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Electrochemical detection of T4 polynucleotide kinase based on target-assisted ligation reaction coupled with silver nanoparticles

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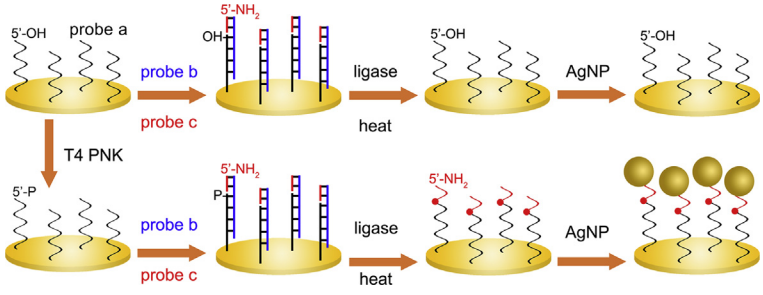
HIGHLIGHTS

- A sensitive and selective electrochemical approach is developed for the detection of T4 polynucleotide kinase activity.
- Silver stripping peak of silver nanoparticles is used for signal generation.
- A smart ligation reaction is designed for further adsorption of silver nanoparticles.
- This method can be used to screen inhibitors of T4 polynucleotide kinase.

GRAPHICAL ABSTRACT



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ABSTRACT

Phosphorylation of DNA is a critical event for normal cell processes such as DNA replication, DNA repair and DNA recombination. It is important to develop accurate and convenient approach for the detection of kinase and inhibition. In this work, we have described a novel sensing strategy for T4 polynucleotide kinase. A single-stranded DNA probe is firstly immobilized on the surface of the electrode. After phosphorylated by target kinase, it could be ligated with amino group labeled DNA probe by certain hybridization. Denaturation process is then proceeded to release unligated DNA probes, which may help reduce the background signal. The remained amino groups could be used to adsorb silver nanoparticles (AgNPs) as electrochemical species for the quantification of T4 polynucleotide kinase. This biosensor shows high sensitivity and selectivity. It may also be used for the screening kinase inhibitors. Therefore, it shows great potential utility in biological process investigation, drug discovery and clinical diagnosis.

1. Introduction

T4 polynucleotide kinase (T4 PNK) belongs to the 5′-kinase family, which catalyzes the transfer and exchange of phosphate group from ATP to the 5′-hydroxyl terminal of polynucleotides [1]. This property is the prerequisite for the repair of broken termini of DNA/RNA induced by external or internal agents [2]. It is not only

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widely used as a tool enzyme in molecular biology, but also plays important roles in a majority of normal cellular events such as DNA replication, DNA repair and DNA recombination [3,4]. The cellular nucleic acid metabolism is closely related to many disorders including Werner syndrome [5], Myelodysplastic syndrome [6], Huntington's disease [7], cardiovascular disease [8] and so on. As a result, accurate and convenient detection of T4 PNK and its potential inhibitors are of great importance in nucleic acid metabolism research and clinical diagnosis [9,10].

Traditional methods to estimate kinase activity rely on ^{32}P-labeling, polyacrylamide gel electrophoresis or autoradiography. Nevertheless, these methods suffer serious drawbacks such as radioactive or biotoxic hazards, complicated operation and expensive instruments. To circumvent them, many convenient biosensors have been developed in recent years mainly based on the techniques of electrochemistry [11], colorimetry [12] and fluorescence [13]. For example, Hou et al. combined λ exonuclease cleavage reaction and recycling assembly of molecular beacons for amplified fluorescent detection of T4 PNK [14]. Cui et al. utilized enhanced quasi-reversible redox signal of prussian blue generated by self-sacrificial label of iron metal-organic framework in an electrochemical biosensor [15]. Liu et al. developed a label-free colorimetry approach for T4 PNK assay by introducing G-quadruplex/hemin DNAzyme [16]. Li et al. fabricated another λ exonuclease-assisted T4 PNK biosensor based on the fluorescence variations from DNA-templated silver nanoclusters (AgNCs) [17]. However, there is still much room for improvement. For example, the linear range of Liu's work was too narrow; Li's method belonged to a signal-off mode, which might suffer the problem of false positive results. In this contribution, we have employed silver nanoparticles (AgNPs) as the sensitive electrochemical sources [18], and designed a target-assisted ligation reaction for selective analysis of T4 PNK and its inhibitors. The peak of AgNPs is much narrower than those of most other signal sources including the fluorescence peak of AgNCs, which is suitable for ultrasensitive analysis. In addition, the analytical performances of this signal-on biosensor are excellent without any signal amplification process, which may be applied for biological process investigation, drug discovery and clinical diagnosis.

