One-step highly sensitive florescence detection of T4 polynucleotide kinase activity and biological small molecules by ligation-nicking coupled reaction-mediated signal amplification

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ARTICLE INFO

Article history:
Received 21 December 2012
Received in revised form 15 March 2013
Accepted 15 March 2013
Available online 22 March 2013

Keywords:
T4 polynucleotide kinase
Florescence
Ligation-nicking coupled reaction
Signal amplification
Small molecules

ABSTRACT

DNA phosphorylation, catalyzed by polynucleotide kinase (PNK), plays significant regulatory roles in many biological events. Herein, using T4 PNK as a model target, we describe a one-step, highly sensitive, simple and rapid florescence approach for monitoring its activity and inhibition. This innovative strategy is inspired by the great amplification capability of ligation-nicking coupled reaction-mediated signal amplification. In the presence of T4 PNK, one of two short oligonucleotides complementary to the loop sequence of molecular beacon (MB) are phosphorylated, and then ligated with the other by DNA ligase. Upon formation of the stable duplex between the ligated DNA and MB, the fluorescence is restored and further significantly amplified through nicking endonuclease assisted cleavage of multiple MBs. Meanwhile, the cleavage of MBs will also generate new nicks to initiate the ligation reaction. Eventually, a maximum fluorescence enhancement is obtained when the ligation and nicking process reached a dynamic equilibrium. As compared to those of the existing approaches except for the assay based on signal transduction function, the sensitivity (0.00001 U/mL) of the proposed strategy is 100–1700 times higher. The application of the sensing system in complex biological matrix and screening of T4 PNK inhibition are demonstrated with satisfactory results. Moreover, this approach is also successfully used to detect biological small molecules such as adenosine triphosphate (ATP), and can be further extended for nicotinamide adenine dinucleotide (NAD+) detection.

1. Introduction

DNA phosphorylation plays an essential regulatory role in many cellular processes such as DNA recombination and DNA replication (Ma and Yeung, 2010; Wang et al., 2002; Whitehouse et al., 2001). In the DNA phosphorylation process, polynucleotide kinase (PNK) can catalyze the transfer of the terminal phosphate of adenosine triphosphate (ATP) to the 5′-hydroxyl termini of nucleic acids (Richardson, 1965), which is indispensable in many nucleic acid repair mechanisms for the repair of the 5′-hydroxyl termini (Wang et al., 2002; Whitehouse et al., 2001). Numerous damaging agents of endogenous or exogenous origins, for example, ionizing radiation (Henner et al., 1983), chemical agents as well as nucleases (Torriglia et al., 1998), usually attack DNA to cause DNA strand breaks followed by the generation of 5′-hydroxyl termini, which is closely associated with spinocerebellar ataxia (Caldecott, 2003) and neurodegenerative disease (Rass et al., 2007).

