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A turn-on fluorescent method for determination of the activity of alkaline phosphatase based on dsDNA-templated copper nanoparticles and exonuclease based amplification

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Abstract The authors describe a method for the determination of the activity of alkaline phosphatase (ALP) that utilizes dsDNA-templated copper nanoparticles (CuNPs) coupled to enzymatic amplification via λ exonuclease. A hybrid of a DNA modified with a phosphate moiety at the 5′-end (P-DNA) and a P-DNA complementary sequence (cP-DNA) is employed as the dsDNA substrate for ALP. In the absence of ALP, the dsDNA is cleaved by the λ exonuclease, which hinders the formation of CuNPs which display fluorescence with excitation/emission peaks at 340/565 nm. However, ALP-mediated hydrolysis of the 5′-phosphoryl end impedes the cleavage of dsDNA by the λ exonuclease, and this promotes the formation of fluorescent dsDNA-templated CuNPs via ascorbate-mediated reduction. Under the optimized experimental conditions, this method exhibits a high specificity to ALP and has a 0.1 $U \cdot L^{-1}$ limit of detection. The strategy also provides the basis for a screening platform for inhibitors of ALP.

Keywords Fluorescence · Nanoprobe · CuNPs · Enzyme activity assay · ALP inhibitor

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Introduction

Alkaline phosphatase (ALP) is found in almost all living organisms and in a variety of tissues (liver, bone, intestine, kidney, and placenta) [1]. Since ALP plays a pivotal role in cell cycle, growth, apoptosis, and signal transduction pathways, it is commonly used as a biomarker for the development of phosphorylation-related biochemical behaviors and clinical laboratory tests [2]. Studies have shown that abnormal serum levels of ALP are closely related to a variety of human diseases, especially those of the liver and bone [3]. Currently, serum ALP level is routinely used as a diagnostic index for several human diseases such as bone disorders (Paget's disease and osteomalacia), cancer (breast cancer and prostatic cancer), diabetes, and liver dysfunction and the normal concentration of ALP in human serum is in the range of 20–140 U/L [4–6]. Considering the significance of ALP levels in diagnosis of human diseases, the development of a simple, sensitive, yet inexpensive method for detection of ALP activity and its inhibition is required.

Several novel analytical strategies have been developed for detection and quantitation of ALP activity, such as colorimetric assays [7–9], electrochemical detection assays [10–12], and assays based on surface enhanced resonance Raman scattering (SERRS) assay [13]. Although these novel analytical strategies are usually highly sensitive and require small sample volume, they are expensive and require tedious sample preparation compared to the fluorescence-based methods. So far, some facile, low-cost, and sensitive fluorescence-based strategies have been used to assay the activity of ALP via its ability to remove phosphate groups from different types of substrates such as nucleic acids, proteins and small molecules [14–29]. Among these substrates, nucleic acids have been extensively used to set up fluorescence-based methods for detection of ALP activity. For example, Wang et al. reported

a promising new fluorescence-based sensing strategy for ALP detection that utilizes the polycation-induced noncovalent perylene DNA probe self-assembly [21]. However, this approach involves complicated procedures for probe synthesis. Two fluorescence assays that use the graphene oxide platform and nucleic acid substrates for ALP detection have also been reported [18, 25]. However, the synthesis of graphene oxide is time-consuming and expensive. Du and co-works reported a new method for detection of ALP activity based on a DNA hairpin and thioflavin T (ThT) probes [14]. Although this method is low-cost and facile, and has poor sensitivity, which impedes its application. Thus, development of a simple, sensitive, cost-effective, and robust method to assay ALP activity is required.

DNA-templated metal nanoparticle—a new fluorescent probe—has attracted interest in the field of analytical sciences due to its inherent advantages such as superior optical properties, low toxicity, and high sensitivity [30]. Compared to the DNA-templated silver nanoparticles, the DNA-templated copper nanoparticles (CuNPs) possess the inherent advantage of being inexpensive and simple to synthesize. The DNA-templated CuNPs have been widely used for biomarker detection owing to their specificity towards the DNA templates and generation of relatively low background from biomolecules such as DNA, microRNA, small molecules, metal ions, enzyme and proteins in biological systems [31–35]. For example, two groups reported the detection of ALP activity based on the formation of fluorescent CuNPs employing DNA as a template [36, 37]. In this system, ALP hydrolyzes pyrophosphate and releases free Cu^{2+} ions, which effectively form DNA-templated fluorescent CuNPs.