2. Experimental

2.1. Materials and reagents

T4 PNK and T4 DNA ligase were purchased from Takara Biotechnology Co. Ltd (Dalian, China). Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), lysozyme, trypsin, thrombin, IgG, silver nitrate, sodium borohydride, 6-mercapto-1-hexanol (MCH), adenosine diphosphate (ADP), adenosine triphosphate (ATP), bovine serum albumin (BSA), and ammonium sulfate were purchased from Sigma-Aldrich (USA). cAMP-dependent protein kinase (PKA) and uracil-DNA glycosylase (UDG) were purchased from New England Biolabs (Beijing, China). Serum samples were collected from local hospitals (Suzhou, China). Other reagents were of analytical grade and were used without treatment. All solutions used in this work were prepared with ultrapure water, which was obtained from a Milli-Q water purification system (USA). DNA oligonucleotides were synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China). The sequences were shown in Table 1.

2.2. Synthesis of AgNPs

Bare AgNPs were prepared by borohydride reduction of Ag^+ according to a previously reported method [19]. We firstly prepared the solution of $AgNO_3$ and trisodium citrate with independent concentrations of 0.25 mM. Secondly, 3 mL of $NaBH_4$ solution with the concentration of 10 mM was prepared, which was then blended with 100 mL of $AgNO_3$ and trisodium citrate solution. After reduction reaction of 0.5 h, the mixture was stood still overnight in the dark. The formed AgNPs with bright yellow color were then purified by three cycles of centrifugation at 12 000 g for 30 min.

Table 1
DNA sequences used in this work.

Name	Sequence (from 5′ to 3′)
probe a	*GCAAGAATTTCGACATGGCGTG*-SH
probe b	*CACGCCATGTCGAAATTCTTGC*GTGCCTAT
probe c	NH_2-TTTATAGGCAC
probe d	P-GCAAGAATTTCGACATGGCGTG-SH
probe e	TTTATAGGCAC

2.3. Electrode treatment and DNA modification

The substrate gold electrode (2 mm) was pretreated before further modification. Generally, it was soaked in piranha solution (98% H_2SO_4:30% H_2O_2 = 3:1) for 5 min (*Caution: highly corrosive*). Next, it was rinsed with double-distilled water and then polished to a mirror-like surface with P5000 silicon carbide paper and 1, 0.3, and 0.05 μm alumina slurry, successively. Subsequently, the electrode was sonicated in ethanol and then double-distilled water for 5 min, respectively. The electrode was then electrochemically cleaned with 0.5 M H_2SO_4 and dried by nitrogen. The pretreated electrode was incubated with 0.5 μM probe a (10 mM Tris-HCl, 10 mM TCEP, 1 mM EDTA, 0.1 M NaCl, pH 7.4) for 10 h. Then, it was treated with 0.1 M MCH for 0.5 h in order to obtain well aligned DNA monolayer [20].