To date, several traditional bioassays, including polyacrylamide gel electrophoresis (PAGE), radioactive 32P-labeling and autoradiography (Amitrur et al., 1987; Karimi-Busheri et al., 1999; Meijer et al., 2002; Wang and Shuman, 2001; Wilson and Thompson, 1997), have been developed to achieve the reliable detection of PNK activity, respectively. Although these approaches are well-established and have been widely used, they are laborious, DNA-consuming, unwieldy, discontinuous or costly. Furthermore, some of them require radio-isotope labeling which necessitates stringent safety measures to control radiographic exposure. These intrinsic drawbacks significantly impede the broad application of these techniques and lead to an urgent demand to develop alternative, robust and convenient methods for monitoring the activity and inhibition of T4 PNK. Recently, some intriguing techniques have been explored for overcoming the above-mentioned shortcomings. By using titanium ion (Ti4+) mediated signal transition coupled with signal amplification of single wall carbon nanotubes (SWCNTs), a novel and label-free electrochemical sensing platform has been constructed for detection of T4 PNK activity and inhibition with relatively high sensitivity (Wang et al., 2011). Nevertheless, it suffers from the requirement of multiple washing steps and the complex preparation process of DNA/ferrocene-SWCNT bioconjugates. Owing to the significant merits of simplicity, real-time...
detection, high-throughput ability and ease of automation, several fluorescence-based sensing systems have been developed for the detection of PNK activity based on the λ exonuclease cleavage reaction combined with graphene oxide (GO) (Lin et al., 2011), a singly labeled DNA-hairpin smart probe (Song and Zhao, 2009), and a perylene probe (Jiao et al., 2012). Despite many advances in these methods, the low sensitivity still remains as a major drawback, which is caused by the nonspecific cleavage of 5′-hydroxyl termini by λ exonuclease (Little, 1967; Subramanian et al., 2003). Taking advantage of the high knowledge, no reports are available for monitoring the activity and theirs high sensitivity, simplicity and rapidity. However, to our amplification of non-specific DNA (ssDNA), double-stranded DNA (dsDNA) and stem-loop) and GO, π systems rely on the different interactions such as hydrogen bonding or toward the target with a detection limit down to 0.001 U/mL (Wu et al., 2012). Recently, Fan and his co-workers constructed a GO-based nano-beacon for direct T4 PNK analysis without the need for hairpins dual labeling. Thanks to the super fluorescence quenching efficiency of GO, the proposed biosensor exhibits a significantly improved sensitivity toward the target with a detection limit down to 0.001 U/mL (Wu et al., 2011). However, the synthesis of GO is complex, time-consuming and laborious. Moreover, since the GO-based detection systems rely on the different interactions such as hydrogen bonding or π–π stacking between various DNA structures (e.g. single-stranded DNA (ssDNA), double-stranded DNA (dsDNA) and stem-loop) and GO, the non-specific adsorption of biomolecules on GO surfaces may pose a challenge for their application in complex biological samples. In addition, the 1:1 signal transduction function involved in all reported approaches, namely each target-triggered DNA phosphorylation event only producing one signal response, greatly limits the sensing sensitivity. Consequently, the establishment of a highly sensitive, simple and rapid fluorescence sensing approach for the amplified detection of T4 PNK activity is greatly desirable.

With the aim of improving the sensing performance, several isothermal signal amplification strategies have been adapted for the analysis of enzyme activities (Chen and Zhao, 2013; Li et al., 2010; Zhao et al., 2013), DNA (Kong et al., 2011; Xu et al., 2011), proteins (Xue et al., 2012; Zheng et al., 2012), metal ions (Qi et al., 2012; Zhu et al., 2009), as well as small molecules (Lu et al., 2011; Zhao et al., 2012). Among them, the nicking endonuclease mediated amplification methods have received considerable attention for their high sensitivity, simplicity and rapidity. However, to our knowledge, no reports are available for monitoring the activity and inhibition of enzyme by utilizing the unique amplification technique. Recently, we are surprised to find an obviously amplified fluorescence response by nicking endonuclease assisted cleavage of multiple MBs in the presence of DNA ligase. Inspired by this observation, using T4 PNK as a model enzyme which is the first discovered PNK and has become a powerful research tool in biology, molecular biology and bioengineering (Bernstein et al., 2005; Wang et al., 2002), we herein present a novel ligation-nicking coupled reaction-mediated-amplification sensing platform to highly sensitive and rapid analysis of T4 PNK activity and inhibition. To demonstrate the potential application of the developed biosensor in complex biological samples, human serum spiked with different concentrations of T4 PNK was measured. Furthermore, expansion of the assay to detect biological small molecules such as ATP with high sensitivity and selectivity was also verified.

