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ORIGINAL PAPER

Colorimetric determination of the activity of alkaline phosphatase based on the use of Cu(II)-modulated G-quadruplex-based DNAzymes

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Abstract
A colorimetric detection scheme is introduced for the determination of alkaline phosphatase (ALP) activity based on Cu(II)-modulated G-quadruplex-based DNAzymes. It is exploiting the strong affinity of Cu(II) for pyrophosphate (PPi) upon which the cofactor PPi is trapped by Cu(II). Hence, the activity of the DNAzyme is inhibited. ALP catalyzes the hydrolysis of PPi, causing the release of Cu(II). DNAzyme, in turn, is activated and catalyzes the cleavage of the DNA probe substrate. The released G-rich sequence folds into the G-quadruplex, which can bind hemin and catalyze the oxidation of 2,2′-azinobis (3-ethylbenzothiozoline)-6-sulfonate (ABTS), and this leads to an increase in absorbance at 420 nm. Absorbance increases linearly with increasing ALP activity in 0.07 to 300 $U{\cdot}L^{-1}$ range, with a 70 $mU{\cdot}L^{-1}$ detection limit. The method was applied in ALP inhibition tests and to the determination of ALP activity in spiked serum samples where it gave satisfactory results.

Keywords Pyrophosphate · ABTS · Hemin · Enzyme activity assay · ALP inhibitor

Introduction

Alkaline phosphatase (ALP), a widely distributed enzyme in mammalian tissues, is responsible for dephosphorylation in metabolic pathways [1]. Because the level of serum ALP is linked closely to an extensive range of diseases, including osteopathy, hepatopathy, breast and prostatic carcinoma, cardiac diseases, and diabetes [2, 3], it has been developed to become an important biomarker to help in early diagnosis of hardly-recognized diseases. Beside its high potential in clinical field, it is also advantageous to forensic investigations [4]. Hence, progressive exploration of a strategy for ALP detection that has high velocity, sensitivity, facility, and adjustable dynamic range is undoubtedly in considerably high demand.

Over the past decades, various methods have been put forward to detect ALP activity, and some of these have made some progress in their high sensitivities based on techniques, such as fluorometry [5–12], electrochemistry [13–17], chromatography [18], colorimetry [19–21], and surface-enhanced Raman spectroscopy [22]. Despite some progress, some of these methods have disadvantages: they are time-consuming, high-cost, and incapable of convenient and/or real-time measurement. It is worth mentioning that among these various methods, the fluorometric methods, in which different types of fluorescence probes are mainly utilized, has its advantage and is of many researchers' interests owing to its rapid response, relatively high sensitivity, and convenience. Thousands of papers have established numerous fluorescence probes, and a large portion of which, probes are designed to detect ALP activity. These probes can include conjugated polyelectrolytes, small molecule organic probes, metal nanoclusters, nanosheets, DNA-templated nanoparticles, and quantum

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dots (QDs) [5–10]. Although these fluorescent strategies have indeed made an unignorable contribution to ALP detection, their disadvantages which cannot be ignored can include poor photostability and water solubility of organic fluorescent dyes, complex synthesis and purification processes of conjugated polyelectrolytes, and high toxicity of QDs. Recently, some late-model methods have been established for the detection of ALP activity, in which carbon dots were used as fluorescent signal [23, 24]. Nevertheless, these fluorescence quenching-based enzymatic assays can possibly produce false positive signals when interfered by environmental stimulus. Qu et al. have developed a novel and sensitive turn-on fluorescence method via carbon dots and MnO_2 nanosheets [10]. Its popularity is, however, restricted by its expensive and time-consuming synthesis of MnO_2 nanosheets. In addition, Sun and co-workers have advanced the fluorescence-based ALP detection with highly fluorescent dots. In contrast to these unfavorable features, a unique visual detection technique that can directly observe such signals without any highly complex and/or costly apparatus has gained increasing attention. A commercial kit has been the dominate detection method for detecting of ALP [25]. However, the sensitivity is sometimes unsatisfactory. Therefore, a facile, sensitive, and low-cost method for the detection of ALP is desired.

