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# A label-free assay for T4 polynucleotide kinase/phosphatase activity and its inhibitors based on poly(thymine)-templated copper nanoparticles

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ABSTRACT

DNA 3′-phosphatase takes an important role in DNA damage repair, replication and recombination. Here, we present a novel label-free fluorescent assay for T4 polynucleotide kinase/phosphatase (T4 PNKP) activity and its inhibitor screening by using poly(thymine)-templated fluorescent copper nanoparticles (CuNPs) as a fluorescent indicator. In this assay, we designed a simple T-rich hairpin primer with a 3′-phosphoryl end, which can serve as both the substrate for T4 PNKP and DNA template for the formation of fluorescent CuNPs. Once the phosphorylated hairpin primer was hydrolyzed by T4 PNKP, the resulting hairpin primer with a 3′-hydroxyl end was immediately elongated to form a long double-strand product by DNA polymerase, which prohibited the formation of fluorescent CuNPs due to the lack of poly T single-stranded DNA template. This new strategy provides a sensitive, selective, and cost-effective manner for T4 PNKP analysis, which holds a great potential in the study of DNA damage repair mechanisms.



## 1. Introduction

Damage to cellular DNA is considered to be a significant factor in aging, cancer etiology and treatment, and neurological disorders [1–5]. DNA terminated with a 3′-phosphate is a common product of DNA damage resulting from exposure to both external genotoxic agents and endogenous sources of stress, which can be mutagenic and detrimental to survival [6,7]. Since the discovery of T4 polynucleotide kinase/phosphatase (PNKP) in 1965, it has become a powerful tool in molecular biology research. T4 PNKP can act as a DNA 3′-phosphatase with the capacity to catalyze the hydrolytic removal of the 3′-phosphoryl of DNA, RNA and deoxynucleoside 3′-monophosphates [8]. T4 PNKP is very important for cellular nucleic acid metabolism, particularly in the cellular responses to DNA damage, which relates to many human disorders such as Werner syndrome, Bloom's syndrome, and Rothmund-Thomson syndrome [9]. T4 PNKP has also been widely applied to the detection of DNA adducts or oligonucleotides and the repair of DNA lesions [10–12]. Therefore, due to its clinical and biological importance, the development of simple and sensitive methods for the monitoring of phosphatase activities of T4 PNKP is highly desirable.

Traditionally, radioisotope $^{32}$P-labeling, polyacrylamide gel electrophoresis (PAGE), and autoradiography technology were widely applied to detect DNA dephosphorylation as well as analyze T4 PNKP activity [13–15]. These methods, however, have some shortcomings of being time consuming, laborious, not sensitive, or requiring the radiolabeling of substrates [13–15]. Many convenient and sensitive methods have been developed to solve these problems in recent years. For example, several methods based on fluorescence strategies have been reported [16–18]. Although these fluorescent methods have been proved more advantageous than traditional T4 PNKP assays, their shortcomings, such as the high cost of fluorescent modification and complicated design of molecular beacons, are still unavoidable. Consequently, the development of simple, sensitive, and low-cost methods for T4 PNKP assay is highly desirable.

Recently, nanomaterial-based probes are attracting more interest due to their inherent advantages such as simplicity, sensitivity and low cost, as demonstrated in their wide applications in different fields [19–22]. During recent years, many efforts have been made in the development of simple and fast methods for assay of T4 PNKP activity. Huang and coworkers reported a

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fluorescence polarization (FP) nanosensor for studying T4 PNKP activity and inhibition by combining exonuclease reaction with the strong FP enhancement effect of gold nanoparticles (AuNPs) [23]. Lin et al. developed a sensitive and rapid method to study T4 PNKP activity and inhibition based on exonuclease reaction and a graphene oxide platform [24]. Liu et al. successfully screened the DNA dephosphorylation process by using $WS_2$ nanosheets as a quencher [25]. Nevertheless, all these methods require the design of fluorescence labeling DNA probe, which often suffers from problems such as high cost, low yield, and complex purification steps. Therefore, it is very urgent to establish a sensitive, simple, and label-free fluorescent strategy for detecting T4 PNKP activity.

