



Detection of Pyrophosphate and Alkaline Phosphatase Activity Based on PolyT Single Stranded DNA - Copper Nanoclusters

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Abstract

The determination of pyrophosphate and alkaline phosphatase activity plays a significant role in medical diagnosis. In this work, a label-free “ON-OFF-ON” fluorescence strategy is developed for the analysis of pyrophosphate and alkaline phosphatase activity. Using PolyT single strand DNA as templates to synthesize fluorescent copper nanoparticles, the coordination effect of pyrophosphoric acid on Cu^{2+} inhibited the generation of fluorescence. Afterwards, the addition of alkaline phosphatase into hydrolyze pyrophosphoric acid resulted in the release of Cu^{2+} , whereby the fluorescence intensity could be recovered. Thereupon enhanced-sensitivity for alkaline phosphatase was obtained (0.1 mU/L), much better than previously reported methods. Meanwhile, it could be performed directly in homogeneous solution, which was very close to the actual activity level of alkaline phosphatase under physiological conditions. Likewise, satisfactory results were also obtained in specificity assessment, which demonstrated its potential application in clinical diagnosis. Notably, a new, sensitive, low-cost, short-time, and high-sensitivity platform for alkaline phosphatase detection was constructed, and the design of biosensor using DNA-templated Copper nanoclusters (CuNCs) was instructed in this study.

Keywords Copper nanoclusters · Pyrophosphate · Alkaline phosphatase · PolyT DNA · Fluorescent sensor

Introduction

Alkaline phosphatase (ALP) is a widespread membrane-bound enzyme found in various tissues. With broad substrate specificity, ALP can hydrolyse a variety of phosphorylated compounds in vivo and in vitro, and can catalyse the dephosphorylation of proteins, nucleic acids and small molecules [1]. Dephosphorylation of different natural substrates reflects ALP's different physiological functions, which plays an important role in various biological reactions of organisms. Therefore, monitoring ALP levels is relevant to

the diagnosis of a variety of diseases such as kinetic bone disease [2], hepatitis [3] and prostate cancer [4]. Exploiting simple, sensitive and selective methods is essential for detecting ALP activity. Pyrophosphate (PPi) is a confirmed natural substrate for ALP, existing throughout human body, with a physiological concentration of about 3 $\mu\text{mol/L}$ in healthy individuals. When the PPi content exceeds the normal level, it means that the body may suffer from some diseases [5]. For example, excessive PPi content in synovial fluid is closely related to the accumulation of calcium pyrophosphate dihydrate crystals and some diseases' pathogenic mechanism, including cartilage calcification and hypophosphatemia [6]. As a result, it is also vital to detect PPi levels efficiently.

There are a variety of assays existing for the qualitative, quantitative and structural analysis of various biological substances, such as colorimetric methods [7], spectrophotometric methods [8], high performance liquid chromatography [9], capillary electrophoresis [10], electrochemical assay [11], mass spectrometry [12], Raman spectroscopy [13], etc. Each method does have its own unique advantages. However, it also suffers from the limitations of expensive precision instruments equipment, cumbersome detection

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steps, and long detection time, etc. Moreover, colorimetric methods and fluorescence methods are most common used for rapid ALP detection. Colorimetric methods have many advantages such as no need for large equipment and visible assay process, but it has a high detection threshold [14]. On the contrary, the fluorescence method can reduce the detection limit, and up to present, different types of fluorescent probes for detecting ALP have been widely reported, such as cyanine dyes [15–18], coumarins [19] and BODIPA (Dipyrrromethene Boron Difluoride) [20]. However, to some extent, fluorescent dyes' poor solubility in aqueous solutions leads to low sensitivity, poor specificity and complex sensing mechanisms requirement. In addition, fluorescent sensors established for the detection of ALP, which are based on organic molecules [21], fluorescent polymers [22, 23] and inorganic quantum dots [24, 25] have their own drawbacks, such as fluorescent polymer's complex synthesis process and the quantum dot's toxicity.

