Inhibition of dsDNA-Templated Copper Nanoparticles by Pyrophosphate as a Label-Free Fluorescent Strategy for Alkaline Phosphatase Assay

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Supporting Information

ABSTRACT: On the basis of the inhibition of double strand DNA (dsDNA)-templated fluorescent copper nanoparticles (CuNPs) by pyrophosphate (PPi), a novel label-free turn-on fluorescent strategy to detect alkaline phosphatase (ALP) under physiological conditions has been developed. This method relies on the strong interaction between PPi and Cu2+, which would hamper the effective formation of fluorescent CuNPs, leading to low fluorescence intensity. The ALP-catalyzed PPi hydrolysis would disable the complexation between Cu2+ and PPi, facilitating the formation of fluorescent CuNPs through the reduction by ascorbate in the presence of dsDNA templates. Thus, the fluorescence intensity was recovered, and the fluorescence enhancement was related to the concentration of ALP. This method is cost-effective and convenient without any labels or complicated operations. The present strategy exhibits a high sensitivity and the turn-on mode provides a high selectivity for the ALP assay. Additionally, the inhibition effect of phosphate on the ALP activity was also studied. The proposed method using a PPi substrate may hold a potential application in diagnosis of ALP-related diseases or evaluation of ALP functions in biological systems.
using dsDNA as templates at a low concentration of Cu^{2+} was reported by Rotaru et al. CuNPs have been used as fluorescence indicators in the field of bioanalysis. Scheme 1 outlines the design principle of the CuNP-based fluorescent label-free ALP assay. Rotaru et al. reported that the first step in dsDNA-templated CuNPs formation is the reduction of copper(II) to copper(I) followed by the disproportionation of copper(I) into copper(II) and copper(0). Then, the formed copper(0) is clustered on dsDNA. Additionally, inspired by the fact that PPi has a strong affinity for Cu^{2+}, we speculated that the chelation between PPi and Cu^{2+} would result in the disturbance of the effective Cu^{2+} to Cu^{0} transformation in the CuNPs synthesis process, leading to a low fluorescence. After the treatment by ALP, PPi was hydrolyzed to P, disabling the complexation between Cu^{2+} and PPi. The fluorescent CuNPs can then be formed effectively through the reduction by ascorbate using dsDNA as templates. Thus, the fluorescence intensity was recovered and enhancement was directly related to the ALP concentration.

It is noteworthy that a major advantage of the developed assay is the use of natural substrate PPi with a best assay condition consistent with the physiological condition. This implies the method may provide the actual activity level of ALP in physiological conditions, which allows more accurate evaluation of ALP functions in biological systems. Other advantage is that this method is label-free and does not require a specific synthetic substrate. Additionally, previous studies have shown that ALP-catalyzed PPi hydrolysis has a low pH optimum compared to that for other substrates. It is also reported that ALP-catalyzed PPi hydrolysis will be inhibited by high Mg^{2+} concentration and displays a higher activity in Tris-HCl buffer. Therefore, 10 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl was used throughout the present work.

The effect of PPi and P on the formation of CuNPs was explored first to verify the design principle. As the hydrolysis product of PPi, P exists in an equilibrium mixture of HPO_4^{2-} and H_2PO_4^- in buffer solution (pH 7.4). To study their effects, Cu^{2+} (200 μM) was pretreated with 250 μM PPi, 400 μM HPO_4^{2-}, and 400 μM H_2PO_4-, respectively, at 37 °C for 10 min, and the ascorbate-contained dsDNA was then added to synthesize CuNPs. The fluorescence intensity of obtained CuNPs was used to investigate the effects on CuNPs formation. From Figure 1, in the absence of PPi and P, a strong peak intensity at 565 nm can be observed when excited at 335 nm (curve a), indicating that the CuNPs were formed effectively at a low concentration of Cu^{2+}, through the reduction by ascorbate. When Cu^{2+} was pretreated with PPi, an obvious fluorescence decrease was observed (curve e). However, HPO_4^{2-} and H_2PO_4^- affected the fluorescence intensity only slightly (curves b and c). These results indicate that PPi can strongly chelate with Cu^{2+}, whereas P has a low affinity to Cu^{2+}. Additionally, after the hydrolysis of PPi by ALP, a recovery of fluorescence intensity was clearly observed (curve d). On the basis of the observed different fluorescence inhibition ability of PPi and P, a simple and label-free fluorescent assay for ALP based on the PPi-induced inhibition of CuNPs formation can be achieved.