Lately, Qing et al. reported that single-stranded poly-(thymine) (poly T) DNA could act as an efficient template for the formation of fluorescent copper nanoparticles (CuNPs) [26]. Attractively, the producing of DNA-templated CuNPs is highly efficient and can be completed within just several minutes. Meanwhile, poly T-templated CuNPs exhibit a maximum $\lambda_{em}$ at 615 nm with large MegaStokes shifting (up to 275 nm), which is well-suited as a fluorescent probe for signal transducing in biochemical analysis, as the MegaStokes shifting of fluorophore can enable the removal of strong background signal from complex biological systems [27]. Wang group reported that poly T-templated CuNPs could be used as a fluorescent probe for signal transducing in biochemical analysis [28]. To the best of our knowledge, the exploration of poly T-templated CuNPs is still at a very early stage and has a great potential to be utilized in biochemical applications.

Herein, we proposed to develop a sensitive, simple, low cost, and label-free approach for T4 PNKP assay based on poly T-templated fluorescent CuNPs. This protocol utilized a T-rich DNA probe acting as the substrate for T4 PNKP. The probe is a single-strand DNA molecule that forms self-complementary structure at one end. The 3′-end is modified with a phosphate group and serves as the substrate of the target phosphatases. T-rich part of the probe can be used as a template for the formation of CuNPs. Upon the addition of T4 PNKP, the 3′-phosphoryl of the probe is hydrolyzed to a hydroxyl group and the resulting 3′-hydroxyl is immediately extended by the DNA polymerase in the presence of dNTPs. Additionally, by the fact that the double-strand DNA product does not support CuNPs' formation and so leads to a low fluorescence signal. Thus, the T4 PNKP activity could be identified by CuNPs' fluorescence changes. Compared with the traditional methods, the proposed strategy is convenient, exhibiting high analytical performance, and avoiding the trouble to design complicated fluorescent DNA probes. The method was used as an effective approach for the measurement of the activity of T4 PNKP and the inhibition effects of heparin on T4 PNKP. Furthermore, the proposal has been successfully applied to quantify the phosphatase activity in the extract of A549 human lung adenocarcinoma cells and showed great potential in the analysis of phosphatases in biological samples without any complex pretreatment process.

## 2. Experimental

### 2.1. Chemicals and apparatus

Purified oligonucleotides were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China). The DNA sequences used in this work were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. The sequences of the DNA oligonucleotide were as follows, where the loop sequence were indicated in italics, and complementary sequences were underlined:

Probe1 (P1): FAM- 5′- TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT CGC ACC *TAAA* GGG TGC G-phos-3′

Probe2 (P2): 5′- TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT-3′

Probe3 (P3): 5′-AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA -3′

T4 polynucleotide kinase/phosphatase, Klenow fragment polymerase (KF polymerase, without 3′ to 5′ exonuclease activity), and deoxyribonucleoside triphosphates (dNTPs) were obtained from New England Biolabs (Ipswich, MA, USA). $CuSO_4$, 3-(N-Morpholino) propanesulfonic acid (MOPS), BSA, IgG, Concanavalin A (Con A), Glucose Oxidase ($GO_x$) and sodium ascorbate were bought from Sigma-Aldrich (Shanghai, China). The reaction buffer solution employed in this work was 10 mM Tris–HCl, 10 mM $MgCl_2$, and 50 mM NaCl (pH 7.9). The MOPS buffer (10 mM MOPS, 150 mM NaCl, pH 7.6) was used for the formation of fluorescent CuNPs. All reagents were used as received without any further purifications. All solutions were prepared by ultrapure water obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) with an electric resistance $> 18.2$ MΩ.

The fluorescence measurements were carried out on an FL-4600 spectrometer (Hitachi, Japan). The fluorescence emission spectra were collected from 520 nm to 660 nm at room temperature with a 340 nm excitation wavelength.

### 2.2. Assay procedures

Briefly, a total volume of 25 μL solution containing varying concentrations of T4 PNKP, P1 (1 μM), KF polymerase (25 U $mL^{-1}$), and dNTPs (200 μM) were incubated at 37 °C for 80 min. Then, 55 μL MOPS buffer (10 mM MOPS, 150 mM NaCl, pH 7.6), 10 μL of sodium ascorbate (50 mM), and 10 μL of $CuSO_4$ (2 mM) were added into the solution to give final volumes of 100 μL and was allowed to react for 15 min at home temperature (25 °C), followed by the fluorescence measurement with the excitation wavelength of 340 nm.

