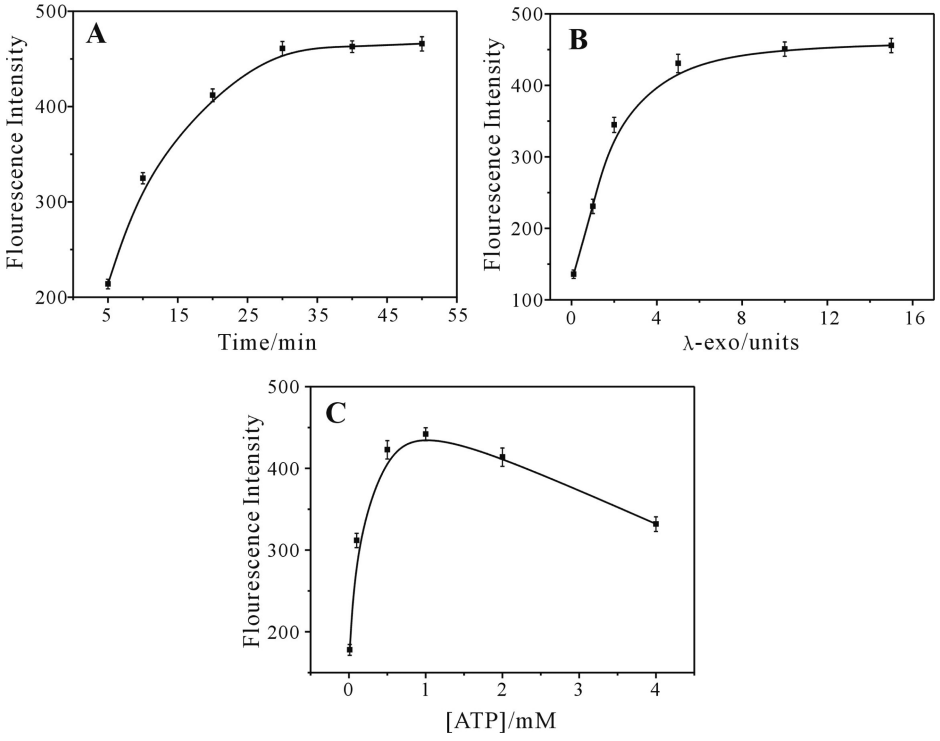

Figure 2. Optimization of (A) reaction time, (B) λ exo amount, and (C) ATP concetration. The assays were carried out in the reaction buffer, containing 50 nM HP and 5 U/mL PNK.

PNK-Catalyzed Phosphorylation and Assay Optimization. In a typical phosphorylation and cleavage assay, 50 nM HP, 1 mM ATP, 10 units of λ exo, and a certain amount of PNK were put into a 50 μL reaction buffer (70 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, pH 8). After this, the incubation of PNK-catalyzed phosphorylation was carried out at 37 °C for 30

dx.doi.org/10.1021/ac403458b | *Anal. Chem.* 2014, 86, 884−890

min. Subsequently, the phosphorylation solution was heated to 90 °C for 2 min, cooled to 37 °C, and kept at this temperature for 1 h. Then, 10 μL of 3 μM MB1, 10 μL of 3 μM MB2, and 230 μL of buffer solution (70 mM Tris-HCl, 10 mM $MgCl_2$, pH 8) were added into the above phosphorylation solution and kept at 37 °C for 2 h. The concentration optimizations of λ exo, ATP, and Mg^{2+} were 0.1−15 units, 0.01−4 mM, and 0.1−25 mM, respectively. The reaction pH was optimized from 7 to 9.

Kinase Inhibitor Evaluation. In the inhibition assay, for the investigatation of the effects of inhibitors on the PNK-catalyzed phosphorylation process, several inhibitors, including adenosine diphosphate (0.1−6 mM), $(NH_4)_2SO_4$ (1−30 mM), and Na_2HPO_4 (10−50 mM), were also contained in the phosphorylation reaction buffer, respectively. After addition of 50 nM HP, 1 mM ATP, 10 units of λ exo, and 5 U/mL of PNK, the reaction was kept at 37 °C, and the procedures were similar to that mentioned above.

Instruments. Fluorescence measurements were performed using a Hitachi F-4500 spectrofluorimeter with a scan rate at 1200 nm/min (Tokyo, Japan). The excitation wavelength was set to 490 nm, and the 24 photomultiplier tube voltage was set to 700 V. The slits for excitation and emission were set at 5 nm/5 nm.