Herein, we report a colorimetric assay system based on Cu^{2+}-modulated G-Quadruplex-based DNAzymes for the detection of ALP activity [26]. Owing to the advantage of strong binding ability of Cu^{2+} and pyrophosphate (PPi) [27–29], cofactor Cu^{2+} can be trapped and lead to effective inhibition of DNAzyme activity. Because ALP can catalyze the hydrolysis of PPi, ALP can cause the release of Cu^{2+}, and in turn activate DNAzyme, which catalyzes the cleavage of DNA probe substrate. These events result in increased absorbance of 2,2′-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid)-H_2O_2 system [30–32].

Experimental

Materials and methods

Alkaline phosphatase (ALP), Uracil DNA glycosylase (UDG), 8-hydroxy guanine DNA glycosidase (hoGG1), bovine serum albumin (BSA), T4 polynucleotide kinase (T4 PNK),and Lambda exonuclease (λ Exo) were purchased from Takara Biotechnology Co., Ltd. (http://www.takarabiomed.com.cn/) (DaLian, China). HPLC-purified DNA probe(agc ttc ttt cta ata cgg tgg gta ggg cgg gtt ggg cta ccc acc tgg gcc tct ttc ttt tta aga aag aac)was obtained from Sangon Biotechnology Co., Ltd. (http://www.sangon.com/) (Shanghai, China). HeEPES, free acid, Sodium chloride (NaCl), Tris (Tris-(hydroxymethyl) aminomethane), hydrochloric acid (HCl), copper sulfate ($CuSO_4$), pyrophosphate (PPi), and hydrogen peroxide (H_2O_2) were purchased from Sinopharm Chemical Reagent Co., Ltd. (http://en.reagent.com.cn/) (Shanghai, China). Hemin and 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) were purchased from Yuanye (http://www.shyuanye.com/) (Shanghai, China). Inorganic phosphate (Pi) was obtained from an equilibrium buffer solution of Na_2HPO_4 and NaH_2PO_4 (pH = 7.4). All other reagents were of analytical-reagent grades. Ultrapure water (18.2 MΩ cm^{-1}) was used in all experiments. All DNA sequences were prepared in TE buffer and stored at −20 °C.

The absorbance was recorded on an Enspire® multimode plate readers (Perkin Elmer, USA) using a 96-well plate. The absorption spectra of the solution were measured at wavelengths from 400 to 470 nm. The absorbance was obtained at 420 nm.

Investigation of feasibility

To investigate feasibility of the method in assaying ALP activity, two types of samples (mixtures A and B) were prepared. In the first sample (mixture A; 35 μL), 60 nM DNA Probe, 10.5 mM NaCl, and 3.5 mM HEPES were first added into 29.5 μL of ultrapure water. The mixture was then heated to 80 °C for 2 min and cooled down to room temperature for 30 min. The second sample (mixture B; 35 μL) was prepared by mixing 4×10^3 U·L^{-1} ALP, 700 μM·L^{-1} PPi, 400 μM·L^{-1} Cu^{2+} in 31 μL tris buffer, and was then incubated at 37 °C for about 30 min. After that, mixtures A and B were mixed and incubated at 25 °C for 15 min, and 1 μL of 100 μM hemin was subsequently added and incubated at 25 °C for 30 min to allow the DNA probe to properly fold and form G-quadruplex/hemin complex. Finally, 15 μL each of 20 mM H_2O_2 and 20 mM ABTS was added into the mixture. After 10 min, the absorption spectra from 400 to 470 nm was measured. Samples, in which ALP were not added, were done in parallel for comparison.

Optimization of analysis conditions

Various concentrations of DNA probe (10–200 nM), PPi (100–800 μM), and Cu^{2+} (100–1000 μM) were tested in order to find an optimal condition.

ALP activity assay

Twelve samples were prepared in the assay of ALP activity under an optimized condition. Mixture A in a reaction buffer (29.5 μL H_2O, 3.5 mM HEPES,

10.5 mM NaCl) containing 60 nM DNA probe, and mixture B in a reaction buffer (10 mM Tris-HCl, 700 μM·L^{-1} PPi, 400 μM·L^{-1} Cu^{2+}, pH 7.5) containing various amounts of ALP were first mixed, and 1 μL of 100 μM, 15 μL of 20 mM H_2O_2, and 15 μL of 20 mM ABTS were then added. The detailed reaction conditions were the same as that used in the investigation of feasibility.