### 2.3. Kinase inhibitor evaluation

T4 PNKP (25 U $mL^{-1}$) was firstly mixed with varying concentrations of heparin and then incubated with P1 (1 μM), KF polymerase (25 U $mL^{-1}$), and dNTPs (200 μM). Then, the reaction buffer was added to give a final reaction volume of 25 μL, and the solution was kept at 37 °C for 80 min. After that, 55 μL MOPS buffer (10 mM MOPS, 150 mM NaCl, pH 7.6), 10 μL of sodium ascorbate (50 mM), and 10 μL of $CuSO_4$ (2 mM) were added into the solution to give final volumes of 100 μL and were allowed to react for 15 min at home temperature (25 °C). Fluorescence detection was performed at room temperature in the same way as for the assay of T4 PNKP.

### 2.4. T4 PNKP activity detection in diluted cell extracts

A549 human lung adenocarcinoma cell lines were cultured in RPMI 1640 medium supplemented with 12% fetal calf serum, 100 μg $mL^{-1}$ streptomycin, and 100 units $mL^{-1}$ penicillin. Cell extracts were prepared according to the previous reports [29]. The collected cells were resuspended in 20 μL of 10 mM Tris–HCl (pH 7.8) containing 150 mM NaCl. With the addition of 20 μL lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, 0.4 mM phenylmethylsulfonyl fluoride, pH 7.5), the mixture was incubated for 1.5 h at 4 °C with occasional shake. Cell debris was removed by centrifugation at 10,000 rpm 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 T4 PNKP detection in the clean reaction buffer.

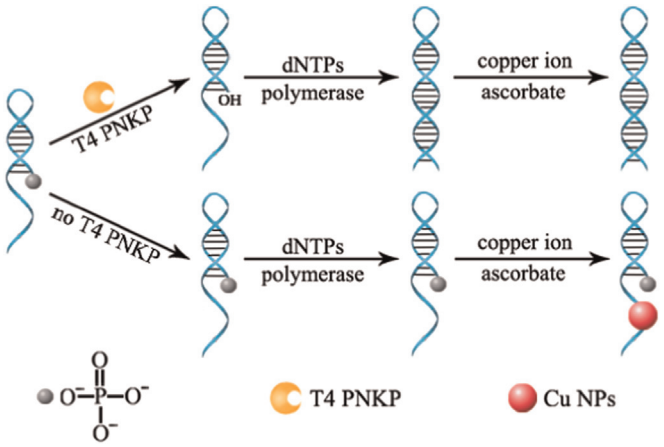
## 3. Results and discussion

### 3.1. Strategy for T4 PNKP activity detection

By taking advantages of the fact that poly T-templated formation of fluorescent CuNPs can be used as fluorescence indicators with an immense potential application to biochemical sensing, a novel label-free method for T4 PNKP detection based on poly T-templated CuNPs has been proposed in this work. The principal design is illustrated in Scheme 1. The primer probe contains two short single stranded complementary sequences which can form a hairpin structure with a 3′-phosphoryl end under appropriate hybridizing conditions. Since the polymerase elongation reaction is only initiated at the 3′-hydroxyl end of the primer, the 3′-phosphorylated DNA probe formed in such a way is an inactive substrate for DNA polymerase in the absence of T4 PNKP. Thus, the T-rich part of primer probe can be used as template for the formation of CuNPs through the reduction of $Cu^{2+}$ by ascorbate, and the formed poly T-templated-CuNPs complexes show high fluorescence. The primer probe, however, can be dephosphorylated into a 3′-hydroxyl end by T4 PNKP, which then can serve as the preferred substrate for DNA polymerase. Therefore, in the presence of T4 PNKP, together with the DNA polymerase and dNTPs, polymerase elongation is initiated to generate a long double-strand DNA product. And the double-strand DNA product does not support CuNPs' formation, leading to a weak fluorescence. Thus, the activity of T4 PNKP can be easily reflected by the fluorescence signal change.