2.4. Quantitative detection

Standard T4 PNK solutions were prepared with a series of concentrations. Probe a modified electrode was then incubated with T4 PNK solutions containing 3 mM ATP at 37 °C for 1 h. Afterward, the electrode was rinsed and then incubated with the mixture of probe b and c (1 μM) for 1 h. It was further treated with 10 U mL^{-1} T4 DNA ligase (50 mM Tris−HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, pH 7.5) at 22 °C for 1 h. After that, the electrode was heated at 95 °C for 5 min and cooled rapidly in an ice bath. The electrode was rinsed and further incubated with AgNPs for 20 min in order to localize AgNPs on the surface of the electrode.

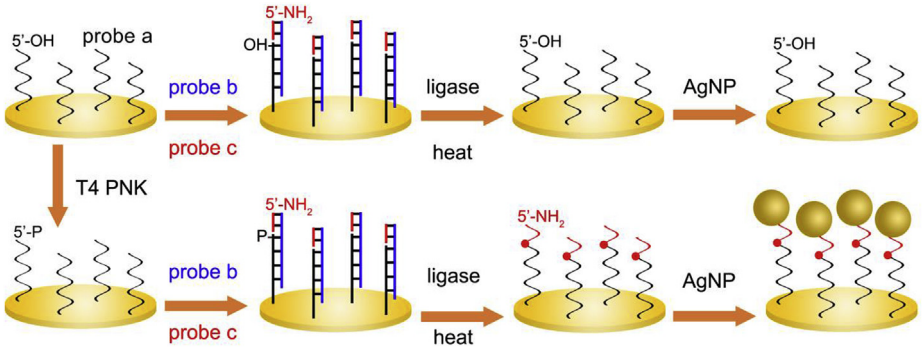
2.5. Electrochemical measurement

All electrochemical experiments were performed on a CHI 660D electrochemical workstation (CH Instruments, China). A traditional three electrode system was employed, which consisted of an Ag/AgCl reference electrode, a platinum wire auxiliary electrode, and a gold working electrode. Electrochemical impedance spectroscopy (EIS) experiments were carried out with the electrolyte of 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 1 M KCl. The parameters included 0.205 V bias potential, 5 mV amplitude, and 0.1–100 000 Hz frequency range. Linear sweep voltammetry (LSV) experiments were performed in the electrolyte of 0.1 M KCl with the scan rate of 0.1 V s^{-1}.

3. Results and discussion

3.1. Sensing principle

The principle of the electrochemical approach for T4 PNK assay is illustrated in Scheme 1. Generally, DNA probe a is immobilized on



Scheme 1. Illustration of the electrochemical detection of T4 PNK.

the electrode interface via gold-sulfur chemistry [21]. In the absence of T4 PNK, ligation reaction cannot be proceeded with the 5′-hydroxyl terminal of probe a. In the presence of T4 PNK, the 5′-hydroxyl terminal of probe a is phosphorylated. After further hybridization with probe b and c, the adjacent probe a and c can be linked by T4 DNA ligase, which keeps probe c on the surface of the electrode. The further denaturation process by heating could release unligated probe c, which reduces background signal significantly. Due to the 5′-amino terminal of probe c, silver nanoparticles (AgNPs) could be localized via silver-amino chemistry on the ligated DNA probes [22]. By analyzing the silver stripping peaks of AgNPs, T4 PNK activity could be evaluated. The successful synthesis of AgNPs could be confirmed by the transmission electron microscopic (TEM) image and the UV–vis spectrum. The size is observed to be about 6 nm and the characteristic absorbance peak at the wavelength of 400 nm (UV–vis spectrum) is due to the surface plasmon excitation of AgNPs (Fig. S1).