2. Experimental

2.1. Reagents and materials

T4 polynucleotide kinase, T4 DNA ligase and ATP were purchased from Takara Biotechnology CO., Ltd. (Dalian, China). Nicking endonuclease Nt.BbvCl was obtained from New England Biolabs Inc. (Beijing, China). Uridine triphosphate (UTP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP), guanosine triphosphate (GTP) and cytidine triphosphate (CTP) were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China), Other chemicals were of analytical grade and were used without further purification. Ultrapure water was obtained through a Milli-Q system (Millipore, Bedford, MA). All oligonucleotides used in this study were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China) and sequences were as follows:

Oligo A, 5′-p-GGAGCTGTA-3′;
Oligo B, 5′-GGCAACAT-3′;
Oligo C, 5′-GGCAACAT-3′;
Oligo D, 5′-GGAGCTGTA-3′;
MB, 5′-(FAM)-(CCACATGCGTCCCGTGG)-(DABCYL)-3′.
The underlined sequence of MB indicates the stem, and the recognition sequence for Nt.BbvCl is highlighted in red. Letter ‘p’ at the 5′ ends of Oligo A and Oligo C represents the phosphate.

2.2. Fluorescence measurement

All fluorescence measurements were carried out on a FluoroMax-4 fluorescence spectrometer (Horiba Jobin Yvon, Edison, NJ) at room temperature. Excitation and emission wavelengths were set at 494 nm and 518 nm with the slit widths of 5 nm, respectively. The emission spectra were obtained by scanning the emission from 510 to 590 nm in steps of 1 nm.

2.3. Amplified assay of T4 PNK activity

In a typical experiment, 250 nM MB, 150 nM Oligo A, 150 nM Oligo B, 1 mM ATP, 6 U of Nt.BbvCl and 17.5 U of T4 DNA ligase were introduced into 20 μL of 1× T4 PNK buffer (50 mM Tris–HCl, 10 mM MgCl2, 5 mM DTT, pH 8.0). After addition of different concentrations of T4 PNK, the solutions were incubated at 37 °C for 30 min prior to fluorescence measurement.

2.4. Detection of the inhibition of T4 PNK

To investigate whether or not inhibitors (sodium hydrogen phosphate, ammonium sulfate and ADP) have influence on both T4 DNA ligase and Nt.BbvCl, a control experiment was carried out. Before the addition of 17.5 U of T4 DNA ligase and 6 U of Nt.BbvCl, inhibitors were introduced into 20 μL 1× T4 PNK buffer containing 1 mM ATP, 250 nM MB, 150 nM Oligo C and 150 nM Oligo D. The solution was incubated at 37 °C for 30 min prior to fluorescence measurement.

For T4 PNK inhibition experiments, the procedure was similar to the detection of T4 PNK activity that noted above except that different concentrations of inhibitors were added together with T4 PNK.

2.5. ATP detection by enzymatic signal amplification

In a typical experiment, the reaction was carried out in 20 μL of 1× T4 PNK buffer containing 250 nM MB, 150 nM Oligo C, 150 nM Oligo D, 6 U of Nt.BbvCl, 4.4 U of T4 DNA ligase and various concentrations of ATP at 37 °C for 30 min. Then, fluorescence emission spectra were recorded as above.