RESULTS AND DISCUSSION

Strategy for PNK Activity Detection. The detection principle for PNK activity is illustrated in Scheme 1. Two MBs (MB1 and MB2), as signal output units, in the absence of trigger DNA (tDNA), can maintain the stem-loop structure due to the binding of the complementary sequences at the ends, and the fluorescence is quenched. Another hairpin probe (HP) with a 5′- hydroxyl group is designed, which contains the sequence of tDNA at the 3′-termini that is caged in the duplex structure of the stem by an interfering sequence at the 5′-termini. The sequences in the toehold region, stem, and part of the loop of MB1 are designed to be complementary with the fragment of tDNA (green color) while the rest sequence of MB1 is complementary to MB2. In the presence of PNK and λ exo, PNK will catalyze the phosphorylation reaction toward the hydroxyl group at the 5′-termini of the HP to yield the 5′-phosphorylated HP, which can be immediately cleaved by λ exo, resulting in the release of tDNA. The amount of released tDNA is positively related to the activity of PNK. It can then hybridize with and open the hairpin structure of MB1, accompanied with the fluorescence restoration of MB1. At the same time, the complementary domain of MB1 to MB2 is exposed and the binding between MB1 and MB2 can occur through a branch migration process.[47−50] Because of that, the MB1−MB2 duplex is more stable than the hybrids between tDNA and MB1; MB2 will replace and free tDNA when it hybridizes with MB1. As a result, the fluorescence of MB2 is restored, which again enhances the fluorescence intensity. Also, the released tDNA becomes available to trigger the successive reaction cycle for the formation of MB1−MB2 duplex. Therefore, the employment of bi-MBs and the recycle of the released tDNA make this strategy appealing in amplifying fluorescence signal for sensitive detection of PNK activity.

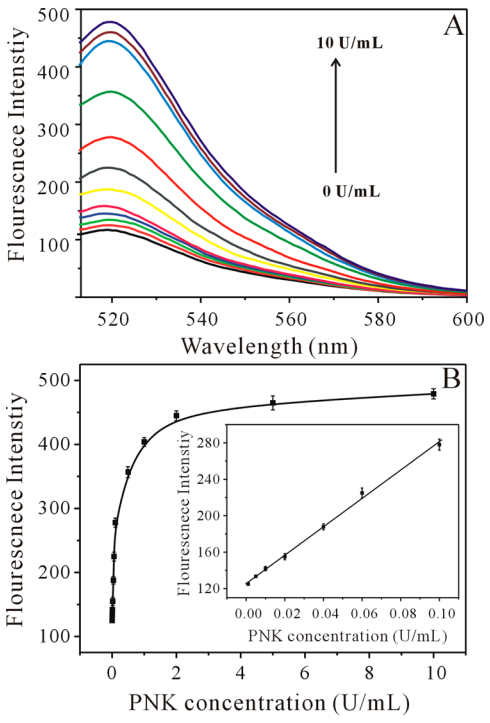

Figure 3. (A) Fluorescence emission spectra of the sensing system upon addition of different concentrations of PNK (from bottom to top): 0, 0.001, 0.005, 0.01, 0.02, 0.04, 0.06, 0.1, 0.5, 1, 2, 5, 10 U/mL. (B) Calibration curve for the fluorescence intensity (at 520 nm) vs PNK concentration. Inset: expanded linear region of the calibration curve.

Monitoring of the PNK-Catalyzed Phosphorylation. The stability of bimolecular beacons in the tested solution was first checked. It can be seen, from Figure 1A, that almost no change of the fluorescence signal for the solution containing MB1 only was observed after an incubation time of 2 h, indicating that the hairpin structure of MB1 was intact (curves c and d). It was also the same phenomenon for the solution containing MB2 only (data no shown). Compared with them, the fluorescence intensity of the solution containing both MB1 and MB2 increased obviously after an incubation time of 2 h (curves a and b), indicating the hybridization between MB1 and MB2 to some extent. It should be noted that the further addition of HP could not induce the evident fluorescence change, indicating that the HP was stable enough in the sensing system and would not interact with MBs. To demonstrate the feasibility of current bimolecular beacon system for fluorescence signal amplification, a mimicking DNA (designed to simulate the product that treated by PNK and λ exo) was employed for the measurement of fluorescence response. As shown in Figure 1B, the MB2-aided amplification method showed a relatively big background signal compared with the conventional method, which employs MB1 only. Fortunately, after addition of a mimicking DNA, the net signal gain of the solution containing MB1 and MB2 (501 ± 18) was significantly larger than that of the solution containing MB1 only (155 ± 13). The signal enhancement by the MB2-aided amplification method can be ascribed to the cycling use of the mimicking DNA and the continuous generation of the MB1−MB2 complex as shown in Scheme 1.