Here, a fluorescence-based strategy is prepared and applied in ALP activity detecting that utilizes the specificity of the fluorescent CuNP probes towards dsDNA templates and the λ exonuclease (λ exo) cleavage reaction. λ exo specifically degrades dsDNA strands with a 5′-phosphoryl termini, generating single-stranded DNA (ssDNA) and mononucleotides. A dsDNA, constructed by hybridizing a P-DNA (modified with a phosphate moiety at 5′-end) and a P-DNA complementary sequence (cP-DNA), was used as the substrate for ALP in our method. In addition, the dsDNA used in this assay was used as templates for synthesizing fluorescent dsDNA-templated CuNPs. In the absence of ALP, the dsDNA is digested by λ exo, which prevents formation of the dsDNA-templated CuNPs. Once the 5′-phosphoryl end is hydrolyzed by ALP, the λ exo cleavage reaction is impeded and the system displays strong fluorescence due to the formation of the dsDNA-templated CuNPs. This new approach provides a low-cost and sensitive platform for detection of ALP and its inhibitors. Furthermore, our method can be also used for detection of ALP in human serum.

Experimental section

Materials and reagents

Alkaline phosphatase from *E. coli* C75 was procured from Takara Biotechnology Co., Ltd. (DaLian, China, http://www.takara.com.cn,). λ exonuclease was purchased from New England Biolabs (Beverly, MA, USA, http://www.neb.sg). Glucose oxidase (GOx) was purchased from MP Biomedicals (Santa Ana, California, USA, http://www.mpbio.com). Thrombin was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA, http://www.sigmaaldrich.com). Bovine serum albumin (BSA) was purchased from Solarbio (Beijing, China, http://www.solarbio.com). 3′-(N-morpholino) propanesulfonic acid (MOPS), copper sulfate ($CuSO_4$), and sodium ascorbate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China, http://www.shreagent.lookchem.com). Inorganic phosphate (Pi), which exists in a buffered equilibrium mixture of Na_2HPO_4 and NaH_2PO_4 (pH 7.4) was used in this assay. All other reagents were of analytical reagent grade. Ultrapure water (18.2 MΩ·cm) from a Milli-Q water purification system (Millipore Corp, Bedford, MA, USA) was used in all the experiments. The two oligonucleotides, P-DNA and cP-DNA, were purchased from Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) and purified using high performance liquid chromatography. The DNA sequences are as follows. P-DNA: 5′-p-CGTGATGA ACGTATGAGCGTAT-3′, cP-DNA: 5′-ATAC GCTCATACGTTCA TCACG-3′. The buffer used are as follows: MOPS buffer (10 mM MOPS, 150 mM NaCl and 10 mM $MgCl_2$, pH 7.5).

Apparatus

All fluorescence measurements were performed on an F-2700 fluorescence spectrophotometer (Hitachi, Japan) with excitation at 340 nm and emission at 500–650 nm for the dsDNA-templated CuNPs. The excitation slits and emission slits were set for 5.0 and 10.0 nm, respectively. Each experiment was carried out in a final volume of 100 μL. All the error bars in this paper were estimated from three replicate measurements.

Determination of ALP

In order to quantitatively measure ALP activity, 500 nM each of P-DNA and cP-DNA were mixed in 100 μL MOPS buffer (10 mM MOPS, 150 mM NaCl and 10 mM $MgCl_2$) and incubated for 10 min at 37 °C to form the dsDNA. Next, different concentrations of ALP were added to the substrate and incubated for 10 min at 37 °C. 45 $U·mL^{-1}$ λ exo was subsequently added to the reaction solution, and incubated for

20 min at 37 °C. 140 μM Cu^{2+} and 1 mM sodium ascorbate were added to the mixture and incubated for 10 min at room temperature before measurement.

For detection of ALP in biological fluids, spiked samples were prepared by addition of different concentrations of ALP to 1% (v/v) human serum.