In order to obtain a high effective analysis performance for the ALP assay, we systematically explored the inhibition effect of PPi on the formation of the CuNPs and optimized some important parameters. Since CuNPs were used as a fluorescence indicator in the present strategy, the concentration...
of Cu\textsuperscript{2+} is obviously important in this study. As shown in Figure 2A, with the increase of Cu\textsuperscript{2+} concentration, the fluorescence intensity in the presence or absence of a certain concentration of PPI both increased gradually and the best response was obtained at 200 \(\mu\text{M}\) Cu\textsuperscript{2+}. Thus, this concentration of Cu\textsuperscript{2+} was used in further experiments.

The fluorescent CuNPs formation was initiated by the reduction of Cu\textsuperscript{2+} by ascorbate and the formed Cu\textsuperscript{0} was then clustered on dsDNA templates. In our design principle, the strong association between PPI and Cu\textsuperscript{2+} would hamper the effective Cu\textsuperscript{2+} to Cu\textsuperscript{0} transformation. Thus, we studied the time dependence of peak intensity after addition of ascorbate-contained dsDNA under various PPI concentrations. The concentrations of PPI from a to i were 0, 25, 50, 100, 150, 200, 250, 300, and 400 \(\mu\text{M}\). Additionally, we further investigated the inhibition effect of PPI on CuNPs formation by studying the relationship between PPI concentrations and peak fluorescence intensity at a fixed reduction time of 4 min. As shown in Figure S1 of the Supporting Information, the peak intensity decreased with increasing PPI concentration. In the concentration range from 0 to 250 \(\mu\text{M}\), the fluorescence intensity exhibits a quasi-linear correlation to PPI concentration with a correlation coefficient of \(R = 0.9918\). When the concentration of PPI was larger than 250 \(\mu\text{M}\), the peak intensity tended to be constant. In accordance with the design principle, the sensitivity improvement of the ALP assay relies on the concentration decrease of PPI used. Thus, 250 \(\mu\text{M}\) PPI was used to obtain enough inhibition of fluorescence intensity for the ALP assay.

All the above discussions demonstrated that PPI can sensitively hamper the effective formation of fluorescent CuNPs, thereby affording the possibility to detect ALP with a good performance. Before the standard ALP assay, the effect of hydrolysis time on the assay performance was also investigated. As shown in Figure S2 of the Supporting Information, one can find that the fluorescence intensity increased with the increase of reaction time and tended to be constant after 50 min. A slightly longer time is needed to complete the reaction, probably due to the intrinsic low hydrolysis rate of ALP when PPI was used as a substrate.\textsuperscript{23} In order to obtain an effective hydrolysis for an ALP assay, 60 min of hydrolysis time was used in the standard ALP assay.