Furthermore, the established MB2-aided signal amplification method was used to evaluate the feasibility for PNK activity detection. As shown in Figure 1C, when the HP was reacted with both PNK and λ exo, a dramatically high fluorescence response was observed (curve d). However, in the presence of λ exo only, the sensing system exhibits a low fluorescence signal (curve c). The main reason for this fact is that PNK catalyzed

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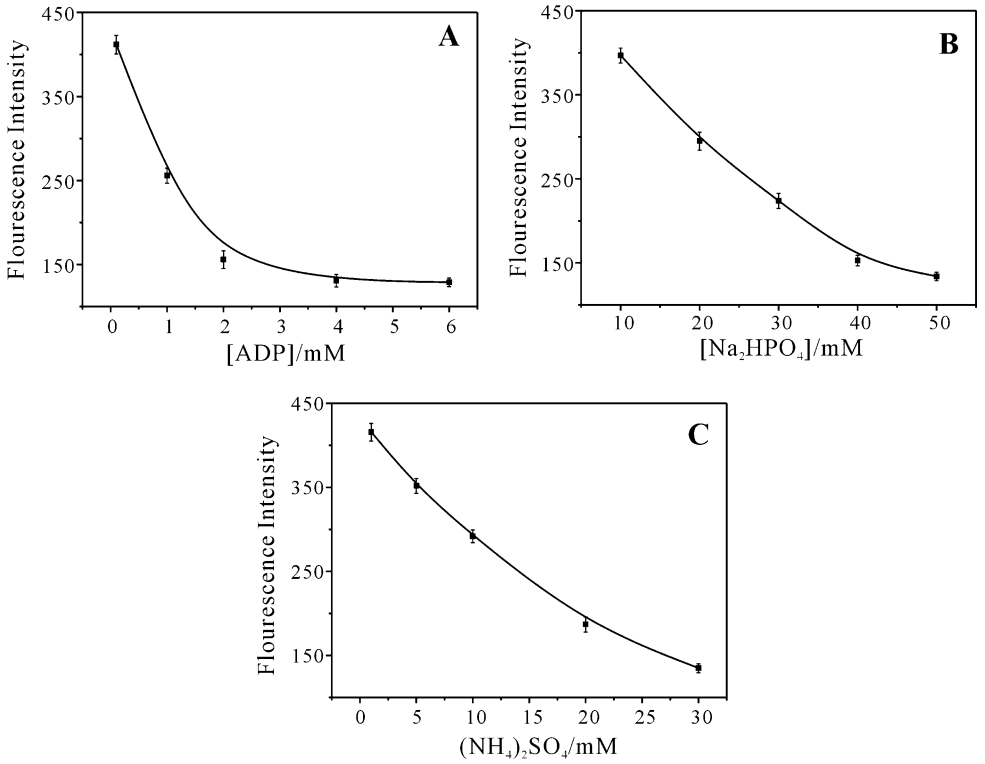

Figure 4. Inhibition effects of (A) ADP, (B) Na_2HPO_4, and (C) $(NH_4)_2SO_4$ on phosphorylation. The assays were carried out in the reaction buffer containing 50 nM HP, 5 U/mL PNK, 1 mM ATP, and 10 units λ exo.

the transfer of γ-phosphate residue of ATP to the 5′-hydroxyl group of HP, yielding a phosphate moiety at the 5′-end of HP. Immediately, λ exo rapidly degrades the duplex part of HP with the 5′-phosphate group, and the λ exo degraded HP fragment will initiate the recycling strand displacement reaction (SDR) process between two MBs, resulting in a high fluorescence signal enhancement. A 5′-phosphorylated-HP was also beforehand synthesized and used for comparison. In the presence of only λ exo but not PNK, the phosphorylated-HP catalyzed by λ exo cleavage showed a very high fluorescence response (curve e). This fully verified the cleavage preference of λ exo for a phosphate moiety at 5′ terminus of dsDNA. Under the same reaction condition, the HP catalyzed by λ exo only can still maintain its original hairpin structure owing to the formation of an inert substrate−enzyme complex.[51] Again, the net signal gain toward PNK activity by MB2-aided amplification was distinctly larger than that by MB1 only, indicating that the proposed signal amplification strategy is effective for determination of PNK activity.