Inhibition of ALP activity

In this assay, Pi, the inhibitor of ALP, was incubated at various concentrations ranging from 0 to 50 μM with 500 nM dsDNA (P-DNA/cP-DNA) in 100 μL MOPS buffer. Then, 50 U·L^{-1} of ALP was added to the substrate and incubated for 10 min at 37 °C. Other experimental procedures were the same as mentioned before.

Results and discussion

Verification of the feasibility of ALP detection using dsDNA-templated CuNPs

The principle of the detection method is illustrated in Scheme 1. Firstly, we designed a P-DNA modified with a phosphate moiety at the 5′-end and a complementary P-DNA sequence (cP-DNA) without any modification. In the absence of ALP, the P-DNA/cP-DNA complexes can be cleaved by λ exo, which hinders the formation of the dsDNA-templated CuNPs. In the presence of ALP, the 5′-phosphate of P-DNA is hydrolyzed to a hydroxyl group, and the λ exo cleavage reaction is impeded. Upon addition of Cu^{2+} and ascorbate to the re-action solution, dsDNA-templated CuNPs were formed that exhibited a strong fluorescence response. ALP levels were subsequently determined by monitoring the variation in fluorescence intensity.

Next, we assessed the feasibility of the proposed method. We observed low levels of fluorescence at 340 nm excitation when single stranded P-DNA or cP-DNA was used to
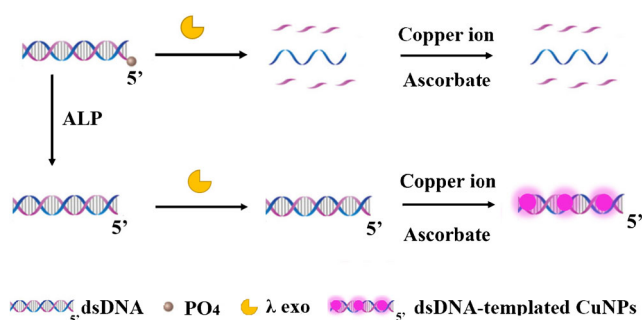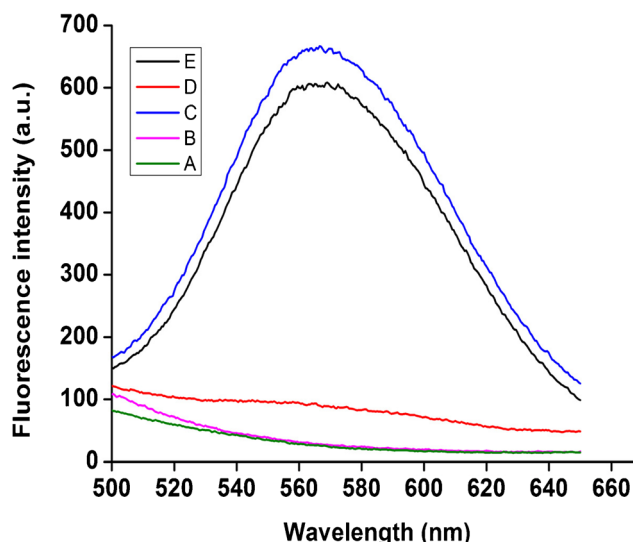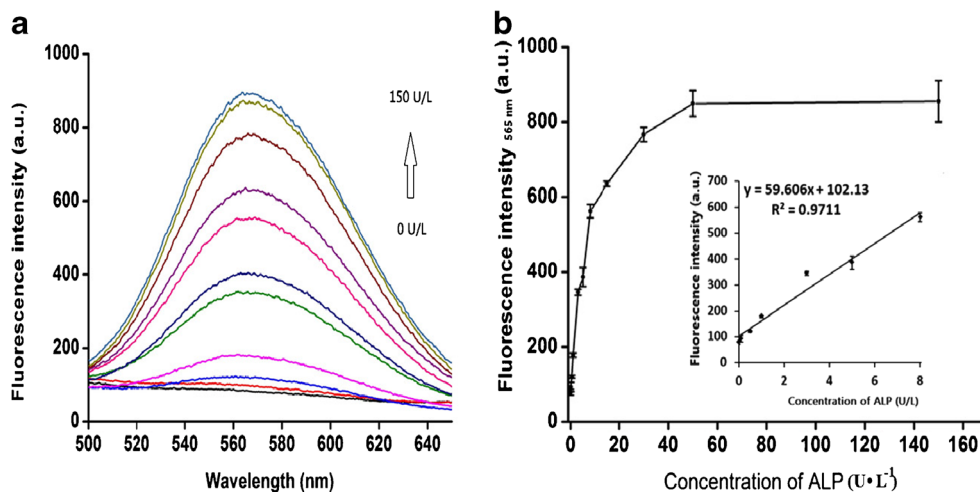