3. Results and discussion

3.1. Principle of T4 PNK activity assay based on ligation-nicking coupled reaction-mediated-signal amplification

Scheme 1.
The basic design of the current sensing system for amplified fluorescence detection of T4 PNK activity is outlined in Scheme 1.
First, a nicking endonuclease, Nt.BbvCI, which can recognize a specific nucleotide sequence in a dsDNA and cleave only one strand at a fixed site with high specific activity (Chan et al., 2011; Higgins et al., 2001), is chosen to facilitate the signal amplification. Second, a MB is specially designed to contain a complete recognition sequence for Nt.BbvCI in the middle of the hairpin loop region and serves as a signal reporter. Finally, two 8-base oligonucleotides, Oligo A carrying a 5′ phosphate group to avoid the undesired target-catalyzed phosphorylation and Oligo B acting as a target recognition probe, are designed to be perfectly complementary to one-half of the loop sequence of MB, respectively. In the absence of T4 PNK, the 5′-hydroxyl termini of Oligo B cannot be phosphorylated, and the subsequent ligation process mediated by T4 DNA ligase will not proceed. Due to the low melting temperature (Tm) values (estimated to be less than 26°C) for the hybrids of Oligo A and B with MB, and the highly specific site recognition of Nt.BbvCI towards the hybridized dsDNA, the extremely low background fluorescence is achieved at the reaction temperature of 37°C. Nevertheless, when the phosphorylation process is catalyzed by target T4 PNK, a ligatable nick can be formed when Oligos (A and B) hybridize to the MB. With the help of T4 DNA ligase, this nick is sealed to generate the 16-base ssDNA, opening the MB’s hairpin structure. This conformational change will result in the restoration of the quenched fluorescence and form the double-stranded recognition sequence for Nt.BbvCI. The nicking endonuclease specifically cleaves the MB strand, producing a new nick. After nicking, the duplex ligated ssDNA/MB becomes less stable which forces the cleaved MB to dissociate from the ligated ssDNA, and the fluorescence signal is further enhanced due to the completely spatial separation of fluorophore from quencher. The released ssDNA is then able to hybridize with another MB and trigger the second cycle of cleavage. Finally, each ligated ssDNA can be repeatedly recycled to facilitate cleavage of multiple MBs. Since T4 DNA ligase is also present in the sensing system, the cleaved MB will be ligated by employing the 16-base ssDNA as temperature and form the thermostable ssDNA/MB duplex again. In practice, the nicking endonuclease assisted cleavage of MBs and the enzymatic ligation reaction for the cleaved MBs are two competitive processes, and the reaction rates exhibit a positive correlation with the their respective substrate concentrations in a certain range. Due to an excess of MB substrates, the nicking process is always predominant and its reaction rate increases with the increase in the concentration of the ligated ssDNA substrate, however when the concentration of MB substrate decreases to a certain extent, the nicking rate begin to decrease. As for the ligation reaction for the cleaved MBs, a continuous increase is expected with the increasing concentration of substrates such as the ligated ssDNA and cleaved MB. Finally, with the whole reaction proceeding, a dynamic equilibrium between this nicking and ligation process will be reached. In this way, each T4 PNK-catalyzed phosphorylation event can be readily converted into a dramatically amplified sensing signal by the ligation-nicking coupled reaction-mediated the fluorescence restoration of multiple MBs.

3.2. Feasibility of the amplified T4 PNK assay

To estimate the amplification function of the novel sensing strategy for screening T4 PNK activity, the target-triggered fluorescence enhancement was measured in the presence and absence of nicking endonuclease, respectively, and the results were depicted in Fig. 1. In comparison with the low background fluorescence without Nt.BbvCI (curve d), there was a negligible fluorescence enhancement in the presence of Nt.BbvCI (curve c), which is attributed to the unstable duplex of short Oligo A and B with MB at the reaction temperature of 37°C and the highly specific site recognition of Nt.BbvCI towards the hybridized dsDNA. However, when the target T4 PNK was present, the fluorescence response by Nt.BbvCI (curve a) was much higher as compared to the system lacking the nicking endonuclease (curve b). In detail, with the introduction of 0.1 U/mL T4 PNK, a (225 ± 19)% increase in the fluorescence signal was readily achieved by the proposed ligation-nicking coupled reaction-mediated signal amplification. In contrast, under the same condition except for the absence of Nt.BbvCI, only a (40 ± 5)% fluorescence
signal enhancement was observed. All the above-mentioned results strongly demonstrated that it is reasonable to expect that one-step highly sensitive assay for T4 PNK activity could be achieved by the proposed amplification strategy based on ligation-nicking coupled reaction.

3.3. Optimization of sensing conditions

In order to achieve the best sensing performance, several vital conditions including T4 DNA ligase concentration, Nt.BbvC1 concentration, Oligo B concentration and the reaction time, were optimized, respectively. Firstly, we investigated the effect of T4 DNA ligase concentration on the performance of the sensing system, and the result was shown in Fig. S1. As the T4 DNA ligase concentration increased, the fluorescence response increased rapidly and reached a maximum at the T4 DNA ligase concentration of 0.875 U/µL. Therefore, 0.875 U/µL was chosen for the following experiment.