### 3.2. Monitoring of the T4 PNKP-catalyzed dephosphorylation

To demonstrate the feasibility of our assay for monitoring T4 PNKP activity, the polymerase elongation was initiated by dephosphorylating the DNA probe using T4 PNKP. A proof of principle experiment was carried out in the absence or in the presence of T4 PNKP. First, the poly T-templated CuNPs were confirmed by transmission electron microscopy (TEM) (Fig. 1). Then, the detection of T4 PNKP was realized by CuNPs' fluorescence monitoring. As shown in Fig. 2, the CuNPs were practically formed once in presence of Probe 1 (curves a) and the high fluorescence intensity was also similar to that of Probe 2 (curves b), which may be due to the same lengths of poly T. Control experiments proved that Probe 2/ Probe 3 duplex strand could not be used as effective template for the formation of CuNPs (curves d), where no fluorescent signal could be detected consequently. Similarly, in the absence of T4 PNKP, the polymerase elongation was not induced and as a result




**Scheme 1.** Schematic representation of poly T-templated CuNPs-based platform for T4 PNKP activity analysis.

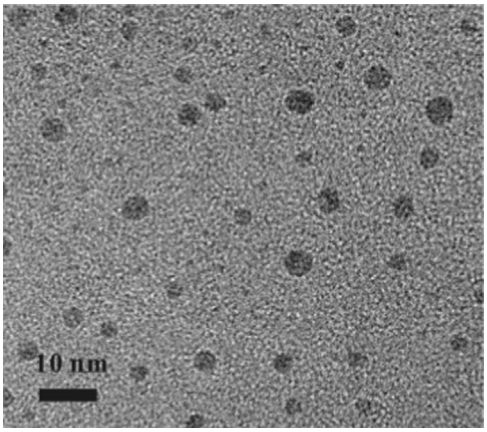



**Fig. 1.** Typical TEM image of poly T-templated CuNPs.

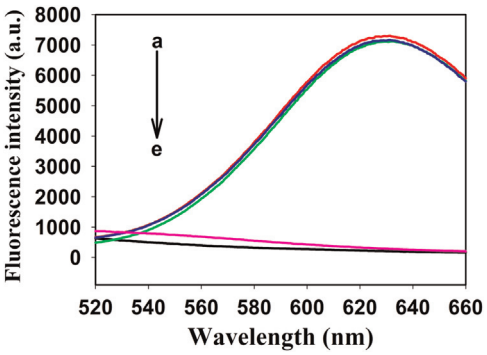



**Fig. 2.** The fluorescence emission spectra of as obtained CuNPs under different conditions: (a) Probe 1+ $Cu^{2+}$ +ascorbate (Probe 1, 1 μM; $Cu^{2+}$, 200 μM; ascorbate, 5 mM; red line); (b) Probe 2+ $Cu^{2+}$ +ascorbate (Probe 2, 1 μM; $Cu^{2+}$, 200 μM; ascorbate, 5 mM; blue line); (c) Probe 1+ KF polymerase+dNTPs+$Cu^{2+}$ +ascorbate (Probe 1, 1 μM; KF polymerase, 25 U $mL^{-1}$; dNTPs, 200 μM; $Cu^{2+}$, 200 μM; ascorbate, 5 mM; green line); (d) Probe 2+Probe 3+$Cu^{2+}$ + ascorbate (Probe 2, 1 μM; Probe 3, 1 μM; $Cu^{2+}$, 200 μM; ascorbate, 5 mM; purple line); (e) Probe 1+ T4 PNKP+KF polymerase+dNTPs+$Cu^{2+}$ +ascorbate (Probe 1, 1 μM; T4 PNKP, 25 U $mL^{-1}$; KF polymerase, 25 U $mL^{-1}$; dNTPs, 200 μM; $Cu^{2+}$, 200 μM; ascorbate, 5 mM; black line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

rather high fluorescence signals were obtained (curves c). In the presence of T4 PNKP (25 U $mL^{-1}$), weaker fluorescence signal was observed with a signal-to-background ratio of more than 15 times (curves e), implying the formation of the long double-strand elongation product. These experimental results demonstrated the feasibility of the proposed strategy for the detection of DNA 3′-phosphatase.