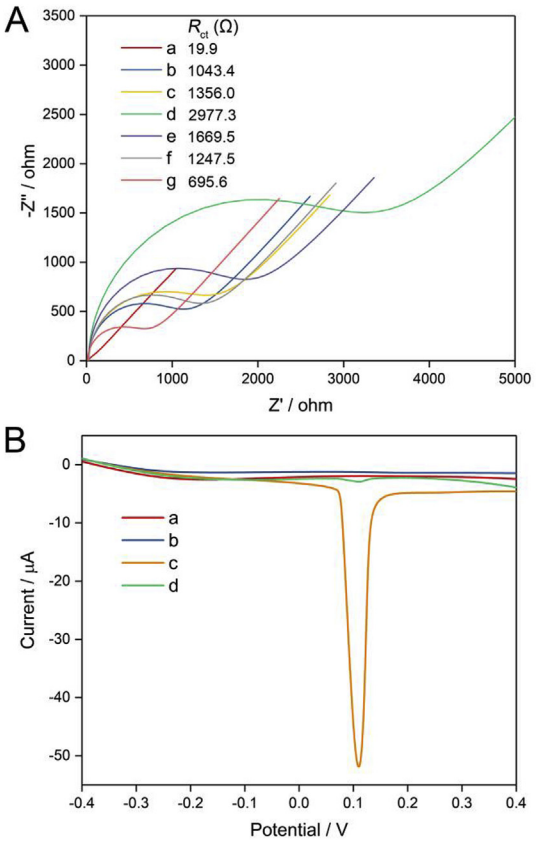


Fig. 1. (A) Nyquist plots corresponding to (a) bare gold electrode, (b) probe a modified electrode, (c) probe d modified electrode, (d) probe a modified electrode after hybridized with probe b and c, after ligation reaction in the (e) presence and (f) absence of T4 PNK, (g) probe d modified electrode after incubation with AgNPs. (B) linear sweep voltammetry curves with AgNPs incubation corresponding to (a) bare gold electrode, (b) probe a modified electrode, after hybridization and ligation reactions in the (c) presence and (d) absence of T4 PNK. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Qualitative experiments

T4 PNK-catalyzed phosphorylation is firstly confirmed by EIS. Negatively charged DNA probes could decelerate electron transfer for the electrochemical species of $[Fe(CN)_6]^{3-/4-}$ [23]. As shown in Fig. 1A, the impedance spectrum of bare electrode contains no semicircle portion, indicating excellent electron transfer rate of the substrate electrode (curve a). However, after the modification of probe a, an obvious semicircle appears with a large charge transfer resistance (curve b). Since the sequence of probe d is the same with probe a, the corresponding modified electrode shows similar semicircle (curve c). After the hybridization reactions between probe a, b and c, more negatively charged DNAs repels $[Fe(CN)_6]^{3-/4-}$, which increases the charge transfer resistance (curve d). With T4 PNK-catalyzed phosphorylation, probe a and c could be ligated. After denaturation, the elongated DNA probe contributes to the larger charge transfer resistance compared with probe a modified electrode (curve e). However, in the absence of target T4 PNK,

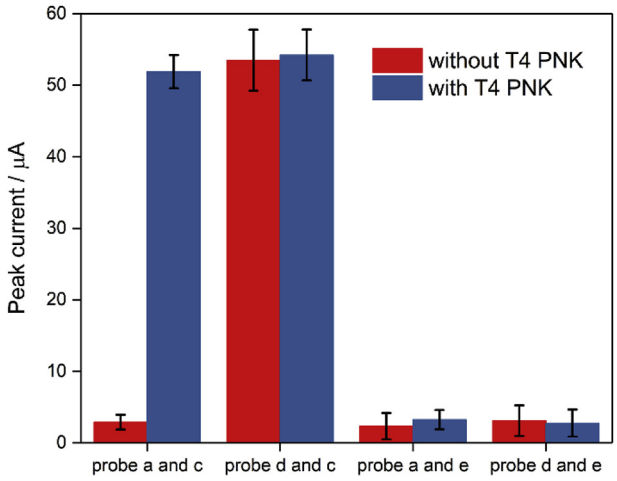


Fig. 2. Linear sweep voltammetry peak intensities for T4 PNK analysis using experimental probes (a, c) and different control probes.