Since the nicking endonuclease mediates the cleavage of MB that will significantly affect the signal amplification, the effects of different concentrations of Nt.BbvC1 ranging from 0.1 to 0.4 U/µL were investigated and the results were depicted in Fig. 2A. It was observed that the fluorescence response increased rapidly with the increasing concentration of Nt.BbvC1 implying the enhanced cleavage of MB, and reached a maximum value at 0.3 U/µL. In the meantime, the corresponding background signals were also measured in the absence of target and the ignorable variations were observed for different conditions (data not shown), which demonstrated the nicking endonuclease did not result in the cleavage of MB even at high concentration. Finally, 0.3 U/µL Nt.BbvC1 was chosen to ensure the efficient cleavage reaction.

In principle, a higher concentration of the target recognition probe (Oligo B) enables a more rapid DNA phosphorylation progress, resulting in a stronger fluorescence response for the detection of target at a fixed assay time. Using the 250 nM MB, the effect of concentration of Oligo B on the fluorescence response of sensing system was further tested with the fixed molar ratio of Oligo B to Oligo A of 1:1. As shown in Fig. 2B, the fluorescence signal enhanced quickly along with the increment of Oligo B concentration, and leveled off when its concentration exceeded 150 nM. Furthermore, the corresponding control experiments were also carried out in the absence of T4 PNK. It can be seen that increased Oligos concentration lead to a slight increase in the background signals. Finally, 150 nm Oligo B was selected for the following experiments owing to maximum signal-to-noise ratio.

The influence of the reaction time was also examined. As illustrated in Fig. 2C, a rapid increase in fluorescence intensity was seen. The signal nearly approached steady state in 30 min, suggesting that a dynamic equilibrium between the nicking endonuclease assisted cleavage of MBs and the enzymatic ligation reaction for the cleaved MBs was reached. As a consequence, the optimal assay time was set to 30 min for our sensing system, which makes the sensing system suitable for rapid screening of T4 PNK activity.

3.4. Amplified detection of T4 PNK activity

The ability of the developed sensing strategy for amplified detection of T4 PNK activity was further tested. A series of sample containing different concentrations of target were analyzed under the optimized conditions. Fig. 3A displayed the fluorescence emission spectra of the sensing system following the successive addition of T4 PNK. As expected, a gradual increase in fluorescence signal was clearly observed along with the increase of T4 PNK concentration ranging from 0 to 1 U/mL. The relationship between the fluorescence intensities at 518 nm and the concentrations of T4 PNK was shown in Fig. 3B. The inset of Fig. 3B clearly revealed that the fluorescence intensity is linearly dependent on the T4 PNK concentration up to 0.1 U/mL. The detection limit was estimated at 0.00001 U/mL in terms of the rule of 3 times standard deviation over the blank response. As compared to those of the previously reported methods except the assay using a relatively expensive epifluorescence microscopy with an electron-multiplying charge-coupled device camera, all limited to 1:1 signal transduction function, the detection limit is lower. For further comparison, the experiments with T4 PNK at various concentrations in the absence of nicking endonuclease were also performed under the same conditions and the results were presented in Fig. S2. A detection limit of only 0.00015 U/mL was obtained, which is 15 times higher than that of the proposed amplification strategy. This significantly improved sensitivity is due to the amplification by nicking endonuclease assisted cleavage of MBs. Table S1 summarizes the performances of the existing approaches in comparison to our sensing platform.

A big challenge for an excellent sensing system is its practical applicability in complex biological matrixes. In order to evaluate

![Image](Image)
the viability of the developed approach in complex biological matrixes, three different amounts of target T4 PNK were spiked to serum (diluted in 1:20 ratio with T4 PNK buffer). As can be seen in Fig. S3, comparable responses were found for T4 PNK in both T4 PNK buffer and serum. The fluorescence signals obtained in serum were all higher than those of in T4 PNK buffer. This increment of fluorescence intensity may be ascribed to the nonspecific binding of proteins in human blood serum to the molecular beacon, which caused the disruption of the molecular beacon’s hairpin structure. Also, some DNA nucleases contained in serum could increase the fluorescence intensity by the degradation of the molecular beacon. Furthermore, the relative standard deviations (RSD) for three repeated measurements of the spiked T4 PNK in serum were less than 5%, indicating that the assay is quite reproducible. Therefore, all the results revealed that the proposed sensing system holds great promise for real sample analysis.