Optimization of Assay Conditions. In current study, the reaction time is a crucial parameter for the PNK-catalyzed phosphorylation and the coupled λ exo cleavage reaction process. An excess of reaction time may induce the false positive fluoresence signal as λ exo would also degrade HP with 5′- hydroxyl slowly.[52] As shown in Figure 2A, the fluorescence intensity increased gradually with the increase of reaction time and then reached equilibrium in 30 min, suggesting the complete phosphorylation and cleavage process. The fluorescence intensity of the sensing system was also found to increase with the amount of λ exo and almost reach the saturation value at an amount of 10 units of λ exo (Figure 2B). Consequently, the optimal reaction time and the amount of λ exo were chosen to be 30 min and 10 units. The ATP concentration also has an important effect on the DNA phosphorylation efficiency, and the lack of ATP may block the DNA phosphorylation.[4] As can be seen from Figure 2C, the fluorescence intensity increased with the increase of ATP concentration and reached its maximum at the ATP concentration of 1 mM, and then decreased again with the further increase of ATP concentration. Thus, the optimized ATP concentration was chosen to be 1 mM. Furthermore, the effects of pH and Mg^{2+} on the coupled enzyme reaction were also studied. As shown in Figure S1 in the Supporting Information, the maximum fluorescence intensity were obtained at pH 8 and 10 mM Mg^{2+}, respectively.

Fluorescence Measurement of PNK Activity. The sensitivity of fabricated biosensor toward PNK activity was then investigated under the optimized conditions. As shown in Figure 3A, the fluorescence intensity at 520 nm gradually increased with increasing PNK concentration, indicating that the fluorescence biosensor was effective for the detection of PNK activity. The calibration plot obtained for the determination of PNK with different concentrations was shown in Figure 3B. The fluorescence intensity of the sensing system varies linearly over the PNK concentration from 1 mU/mL to 0.1 U/mL. The directly measured detection limit for PNK was obtained at 1 mU/mL, which was superior or comparative with the reported assays (Supporting Information Table S1).[4,21,30,34] The linear relationship can be described as $F = 125.5 + 1.56 \times 103C$, where F is the fluorescence intensity, and C is the PNK concentration.

PNK Activity Inhibition Evaluation. Furthermore, the capacity of our fluorescence biosensor to screen the inhibition of PNK activity was also investigated by using three known kinase inhibitors, namely adenosine diphosphate (ADP), ammonium sulfate, and sodium hydrogen phosphate, which

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were considered to have no inhibition effect on the activity of λ exo. Each inhibition assay was carried out with inhibitor at different concentrations in the reaction buffer. As shown in Figure 4A, the fluorescence intensity of sensing system decreased upon the increase of ADP concentration, indicating the inhibition of DNA phosphorylation. The addition of about 0.8 mM ADP had caused a 50% decrease in DNA phosphorylation. The inhibition effect of ADP possibly resulted from the reversible phosphorylation reaction when ADP and 5′-phosphoryl nucleic acids coexisted in the reaction buffer.[4,26] Furthermore, the salt effects of sodium hydrogen phosphate and ammonium sulfate on PNK activity were also evaluated. As shown in Figure 4B,C, the increasing salt concentrations led to the gradual reducing of fluorescence signals, and merely 20 mM Na_2HPO_4 and 10 mM $(NH_4)_2SO_4$ effectively suppressed about 50% PNK activity. The half inhibition concentrations of these two inhibitors were approximate to those previously reported.[4,19,30] Generally, the reason for the salt effects on PNK activity is attributed to the facts that high salt concentration leads to more stable structure HP with the inhibition of the reactivity of 5′-hydroxyl group, and also leads to the change of conformation of PNK with reduced activity.[24] The above-mentioned results show that the proposed strategy is promising in the evaluation of the effects of kinase inhibitors.

CONCLUSIONS

In conclusion, we have developed a novel amplified fluorescence sensing system for sensitive detection of PNK activity and inhibition. By coupling exonuclease enzyme reaction and bimolecular beacon-based signal amplification, this approach exhibits a very high sensitivity for PNK activity screening, with a low detection limit down to 1 mU/mL. Additionally, the present approach uses a simple separation-free procedure in which the enzyme assay is conducted in homogeneous solutions. Also, bimolecular beacon-based signal amplification strategy avoids the use of protein enzyme. Furthermore, the inhibition effects of ADP, Na_2HPO_4, and $(NH_4)_2SO_4$ on PNK activity can be successfully evaluated. Considering the high sensitivity, as well as the simple-to-implement features, the proposed method holds great potential in the research of DNA phosphorylation related process, drug discovery, and clinical diagnostics. It can also be easily extended for the detection toward many other nucleic acid enzymes and may find widespread applications.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Nos. 21375072, 21175076, and 21005043), the Basic Research Program of Qingdao (No. 13-1-4-214-jch), and the Open Fund of State Key Laboratory of Medicinal Chemical Biology (Nankai University) (No. 20130420).

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