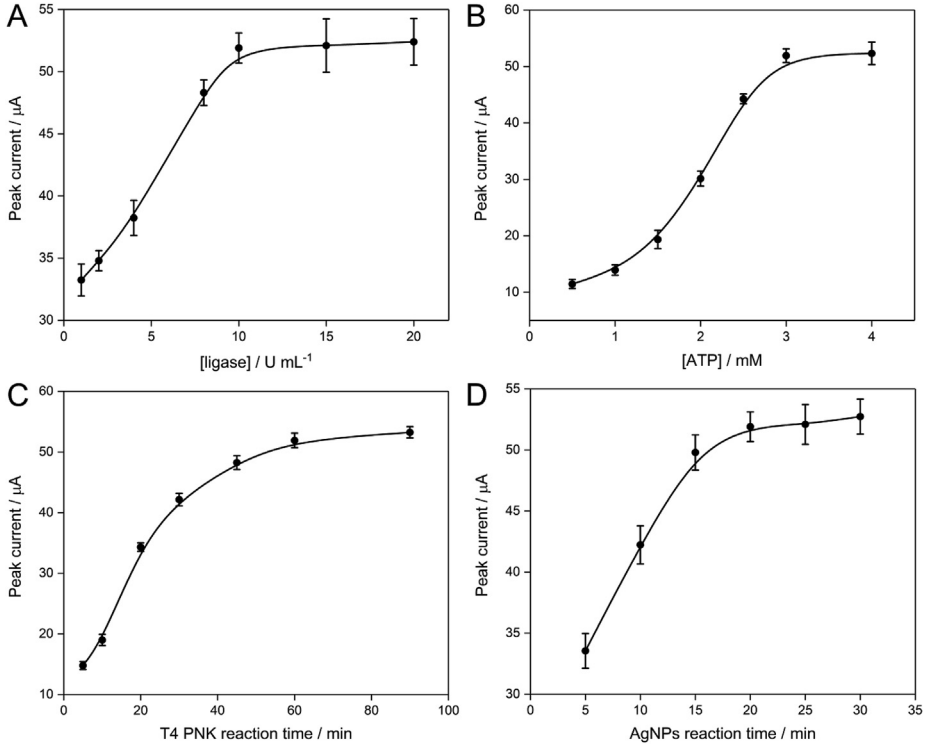


Fig. 3. Optimization of (A) ligase concentration, (B) ATP concentration, (C) T4 PNK reaction time and (D) AgNPs incubation time.

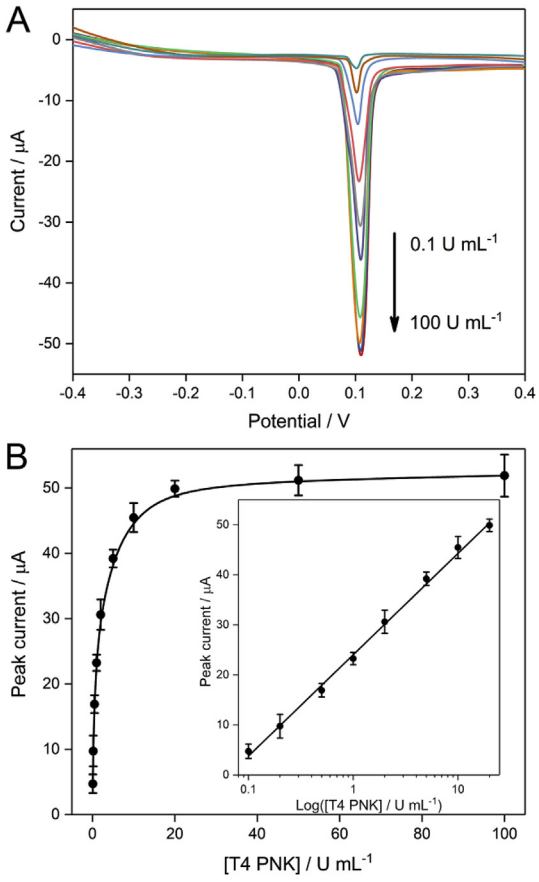


Fig. 4. (A) Linear sweep voltammetry curves for the detection of T4 PNK with the concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 100 U mL^{-1} (from top to bottom). (B) Calibration curve of the electrochemical biosensor. Inset shows the linear range. Error bars stand for standard deviations of three independent measurements.