3.5. Assay of the inhibition on T4 PNK activity

The capacity of the developed method for screening the inhibition of T4 PNK activity was also evaluated by using several known inhibitors, such as ammonium sulfate, sodium hydrogen phosphate, and ADP. Since there were another two enzymes involved in our sensing system, the effects of these inhibitors on T4 DNA ligase and Nt.BbvCI should be eliminated before assay of the inhibition on T4 PNK activity. As shown in Fig. S4, almost the same fluorescence responses were obtained in the absence and presence of inhibitors. The results clearly indicated that the influence of ammonium sulfate, sodium hydrogen phosphate, and ADP on T4 DNA ligase and Nt.BbvCI could be neglected when their concentrations reached 2.5 mM, 2.5 mM and 0.1 mM, respectively, which ensured accurate assay of T4 PNK inhibition.

When the concentrations of the several inhibitors were kept under those mentioned above, the inhibition assays were carried out with target analyte at a fixed concentration of 0.5 U/mL. As can be seen in Fig. 4A and B, the fluorescence intensity gradually decreased with increasing concentrations of ammonium sulfate and sodium hydrogen phosphate, and 1.7 mM ammonium sulfate and 1.4 mM sodium hydrogen phosphate caused a 50% decrease in fluorescence intensity, respectively. This was in accordance with the dose-dependent inhibition of salts. The effect of salts on T4 PNK activity may be attributed to following two factors (Lillehaug and Kleppe, 1975; Lillehaug et al., 1976): (1) a more stable double helix structure of dsDNA can be formed at high concentration of salts, which may make a contribution to the inactivation of the 5′-hydroxyl group; (2) high salt concentrations may seriously affect the conformation of DNA kinase, leading to the reduction of the kinase activity together with the affinity of PNK for its DNA substrate. Moreover, the inhibition of ADP was also estimated.
increased background ligase not only resulted in strong sensing performance was investigated over a concentration range (Rossi et al., 1997). Therefore, the effect of T4 DNA ligase on the amount of adenylated ligase present in commercial T4 DNA ligase, expected background enhancement may be ascribed to the trace extending application of the sensing strategy in ATP detection, the viability and cell injury (Erecińska and Wilson, 1982). For the extended application of the sensing strategy in ATP detection, the simple changes are the uses of phosphate instead of hydroxyl group at the 5′ termini of Oligo B, and Oligo D same as Oligo A except for 5′-hydroxyl termini. The detailed mechanism is depicted in Fig. S5. Unlike T4 PNK activity assay, the high concentration of T4 DNA ligase not only resulted in strong fluorescence signal but also increased background fluorescence for ATP detection. This unexpected background enhancement may be ascribed to the trace amount of adenylated ligase present in commercial T4 DNA ligase, which can catalyze the ligation reaction even in the absence of ATP (Rossi et al., 1997). Therefore, the effect of T4 DNA ligase on the sensing performance was investigated over a concentration range from 0.08 to 0.88 U/μL and the results are shown in Fig. 5A. Finally, 0.22 U/μL T4 DNA ligase was selected for amplified detection of ATP due to the maximum signal-to-noise level. Under the same conditions as for T4 PNK activity assay except for the optimum DNA ligase concentration, the sensitivity of the sensing system was examined by fluorimetric titration. Fig. 5B depicts the variance of fluorescence emission spectra upon the addition of different amount of ATP ranging from 0 to 200 nM. From Fig. 5C, it can be seen that the fluorescence intensity is sensitive to the ATP concentration, and the linear range is from 0.01 nM to 15 nM. Using the unique amplification strategy, a very low detection limit down to 0.005 nM was readily obtained for ATP detection, which is much superior to those of most previously reported fluorescent methods (Li et al., 2012; Li and Ho, 2008; Zhang et al., 2012; Zhen et al., 2010; Zhou et al., 2011).