Fig. 1 The feasibility of the proposed method (emission peaks at 565 nm). (A) Synthesis of CuNPs with 500 nM P-DNA; (B) Synthesis of CuNPs with 500 nM cP-DNA; (C) Synthesis of CuNPs with 500 nM dsDNA (P-DNA/cP-DNA complexes); (D) 500 nM dsDNA and 45 U·mL^{-1} λ exo were incubated for 10 min at 37 °C and then used for CuNP synthesis; (E) 500 nM dsDNA and 45 U·mL^{-1} λ exo were incubated for 10 min at 37 °C after pre-incubation of the dsDNA with 200 U·L^{-1} ALP for 10 min at 37 °C, and then used for CuNP synthesis

synthesize the CuNPs, respectively (Fig. 1, Curve A and Curve B). However, high fluorescence signal was detected when dsDNA (P-DNA/cP-DNA complexes) was used to synthesize the CuNPs (Fig. 1, Curve C). The fluorescence of the CuNPs decreased with addition of λ exo to the dsDNA owing to the λ exo-mediated cleavage of the dsDNA template (Fig. 1, Curve D). Once the 5′-phosphoryl end is hydrolyzed by ALP, the λ exo cleavage reaction is impeded and the system displays strong fluorescence due to the formation of the dsDNA-templated CuNPs (Fig. 1, Curve E). In summary, the results of this experiment demonstrated the feasibility of the proposed strategy for detection of ALP activity.

Optimization of experimental conditions

The following parameters were optimized: concentrations of dsDNA, Cu^{2+}, and λ exo; the reaction time of ALP with the dsDNA; the reaction time between the Cu^{2+}/ascorbate mixture with the dsDNA; and the λ exo cleavage reaction time on the fluorescence signal output. Corresponding data appear in the Electronic Supporting Material (Fig. S1-Fig. S6). We found the following experimental conditions gave best results: (1) 500 nM dsDNA; (2) 140 μM Cu^{2+}; (3) 10 min reaction time between the Cu^{2+}/ascorbate mixture with the dsDNA; (4) 10 min reaction time between ALP and its substrate; (5) 45 U·mL^{-1} λ exo; (6) 20 min λ exo cleavage reaction time. These were used throughout the experiment.

Scheme 1 Schematic illustration of alkaline phosphatase activity assay based on dsDNA-templated copper nanoparticles coupled with λ exonuclease cleavage

Quantitative measurement of ALP activity

To evaluate the sensitivity of the proposed method, a series of
ALP concentrations ranging from 0 to 150 U·L^{-1} (0, 0.1, 0.5,
1, 3, 5, 8, 15, 30, 50, 150 U·L^{-1}) were used under the optimal
conditions described above. As shown in Fig. 2a, the
fluorescence intensity at 565 nm dynamically increased
with increase in the concentration of ALP. Figure 3b
shows the relationship between fluorescence intensity
and the concentration of ALP. The inset of Fig. 2b
shows that the fluorescence intensity had a linear rela-
tionship (R^2 = 0.9711) with ALP concentration in the
concentration range of 0–8 U·L^{-1} and the regression
equation was F = 59.606C + 102.13 (C, U·L^{-1}), where
F was the fluorescence intensity at 565 nm and C was
the ALP concentration. The limit of detection (LOD) of
the proposed strategy was estimated to be 0.1 U·L^{-1},

which was lower than those of previously reported
ALP detection methods (Table 1). Furthermore, the total
reaction time in our method only required 40 min,
which was much shorter than most existing methods
(Table 1). Therefore, the method we developed can de-
tect ALP with high sensitivity and simplicity.