Determination of ALP activity in human serum samples

ALP activity was assayed under the optimal experimental conditions. Human serum samples (1%) and different ALP activities (in the working range of this method) were thoroughly mixed, and the reaction was allowed to take place at room temperature for 3 min. While mixture A was

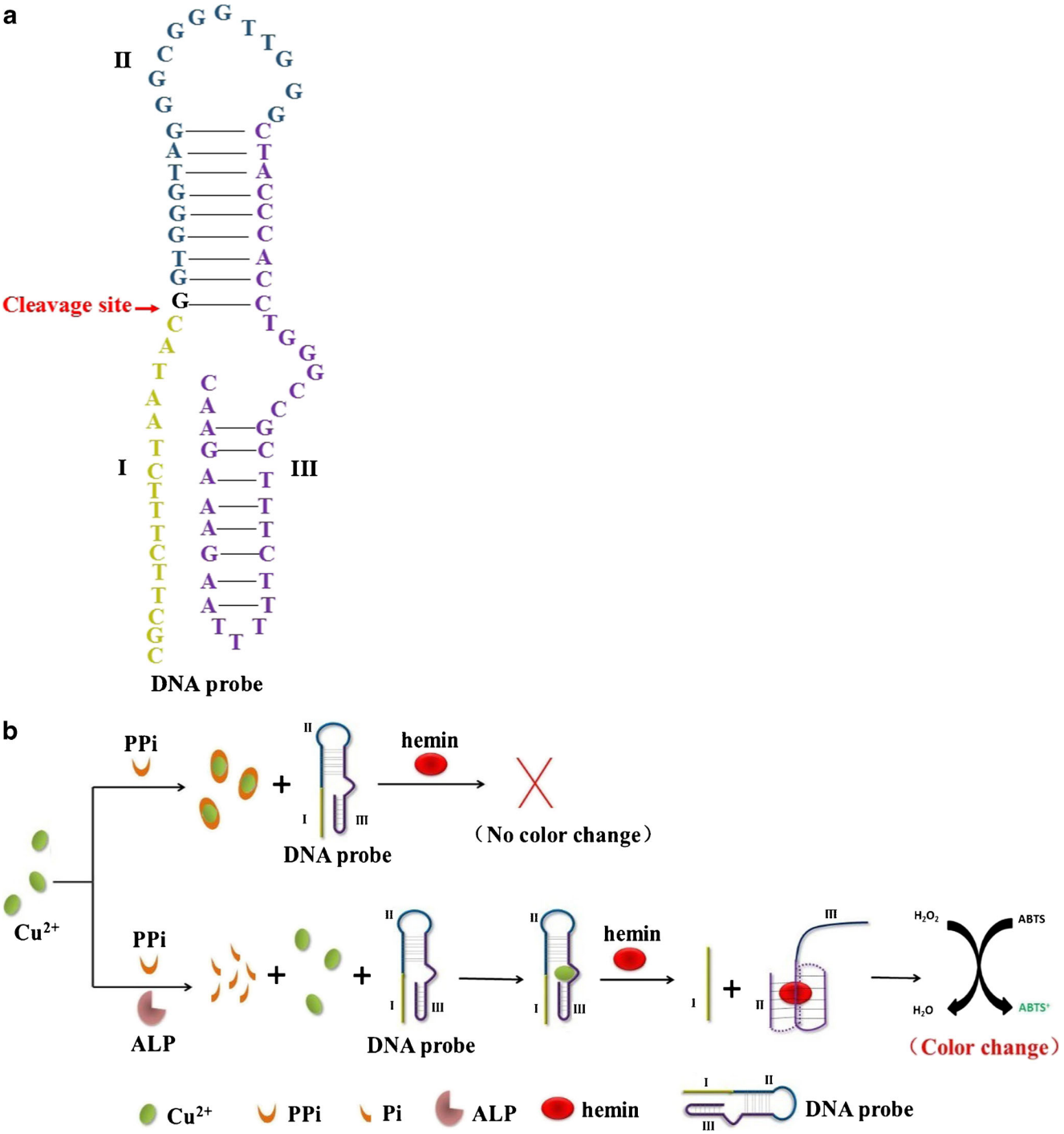

Fig. 1 **a** The structure of the DNA porbe. **b** Schematic illustration of ALP activity detection principle