Besides the detection sensitivity, the ability to achieve specific response to the interested analyte is also very crucial for ideal biosensors with applications to real samples. As we know, one of the major limitations of aptamer-based ATP assays is the inability to distinguish ATP from ADP and AMP, because they share a same aptamer (Du et al., 2011). The selectivity of the sensing strategy was evaluated by challenging it with the analogs of ATP, such as ADP, AMP, UTP, GTP and CTP. As presented in Fig. 5D, a remarkable enhancement in fluorescence intensity was observed only when ATP was tested. The results clearly demonstrated the high selectivity of the method for ATP, which made it promising for complex biological sample analysis. The excellent specificity was attributed to the intrinsically extreme fidelity of T4 DNA ligase for ATP.

Moreover, since NAD⁺, a ubiquitous biological small molecule, functions as a consumable substrate for E. coli DNA ligase, by only changing the T4 DNA ligase as E. coli DNA ligase, the developed amplification strategy can be further employed for rapid, highly sensitive and selective analysis of NAD⁺. Overall, the proposed

![Fig. 5.](image)

**Fig. 5.** (A) The effect of T4 DNA ligase concentration on the sensing performance for ATP detection. The concentration of ATP was 50 nM. (B) Fluorescence emission spectra in the presence of different concentrations of ATP: (a-k) 0, 0.01, 0.25, 1, 5, 10, 15, 25, 50, 100 and 200 nM. Inset: Fluorescence responses to ATP at low concentrations. (C) Variance of fluorescence peak intensities as a function of ATP concentration. The inset shows linear relationship between the fluorescence response and the low ATP concentration. (D) Selectivity of the proposed amplification strategy for ATP compared to its analogs (ADP, AMP UTP, GTP and CTP). The concentration of all analytes is 50 nM. The error bar indicates the standard deviation from three independent experiments. The RSD is less than 5.4%.
sensing system not only can be used for the highly sensitive detection of T4 PNK, but also can be expanded to detect biological small molecule such as ATP and NAD$^+$ with minor modification of the sensing system.

4. Conclusions

In summary, we have demonstrated a novel one-step amplified fluorescence sensing system for assaying T4 PNK activity and inhibition. This sensing strategy relies on the fluorescence restoration of MB as a signal reporter by the competition between the enzymatic ligation reaction and nicking endonuclease assisted recycling cleavage reaction. Unlike all existing T4 PNK activity assays, each event of target-triggered phosphorylation of 5’-hydroxyl termini of DNA can be converted into multiple signal output through simple mixing and one-step isothermal incubation within relatively short assay time (30 min) by the proposed method, thereby affording a dramatically amplified fluorescence signal for target T4 PNK. With a common fluorophotometer, a very low detection limit down to 0.00001 U/mL is achieved with a dynamic detection range of 5 orders of magnitudes. Except for that of the fluorescence assay based on single nanoparticle counting, the sensitivity is superior to those of previously reported methods. Sample assays of spiked T4 PNK in human serum confirm the reliability and practicality of the protocol, indicating a good prospect of this platform for complex biological sample analysis. Also, it is successfully applied to detect the inhibitions of several known inhibitors including ammonium sulfate and sodium hydrogen phosphate, and ADP on T4 PNK. Moreover, this method has been extended for the detection of biological small molecules such as ATP with excellent sensitivity and selectivity, and can be further utilized for NAD$^+$. Altogether, the developed strategy has been proved as a highly sensitive, simple and rapid sensing platform to detect T4 PNK activity, inhibition as well as biological small molecule, and may be promising in the researches of DNA phosphorylation-relevant process, drug developments, and clinical diagnostic.

Acknowledgments

This research was financially supported by the National Natural Science Foundation of China (21005059) and the Fundamental Research Funds for the Central Universities.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.03.034.

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