Selectivity of the ALP activity assay

In order to investigate the selectivity of the proposed assay,
several proteins such as GOx, thrombin and BSA were tested
under the optimized reaction conditions. Figure 3 shows that
other than ALP, none of these proteins triggers an increase in
fluorescence intensity. These results demonstrated that the
proposed assay was specific for ALP, thereby highlighting
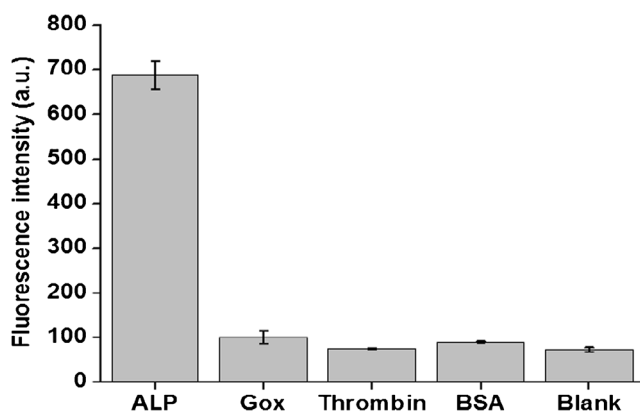its diagnostic potential for detecting ALP activity in biological
samples.

Fig. 3 Fluorescence intensity of dsDNA-CuNP in the presence of ALP,
GOx, thrombin or BSA. Optimized assay conditions were used. Each
data point was determined from three independent experiments. Error
bars were estimated from three replicate measurements

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

Method	LOD (U·L^{-1})	Time (min)	Reference
Thioflavin T	1	10	[14]
Graphitic C$_3$N$_4$	0.08	60	[23]
Graphene Oxide	0.19	70	[18]
Carbon quantum dots	1.1	120	[28]
Phosphate	0.5	40	[16]
Resorufin	0.6	140	[22]
Perylene-Probe	0.3	120	[21]
Graphene Oxide	3	100	[25]
Europium(III)-tetracycline	4	30	[29]
CuNPs	0.1	40	This work

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

Sample	Added (U·L^{-1})	Found	Recovery (%)
1	1	0.977 ± 0.13	97.7
2	5	5.17 ± 0.3	103.4
3	8	7.84 ± 0.28	98

ALP activity inhibition assay

Since inhibition of ALP activity is closely associated with therapy of human diseases, we tested the validity of the proposed assay in evaluating the inhibition of ALP activity. Pi, which can inhibit ALP activity by binding competitively to the ALP active site, was chosen as a model inhibitor in our assay. Addition of Pi up to a concentration of 2.5 mM had no effect on the formation of CuNPs and the activity of λ exo, which suggested that the decrease in fluorescence intensity must therefore be caused by the inhibitory effect of Pi on ALP activity (Fig. S7 and S8). We found that the fluorescence intensity at 565 nm and the relative activity of ALP dynamically decreased with an increase in Pi concentration (Fig. S9) with an IC$_{50}$ value of 3 μM. These results indicate that our method can be potentially used for ALP inhibitor screening.

Application of the proposed assay in biological systems

To evaluate the practical use of the proposed sensing platform, we attempted to detect ALP in 1% human serum [26]. 1 U·L^{-1}, 5 U·L^{-1}, and 8 U·L^{-1} ALP were added to 1% (v/v) human serum, respectively, and detected by the proposed assay. The results are shown in Table 2; the recovery rates of different concentrations of ALP in 1% human serum were 97.7% for 1 U·L^{-1}, 103.3% for 5 U·L^{-1}, and 98% for 8 U·L^{-1}. The results show that the method may have practical applications for ALP detection in biological systems.

Conclusions

In summary, we have successfully demonstrated a turn-on fluorescence method for ALP detection based on dsDNA-templated copper nanoparticles coupled with λ exonuclease cleavage. The advantage of the method is low detection limit (0.1 U·L^{-1}), fast response time (40 min), good selectivity and excellent repeatability. Besides, this method can be used in practical applications involving real biological systems and screening for inhibitors of ALP. We envision that our fluorescence-based strategy for detection of ALP activity may find applications in clinical diagnostics and drug screening.

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