prepared according to that for the ALP activity assay, mixture B was in 10 mM Tris-HCl (pH 7.5), 700 $\mu M \cdot L^{-1}$ PPi, 400 $\mu M \cdot L^{-1}$ Cu^{2+}, and different activities of ALP. Subsequent procedures and absorbance measurements were carried out following the ALP activity assay. Recoveries of ALP from the serum samples were calculated by the regression equation using the absorbance and the activity of ALP.

Results and discussion

Experimental principles

Principle of the proposed detection method is illustrated in Fig. 1b, and the structure of the DNA porbe is shown in Fig. 1a. The DNA probe consists of three main components: domain III represents the DNA-cleaving Cu^{2+}-dependent DNAzyme; domain II contains the sequence of HRP-mimicking DNAzyme, which can give colorimetric signal readout; and domain I is the substrate of DNA-DNAzyme. In the absence of ALP, PPi can strongly chelate and form stable complex with Cu^{2+} ion. As a result, the activity of DNAzyme, which catalyzes the cleavage of DNA probe substrate, is inhibited, leading to a low absorbance background in the assay system. On the other hand, when APL is present, it catalyzes the hydrolysis of PPi into phosphate, causing the release of free Cu^{2+} cofactor, which thus activates DNAzyme to catalyze the cleavage of DNA probe substrate. The released G-rich sequence (domain II) folds into a G-quadruplex that can intercalate hemin and form catalytically active HRP-mimicking DNAzyme, resulting in an increased absorbance of the ABTS-H_2O_2 system, in which ALP levels can subsequently be determined by the variations of absorbance intensity.

Feasibility of the proposed strategy

To verify whether or not the proposed strategy is feasible, two samples were prepared: sample A containing DNA probe, Cu^{2+}, and PPi; and sample B containing DNA probe, Cu^{2+}, PPi, and ALP. Fig. 2 shows the absorption spectra of the detection system in the presence (curve b) and absence (curve a) of ALP. The data showed that when ALP is absent (curve a), the absorbance is markedly lower than that when ALP is present, suggesting that Cu^{2+} may be reduced by PPi and no G-quadruplexes are formed. As displayed in Fig. 2b, absorbance is significant increased in the presence of ALP (4×10^3 $U \cdot L^{-1}$) compared with that in the absence of ALP, indicating that ALP blocks PPi from chelating Cu^{2+}, and in turn G-quadruplexes are formed and led to such enhancement of colorimetric signal. Finally, these results demonstrated that the proposed ALP detection strategy was feasible.