### 3.3. Optimization of assay conditions

In order to optimize the experimental conditions, a series of measurements were researched in this work. It was found that the concentration of $Cu^{2+}$ was an important factor influencing the fluorescent probes of poly T-templated-CuNPs. We first explored the effect of the concentration of $Cu^{2+}$ on the fluorescence of poly T-templated-CuNPs. We found that the fluorescent signal resulting from the metallization increased sharply upon increasing the concentration of $Cu^{2+}$ ions and the best signal-to-background ratio was obtained with 200 μM $Cu^{2+}$ (Fig. S1). Thus, this concentration of $Cu^{2+}$ was used in all experiments.

The kinetic behaviors of the newly designed biosensor were further studied by monitoring the fluorescence intensity of CuNPs as a function of time. We found that the fluorescence generated by

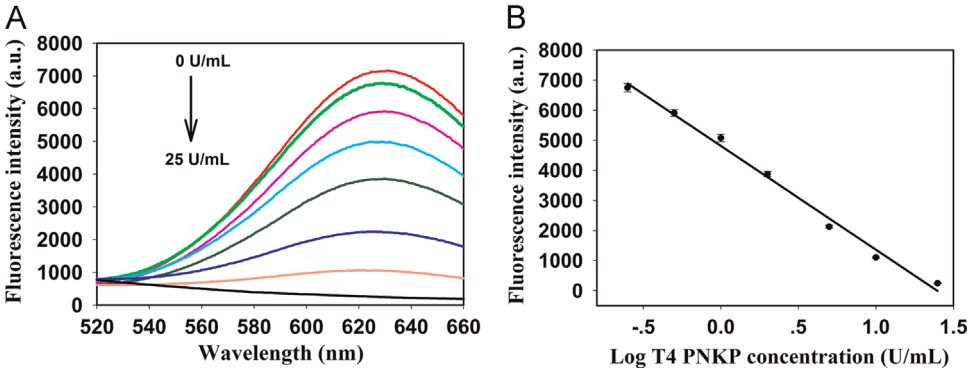



**Fig. 3.** (A) Fluorescence intensity-wavelength curves with different activity units of T4 PNKP (top to bottom, 0, 0.25, 0.5, 1, 2, 5, 10, 25 U $mL^{-1}$) in reaction buffer. (B) Calibration curve for T4 PNKP detection. The error bars represented for standard deviation (SD) across three repetitive experiments. (Probe 1, 1 μM; KF polymerase, 25 U $mL^{-1}$; dNTPs, 200 μM; ascorbate, 5 mM; $Cu^{2+}$, 200 μM).

poly T-templated CuNPs was up to stable within just several minutes from the start of the reaction (Fig. S2), which maintained an acceptable level of fluorescence after two hours with good stability. Therefore, fluorescence spectra were recorded after 15 min in all studies. The results showed that this fast and label-free fluorescent assay for T4 PNKP detection could be carried out reliably based on poly T-templated CuNPs.

Moreover, to achieve the best sensing performance, the concentrations of dNTPs and KF polymerase were also optimized (Fig. S3). Experimental results showed that the following conditions provided the maximum S/N ratio for the sensing system: 25 U $mL^{-1}$ KF polymerase and 200 μM dNTPs.