In summary, based on existing analytical tools, there is still a need to explore a simple, effective, sensitively as well as specifically method to detect biomolecules content and biological processes change. As such, metal nanoclusters (MNCs) are gradually gaining attention. MNCs consist of a few to hundreds of atoms, typically less than 2 nm in size, which is between metal atoms (unique optical properties) and nanoparticles (plasmonics), with many molecular-like properties, unique optical, electronic and chemical properties, including intense luminescence, excellent photostability, good biocompatibility, and sub-nanometer dimensions [26, 27]. These new properties of metal NCs make it an ideal nanomaterials which has broad application prospects in photoluminescence analysis, bioanalysis and imaging, environmental monitoring, industrial catalysis and electronic devices [28–31]. Compared with gold nanoclusters (Au NCs) and silver nanoclusters (Ag NCs), copper nanoclusters (Cu NCs) have more abundant and accessible precursors and incomparable price advantages, furthermore, they are more suitable for various applications due to their good electrochemical properties, fluorescence and biocompatibility without toxicity.

Compared to traditional fluorescence techniques, using natural substrates combined with luminescent materials has more obvious advantages, which can more directly reflect the function of ALP and the relationship between ALP and its natural substrates. However, only some phosphate compounds have been demonstrated ALP natural substrates, such as β -glycerophosphate, pyridoxal-5'-phosphate, and glucose-6-phosphate, etc. [32, 33]. Among them, PPI is a unique natural substrate because it has lower optimal pH and slower hydrolysis rate [34–36]. Since PPI has strong coordination properties and can bind Cu^{2+} to form complexes, in this work, a simple, sensitive, efficient and

feasible label-free fluorescence method for rapid ALP detection was envisaged, through constructing a template dependent DNA-CuNCs fluorescence sensor, using PPI as substrates, adopting an “ON-OFF-ON” strategy. It has been proved that this method can be performed directly in homogeneous solution, and can be very close to physiological ALP content. This method may hold a potential application in biological analysis and diagnosis.

Experimental parts

Instruments and reagents

Ascorbic acid was purchased from Tianjin Fengships Chemical Reagent Technology Co., Ltd. CuSO_4 was purchased from Tianjin Damao Chemical Reagent Factory. Alkaline phosphatase, Bovine serum albumin, trypsin, lipase and various amino acids were purchased from Biotech Bioengineering (Shanghai) Co., Ltd. Other chemicals used in this work were of analytical grade and were used directly without additional purification. The solutions were prepared using ultrapure water which was obtained through a Millipore Milli-Q water purification system (Massachusetts, USA) and had an electric resistance $> 18.2 \text{ M}\Omega$.

All DNA sequences used in this study were synthesized and purified by Shanghai Sangong Biotech Co. Ltd., China., and their sequences were as follows (Table 1):

The fluorescence experiments were performed on FL-7000 spectrometer (Hitachi, Japan). The fluorescence emission spectra of CuNCs were recorded from 500 to 700 nm at room temperature ($\sim 20^\circ\text{C}$) with a 345 nm excitation wavelength.

Preparation of CuNCs

CuNCs were prepared directly using DNA as a template and ascorbic acid as a reducing agent. $20 \mu\text{L}$ 1 mM Cu^{2+} and $10 \mu\text{L}$ $5 \mu\text{M}$ single stranded DNA solution were added to $40 \mu\text{L}$ 1 mM PBS buffer solution ($\text{pH} = 7.4$). After fully mixing, $30 \mu\text{L}$ 10 mM ascorbic acid was added for light-avoidance reaction for 5 min. Fluorescence detection was performed to

Table 1 Oligonucleotide names and sequence

Name	Sequence (5'→3')
A30	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
C30	CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
G30	GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
T30	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
T40	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTT
T50	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTT

determine the emission and excitation spectra. The results obtained by fluorescence spectrometry were obtained from > 3 replicate experiments.

Optimization of experimental conditions

Theoretically, under the same excitation intensity, the higher the fluorescence intensity is and the longer the fluorescence lifetime is, the sensitivity and the stability of the method will be higher. Based on preparation of CuNCs, several experiments were designed to optimize the conditions, successively using PolyT ssDNA with different T base numbers (T30, T40, T50) as templates, adjusting pH value of PBS buffer solution and the DNA: Cu²⁺ ratio, setting a series of ascorbic acid concentrations and controlling the optimal reaction time, etc. The preparation of DNA-CuNCs was repeated several times in multiple groups, through fluorescence detection, the effects of various conditions on the fluorescence intensity and fluorescence lifetime of DNA-CuNCs were explored, and the optimal system for synthesizing DNA-CuNCs was determined.