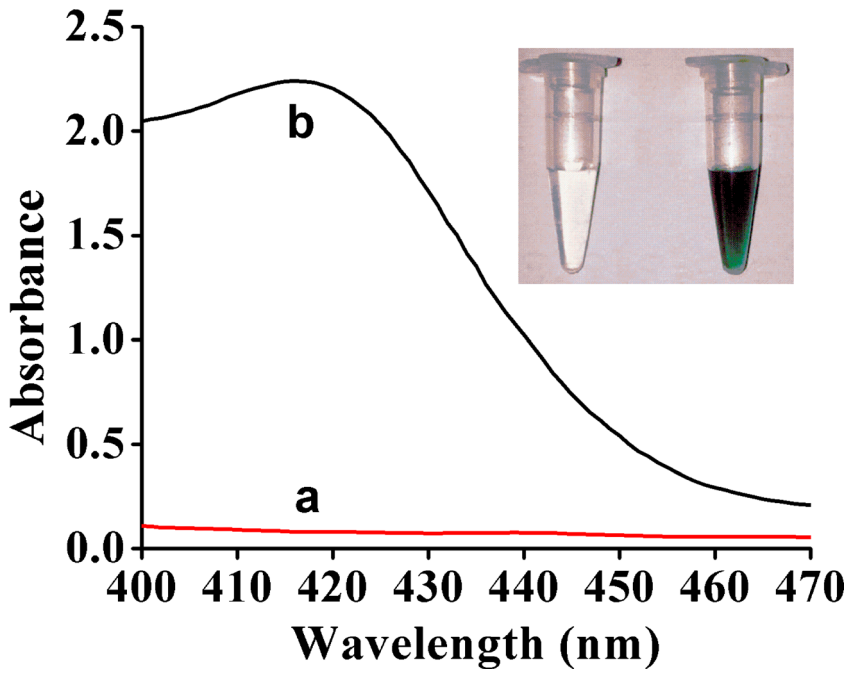

Fig. 2 Absorbance spectra in the absence (**a**) and presence (**b**) of ALP

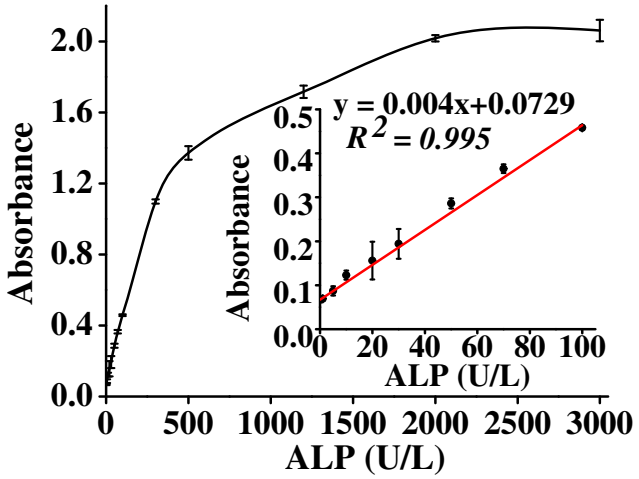

Fig. 3 ALP assay: linear curve of the enhanced absorbance to activity of ALP. The inset shows the linearity of the absorbance respect to ALP activities. ($n = 3$)

Optimization of method

The following parameters were optimized: (a) DNA probe concentration; (b) Cu^{2+} concentration; (c) PPi concentration. Respective data and figures are given in the Fig. S1. The following experimental conditions are found to give best results: (a) A DNA probe concentration of 60 nM; (b) A Cu^{2+} concentration of 400 μM; (c) A PPi concentration of 700 μM.

Quantification of ALP activity

Under the chosen optimal conditions, performance of the proposed strategy, in terms of ALP activity detection, was systematically investigated, in which the activities of ALP are varied from 0 to 3000 $U{\cdot}L^{-1}$ (0.07, 1, 5, 30, 50, 70, 100, 300, 500, 1200, 2000, and 3000 $U{\cdot}L^{-1}$). As shown in Fig. S2, the absorbance increased with increasing activity of ALP and plateaued at 2000 U/L. The inset of Fig. 3 showed that the absorbance has a linear correlation (R^2 = 0.9946) with ALP activity ranges from 0.07 to 300 $U{\cdot}L^{-1}$. The detection limit is estimated to be 0.07 $U{\cdot}L^{-1}$ according to the 3σ rule, which is comparable to or better than those of other methods (Table 1).

Table 1 Comparison of different methods for the detection of ALP activity

Method	Linear range ($U{\cdot}L^{-1}$)	LOD ($U{\cdot}L^{-1}$)	Reference
Cu(II)-phenanthroline	0–200	3.5	[33]
BSA-AuNCs	1–200	0.05	[8]
Thioflavin T	1–200	1	[34]
Graphitic C_3N_4	0.1–1000	0.08	[9]
Carbon Quantum Dots	6.7–782.6	1.1	[23]
Copper nanoparticles	0–8	0.1	[2]
Quantum Dots	0–3	0.1	[12]
Colorimetric method	0.07–100	0.07	This work

Selectivity

A series of enzymes, including UDG, hoGG1, Lambda Exo, and PNK, each at a concentration of 2×10^3 $U{\cdot}L^{-1}$, were tested. Some other molecules, such as lysozyme, BSA, streptavidin (SA), glycine, alanine, arginine, serine, tryptophane, glutamic acid, L-histidine, $C_6H_{12}O_6$ and ATP, each at a concentration of 0.5 μM, were also tested. Fig. 4 shows that none of these proteins led to increased absorbance, in contrast to ALP. The results demonstrate that the method has good selectivity.