The ability of this method for the ALP assay was investigated by testing different concentrations of ALP under optimized conditions. ALP at different concentrations was incubated with PPI at 37 °C for 60 min. After incubation, 200 \(\mu\text{M}\) Cu\textsuperscript{2+} was added into the enzymatic reaction solution followed by the addition of ascorbate-contained dsDNA 10 min later. The fluorescence intensity obtained at 565 nm at a fixed reduction time of 4 min was used to evaluate the performance of the present method. Figure 3A depicts the fluorescence spectra of obtained CuNPs after the hydrolysis of PPI by ALP of various concentrations. It is clearly observed that the peak intensity increased gradually with increasing ALP concentration. As shown in Figure 3B, a plot of the peak intensity versus different concentrations was incubated with 0.3 units/L to 7.5 units/L (The ALP activity was estimated in the Supporting Information). The calibration equation was \(F = 1690.7005 + 1107.5779 \log C_{\text{ALP}}\) (where \(R = 0.9938, F\) is the peak intensity, \(C_{\text{ALP}}\) is the concentration of ALP). A detection limit of \(<0.1\) nM (~0.3 units/L) can be obtained from the calibration curve. This detection limit is lower than those of previously reported assays using PPI substrates.\textsuperscript{14−17} The relative standard deviations (RSDs) of peak intensity were 3.8%, 3.4%, 4.5%, 3.0%, and 3.2% in three repetitive assays of 0.1, 0.25, 0.5, 2.5, and 5 nM ALP. These results demonstrated that the proposed fluorescent method can sensitively detect ALP with good reproducibility.

It was reported that alkaline phosphatase can be inhibited by the phosphate group containing compounds, due to the competitive binding to the ALP active site.\textsuperscript{24} Thus, its specific reaction product, Pi, can also inhibit ALP activity as a competitive inhibitor.\textsuperscript{25} Here, we used the present method to explore the inhibition effect of Pi on ALP activity. The investigation was carried out with 2.5 nM ALP, 250 \(\mu\text{M}\) Pi, and Pi of different concentrations. As can be seen from Figure

![Figure 2](image-url)
S3 of the Supporting Information, with an increase in the Pi concentration from 0 to 4 mM, the fluorescence intensity decreased gradually. One noticed that addition of Pi up to a concentration of 4 mM had only a little effect on the CuNPs formation (data not shown), confirming that the decrease of fluorescence intensity depended on the inhibition effect of Pi on ALP activity. These results demonstrate that this strategy can be used to study the inhibition effect of Pi on ALP activity, and it may be extended to screen other inhibitors of ALP.

The proposed fluorescence turn-on mode will reduce the possibility of false-positive signals associated with some turn-off assays. The selectivity of the present method was demonstrated by respectively testing some nonspecific proteins including thrombin, glucose oxidase (GOx), lysozyme, bovine serum albumin (BSA), and avidin. As shown in Figure S4 of the Supporting Information, the proposed method yielded very weak fluorescence responses to other interfering proteins. The results obviously indicated that this turn-on strategy had a high selectivity toward ALP.

The practicality of this method was also demonstrated by the detection of ALP in diluted human serum (1%). As can be seen from Figure S5 of the Supporting Information, the fluorescence intensity increased with the increase of ALP concentration from 0 to 3.5 nM (0–10.5 units/L). With the calibration curve in serum, six serum samples with added ALP of different concentrations were measured. Satisfactory recoveries between 93.3% and 103.0% were obtained (as shown in Table S1 of the Supporting Information), indicating the potential of this method for applications in complicated real samples.

In summary, based on dsDNA-templated CuNPs, a novel label-free fluorescent turn-on ALP assay has been developed under physiological conditions using a natural substrate, PPi. This study relies on the strong interaction between PPi and Cu$^{2+}$, which would hamper the effective formation of fluorescent CuNPs, resulting in low fluorescence intensity. The ALP-catalyzed PPi hydrolysis disabled the complexation between Cu$^{2+}$ and PPi. The fluorescent CuNPs could then be effectively formed through the reduction by ascorbate in the presence of dsDNA templates, thus the fluorescence was recovered. The proposed method is cost-effective and convenient without any labels or complicated operations. At the same time, this method exhibits a higher sensitivity compared with the previously reported ALP assay using PPi substrate. Additionally, the fluorescence turn-on mode reduces the possibility of false-positive signals associated with turn-off assays and displays a high selectivity for ALP. The proposed assay using PPi substrate may hold potential applications in the diagnosis of ALP-related diseases under physiological conditions or the study of the physiological function of ALP in skeletal mineralization and vascular calcification.

### ASSOCIATED CONTENT

#### Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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### REFERENCES