ligation reaction is inhibited and the DNA probe on the electrode remains the original length. Therefore, the diameter of the semicircle domain does not change significantly (curve f). The effect of adsorbed AgNPs on probe d modified electrode is also studied. Due to the excellent electrical conductivity of AgNPs, the charge transfer resistance is decreased sharply, demonstrating the successful silver-amino chemistry (curve g). We have then employed LSV for investigation. As shown in Fig. 1B, bare electrode and probe a modified electrode cannot adsorb AgNPs, thus, no current peaks are observed. With T4 PNK-catalyzed phosphorylation, amino group modified DNA probe c is linked, which helps localization of AgNPs. Thus, a sharp peak is shown. In the absence of T4 PNK, probe a and c cannot be ligated with the 5′-hydroxyl terminal of probe a. Without the amino group of probe c, no silver stripping peak is available.

The ligation reaction and AgNPs adsorption rely on the phosphate group and the amino group. As shown in Fig. 2, with probe a and c used in the sensing system, the T4 PNK-catalyzed reaction can be distinguished by the variation of LSV peaks. If probe a is previously phosphorylated (probe d), even without T4 PNK, a high peak is achieved, which is similar with the case of T4 PNK, demonstrating the necessity of the phosphate group for the ligation reaction. In addition, if the amino group is removed from probe c (probe e), AgNPs can no longer be localized on the electrode surface, no matter whether the probes are ligated, which are reflected by the low silver stripping peak currents.

3.3. Analytical performance of the proposed method

Before quantitative detection of T4 PNK, several experimental conditions are optimized by comparing the peak currents. As shown in Fig. 3, the peak intensities reach saturation with 10 U mL^{-1} ligase, 3 mM ATP, 60 min T4 PNK-catalyzed reaction and 20 min AgNPs incubation, which are then used as optimized conditions.

Quantitative detection of T4 PNK is then evaluated. Fig. 4A

Table 2
Comparison of the analytical performances of T4 PNK analysis of the proposed method with previous reported methods.

Techniques	Materials	Detection range (U mL^{-1})	LOD (U mL^{-1})	Ref
fluorescence	ligase and reduced graphene oxide	1 to 100	0.2	[24]
colorimetry	HRP-mimicking DNAzyme and λ exonuclease	0.1 to 100	0.06	[25]
fluorescence	graphene oxide and λ exonuclease	0.05 to 10	0.05	[26]
fluorescence	allosteric aptamer and λ exonuclease	0.05 to 0.5	0.05	[27]
fluorescence	dumbbell DNA-templated CuNPs	0.05 to 5	0.05	[28]
fluorescence	nicking enzyme and CuNPs	0.02 to 20	0.02	[29]
fluorescence	WS_2 nanosheets	0.01 to 10	0.01	[30]
colorimetry	G-quadruplex/hemin DNAzyme	0.01 to 0.8	0.01	[16]
DPV	ferrocene-modified SWCNT	0.01 to 0.1	0.01	[31]
LSV	ligase and AgNPs	0.1 to 20	0.01	this work