ALP activity inhibition assays

The inhibition of ALP is closely associated with drug screening and disease therapy; validity of the proposed assay in evaluation of ALP inhibition was thus investigated. Na_3VO_4 as a common ALP inhibitor was employed for inhibiting assays [35]. Fig. 5 shows that the relative activity of ALP drastically decreased with increasing

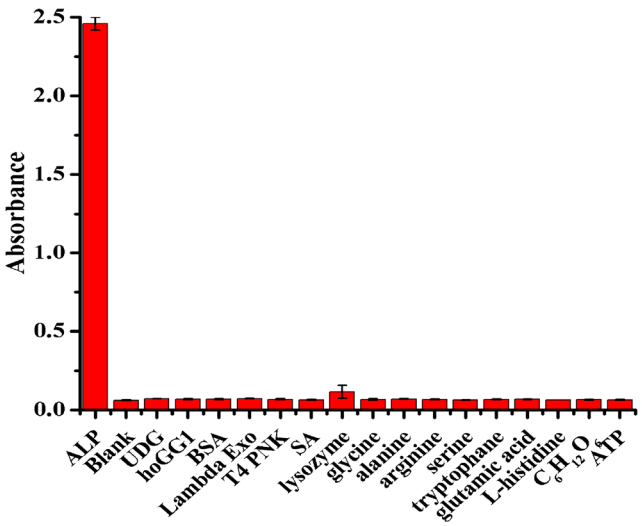

Fig. 4 Selectivity of the proposed assay. Absorbance of ALP in comparison with those of interference molecules: UDG, hoGG1, BSA, Lambda Exo, PNK, lysozyme, SA, glycine, alanine, arginine, serine, tryptophane, glutamic acid, L-histidine, $C_6H_{12}O_6$ and ATP. (n = 3)

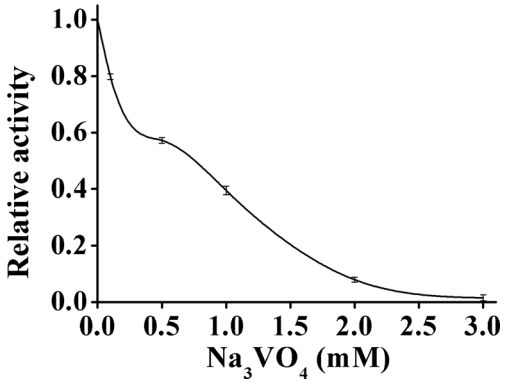

Fig. 5 Inhibitory effect of Na_3VO_4 (0, 5, 10, 20, and 30 mM) on ALP activity. (n = 3)

Na_3VO_4 concentration with an IC_{50} value of 0.9 mM. This result shows that the proposed strategy is suitable for the identification and characterization of enzymatic inhibitors.

ALP activity assays in biological samples

To investigate practical use of the method, ALP was tested in 1% human serum. Table 2 shows the recovery amount of ALP detected by the proposed assay when ALP of 10, 20, and 50 $U{\cdot}L^{-1}$ were added into the biological sample. The recovery rates were 100.6%, 102.5%, and 91% from sample added with 10, 20, and 50 $U{\cdot}L^{-1}$ of ALP, respectively. These results show that the proposed method is highly potential for practical detection in biological systems.

Conclusions

In summary, a convenient colorimetric method based on Cu^{2+}-modulated G-Quadruplex-based DNAzymes, is successfully developed and applied for ALP activity detection. The method exhibites high sensitivity to ALP with a detection limit of 0.07 $U{\cdot}L^{-1}$ under optimal conditions. Moreover, the proposed method is highly selective and successfully applied in quantitative determination of ALP in human serum samples with satisfactory result. This method does not require complicated synthesis and/or modification of the probes. Thus, the present strategy may have potential applications for ALP detection in analytical practice.

Table 2 Recovery experiments of ALP in diluted human serum using this method

Sample	Added ($U{\cdot}L^{-1}$)	Found ($U{\cdot}L^{-1}$)	Recovery (%)
1	10	10.06 ± 0.44	100.6
2	20	20.5 ± 2.06	102.5
3	50	45.5 ± 0.29	91

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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