### 3.4. Fluorescence measurement of T4 PNKP activity

We chose the fluorescence emission at 520 nm to evaluate the performances of the proposed method. The activity of T4 PNKP was quantified based on the optimal experimental conditions. Fig. 3A depicted the typical fluorescence spectral responses of the fluorescent biosensor to T4 PNKP at varying concentrations. It is clearly observed that the peak intensity decreased gradually with increasing T4 PNKP concentration ranging from 0 to 25 U $mL^{-1}$. Fig. 3B indicated that the fluorescence signal was linearly decreased with the T4 PNKP concentration in the range from 0.25 to 25 U $mL^{-1}$ (regression coefficient $R^2=0.994$). The relative standard deviations of peak fluorescence readings were 3.2%, 2.6%, and 1.5% in three repetitive assays of 0.5 U $mL^{-1}$, 1 U $mL^{-1}$, and 10 U $mL^{-1}$ T4 PNKP, respectively, which showed that this proposed method had a good reproducibility. According to the 3σ rule, the detection limit of T4 PNKP activity was estimated to be 0.25 U $mL^{-1}$. The excellent performance in sensitivity was much better than that of the previously reported fluorescent assay based on a universal molecular beacon and quantitative real-time PCR [30]. Meanwhile, compared with the classical $^{32}$P-labeling assay [12], the sensitivity and detection range of the strategy were all significantly improved. Besides, the limit of detection or detection range of the assay was also comparable to the previously reported methods [24,31,32]. We estimated that the higher sensitivity of the present sensor might be attributed to two aspects. In the first place, the formation of CuNPs may be sensitive to the poly T single-stranded DNA template. There is one more point that the label-free design helps to lower the background. The results demonstrated that the proposed method could be used as a highly sensitive fluorescent biosensor for T4 PNKP detection.

### 3.5. Assay selectivity

To demonstrate the selectivity of the present strategy, control experiments using some interfering proteins including BSA, IgG, Con A, and $GO_x$ were respectively tested with the procedures of T4 PNKP assay. With the comparison of peak intensity after treatment by these proteins, it was obviously observed that these interfering proteins did not cause a remarkable fluorescence change, indicating a high selectivity of the proposed poly T-templated CuNPs-based T4 PNKP assay (Fig. 4).

### 3.6. Investigation of T4 PNKP activity detection in diluted cell extracts

The feasibility of the proposed strategy was also tested by the detection of T4 PNKP in complex biological samples. In order to examine the possibility of the as-proposed sensing platform for cellular T4 PNKP activity profiling, A549 human lung adenocarcinoma cell extracts were added into the buffer to simulate the intracellular environment during the test procedure. It was observed that the fluorescence signals decreased when the concentrations of T4 PNKP gradually increased from 0 to 25 U $mL^{-1}$ (Figs. 5A and S4A). The fluorescence intensity and the logarithm of T4 PNKP concentration also exhibited a linear relationship like that operated in Tris–HCl buffer (Figs. 5B and S4B). The above results demonstrated that the as-proposed sensing platform works well in complex mixtures with other possible coexisting interfering species, suggesting that the method could be further used for real sample analysis.

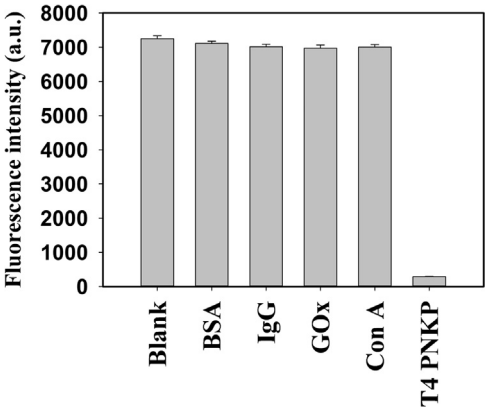



**Fig. 4.** The selectivity of the poly T-templated CuNPs-based strategy for T4 PNKP assay. The concentrations of BSA, IgG, $GO_x$, T4 PNKP, and Con A were 20 mg $L^{-1}$, 20 mg $L^{-1}$, 100 U $mL^{-1}$, 25 U $mL^{-1}$, and 20 mg $L^{-1}$, respectively. Error bars were estimated from three replicate measurements. (P1, 1 μM; KF polymerase, 25 U $mL^{-1}$; dNTPs, 200 μM; ascorbate, 5 mM; $Cu^{2+}$, 200 μM).