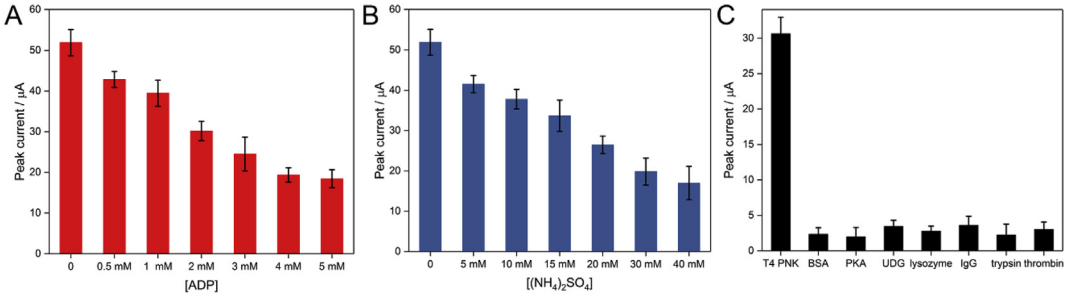


Fig. 5. Inhibition effects of (A) ADP and (B) $(NH_4)_2SO_4$. (C) Selectivity of the electrochemical method for T4 PNA assay.

Table 3
Detection of T4 PNK in serum samples.

Sample	Spiked (U mL^{-1})	Detected (U mL^{-1})	Recovery (%)	Relative error (%)
1	0.5	0.48	96	3.5
	5.0	5.1	102	4.8
2	0.5	0.51	102	5.3
	5.0	5.1	102	4.2

depicts the linear sweep voltammetry responses in the presence of different levels of T4 PNK varied from 0.1 to 100 U mL^{-1}. It is observed that the peak current increases with the increase of T4 PNK concentration. The detailed relationship is shown in Fig. 4B. A linear range is from 0.1 to 20 U mL^{-1} with the equation of $y = 24.009 + 20.245\ x$ ($n = 3$, $R^2 = 0.997$), in which y stands for the peak current, x is the logarithm of T4 PNK concentration. The limit of detection (LOD) is determined to be 0.01 U mL^{-1} based on 3 SD/slope of calibration, which is quite low. After comparing with some representative T4 PNK assay reported previously, the analytical performance of this method shows great superiority (Table 2). Furthermore, we have demonstrated the good reproducibility by challenging different sets of AgNPs and electrodes (Fig. S2).

3.4. Inhibition, selectivity, and practical investigation

It has been disclosed that ADP and $(NH_4)_2SO_4$ are two known inhibitors of T4 PNK [32]. Target enzyme is firstly spiked with different concentrations of inhibitors, which are then used for phosphorylation reaction. As shown in Fig. 5A and B, with more ADP or $(NH_4)_2SO_4$, T4 PNK activity is inhibited and the obtained LSV peak currents drops gradually, the results of which are agreed with previously reported literatures [33,34]. Therefore, it shows great potential use for screening inhibitors of T4 PNK. To verify the high selectivity of this method for T4 PNK assay, we have studied the LSV responses of this method towards various potential interferents including BSA (0.1 μg mL^{-1}), PKA (2 U mL^{-1}), UDG (2 U mL^{-1}), lysozyme (0.1 μg mL^{-1}), IgG (0.1 μg mL^{-1}), trypsin (0.1 μg mL^{-1}) and thrombin (0.1 μg mL^{-1}). These interfering species cannot result in the increase of peak currents. Only target T4 PNK (2 U mL^{-1}) is able to generate a high current peak, which demonstrates the high specificity of this method (Fig. 5C).

To prove the practical utility of this method, different amount of T4 PNK is spiked into human serum samples and the concentrations are then detected by the proposed method, which are consistent with the initially spiked amounts (Table 3). The recoveries are from 96% to 102% and the all relative errors are less than 6%, demonstrating that this method is applicable to complicated biological samples.

4. Conclusions

In summary, a ligation reaction based electrochemical assay, which converts phosphorylation event into silver stripping peak, is developed for sensitive and selective detection of T4 PNK. Under optimal conditions, a low LOD of 0.01 U mL^{-1} is achieved with a linear range of 0.1–20 U mL^{-1}. This method is also used to screen the effects of inhibitors of T4 PNK and shows excellent selectivity. It avoids complicated design and costly labelling, which may provide a facile sensing platform for monitoring enzyme activity in the applications of biological research, drug discovery and clinical diagnosis.

Conflicts of interest

There are no conflicts to declare.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2019.07.072.

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