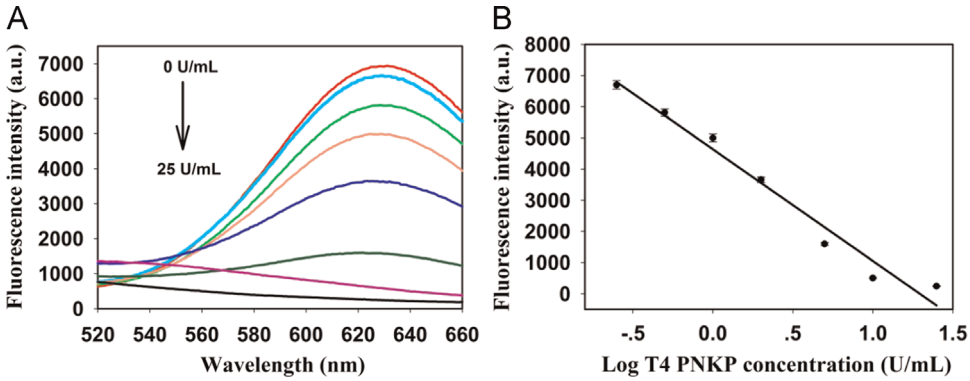



**Fig. 5.** (A) The fluorescence intensity with different activity units of T4 PNKP in reaction buffer containing 1% (v/v) cell extracts. (B) The dependence of fluorescence intensity on the logarithm of the T4 PNKP concentration in reaction buffer containing 1% (v/v) cell extracts. The error bars represent standard deviation (SD) across three repetitive experiments. (Probe 1, 1 μM; KF polymerase, 25 U $mL^{-1}$; dNTPs, 200 μM; ascorbate, 5 mM; $Cu^{2+}$, 200 μM).

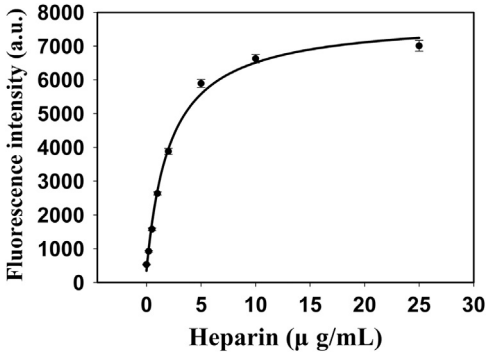



**Fig. 6.** Inhibition effects of heparin on dephosphorylation. The concentration of T4 PNKP is 25 U $mL^{-1}$. The concentrations of heparin are 0, 0.2, 0.5, 1, 2, 5, 10 and 25 μ g $mL^{-1}$, respectively. The error bars represented for standard deviation (SD) across three repetitive experiments.

### 3.7. Assay of the inhibition on T4 PNKP activity

The established strategy can be further adapted to study the effects of inhibitor compounds on the phosphatase activities of T4 PNKP. Heparin has been reported to be inhibitors of T4 PNKP [33]. Fig. 6 showed the effect of heparin on the activity of T4 PNKP. As the heparin concentration increased, the relative activity of T4 PNKP decreased sharply. The half-maximal inhibitory concentration (IC50) of heparin was acquired from the plot of relative activity of T4 PNKP versus heparin concentration and was found to be 1.8 μg $mL^{-1}$. As demonstrated by the above experimental results, the inhibition effect of the inhibitor compounds on the phosphatase activities of T4 PNKP could also be quantitatively evaluated. Potentially, this proposed method could be also readily applied to assaying other DNA 3′-phosphatases.

## 4. Conclusions

In conclusion, based on poly T-templated CuNPs, a novel label-free fluorescent sensing platform for the assay of DNA 3′-phosphatases T4 PNKP and its inhibitors has been developed. The assay relies on the principle that the polymerase elongation reaction is only initiated at the 3′-hydroxyl end of the probe, and the double-stranded of elongation product does not support CuNPs' formation. The successful detection of T4 PNKP with the limit of detection of 0.25 U $mL^{-1}$ demonstrates its potential to be a general method with high sensitivity and selectivity. Our method was also used to investigate the effects of inhibitor compounds on the phosphatase activities of DNA 3′-phosphatases and satisfactory results were obtained. In addition, the proposed strategy was applicable to a real biological sample. Also, we could find that the relative standard deviation (RSD) of results were satisfactory, suggesting that the method was reliable and practical for the assay of T4 PNKP (Table S1). Given the simplicity, selectivity, and sensitivity of this assay, the proposed strategy may become a method of choice for the assay of DNA 3′-phosphatases and would be applicable in the study of DNA damage repair mechanisms.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.08.055.

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