Detection of PPi

In this design, the strong combination of PPi and Cu²⁺ would hinder the efficient conversion of Cu²⁺ to Cu⁰. Therefore, the ability of PPi to inhibit the formation of CuNCs could be investigated by investigating the time dependence of peak intensities after adding dsDNA containing ascorbic acid at different PPi concentrations. According to the optimization results, while adding Cu²⁺ and PolyT ssDNA solution to the buffer solution, a certain amount of PPi was added to react for 20 min, subsequently 30 µL of ascorbic acid was added for light-avoidance reaction for 5 min. Then performing fluorescence detection to observe the change in fluorescence intensity.

Detection of ALP activity

After adding 20 µL of Cu²⁺, a fixed concentration of PPi solution and different concentrations of ALP to the buffer, heating it in a water bath at 37 °C for 1 h, and mixing it with PolyT ssDNA solution and ascorbic acid solution for 5 min, the fluorescence detection was performed again. In the presence of ALP, PPi will be hydrolyzed to Pi, re-release the Cu²⁺ wrapped by PPi, and Cu²⁺ binds to DNA, thus fluorescence restored.

Selective detection

To investigate the selectivity of DNA-CuNCs for PPi over other compounds, we used phosphate compounds (HPO₄²⁻,

H₂PO₄⁻) as well as the following amino acids: arginine, threonine, glycine and glutamic acid for specific assay analysis. The same amount of the above substances as PPi was added to the DNA-CuNCs synthesis system, and the fluorescence spectra were recorded.

Selected proteins/enzymes such as Bovine serum albumin (BSA), Trypsin and lipase were used for comparative tests on the selectivity of ALP. These unrelated proteins/enzymes were added respectively to a mixture of DNA-CuNCs PPi for fluorescence detection, and the changes in fluorescence intensity were observed.

Results and analysis

The principle of assay

A simple, sensitive, efficient and feasible labeling free fluorescence method was developed for the detection of PPi and ALP activity using the "ON-OFF-ON" strategy. As shown in Fig. 1, polyT single strand DNA-CuNCs can form fluorescence under specific excitation. The strong combination of PPi and Cu²⁺ can block the binding of Cu²⁺ to DNA and terminate the fluorescence signal. However, in the presence of ALP, ALP can hydrolyze PPi and form free phosphoric acid group, which cannot continue complexing with Cu²⁺ and recover fluorescence. Moreover, the degree of fluorescence recovery was affected by the ALP concentration.

Feasibility analysis

The feasibility of preparing copper nanoclusters: following the preparation scheme of DNA-CuNCs, after adding A30, C30, G30, T30 DNA to reaction buffer, respectively, detection under UV-VIS spectrophotometer found that the fluorescence emitted by T30 was most obvious, while several others showed almost no fluorescence phenomenon

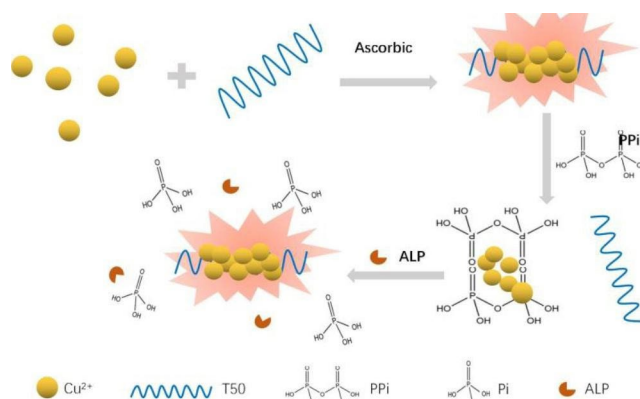


Fig. 1 Schematic diagram of DNA-CuNCs fluorescence detection of PPi and ALP activity

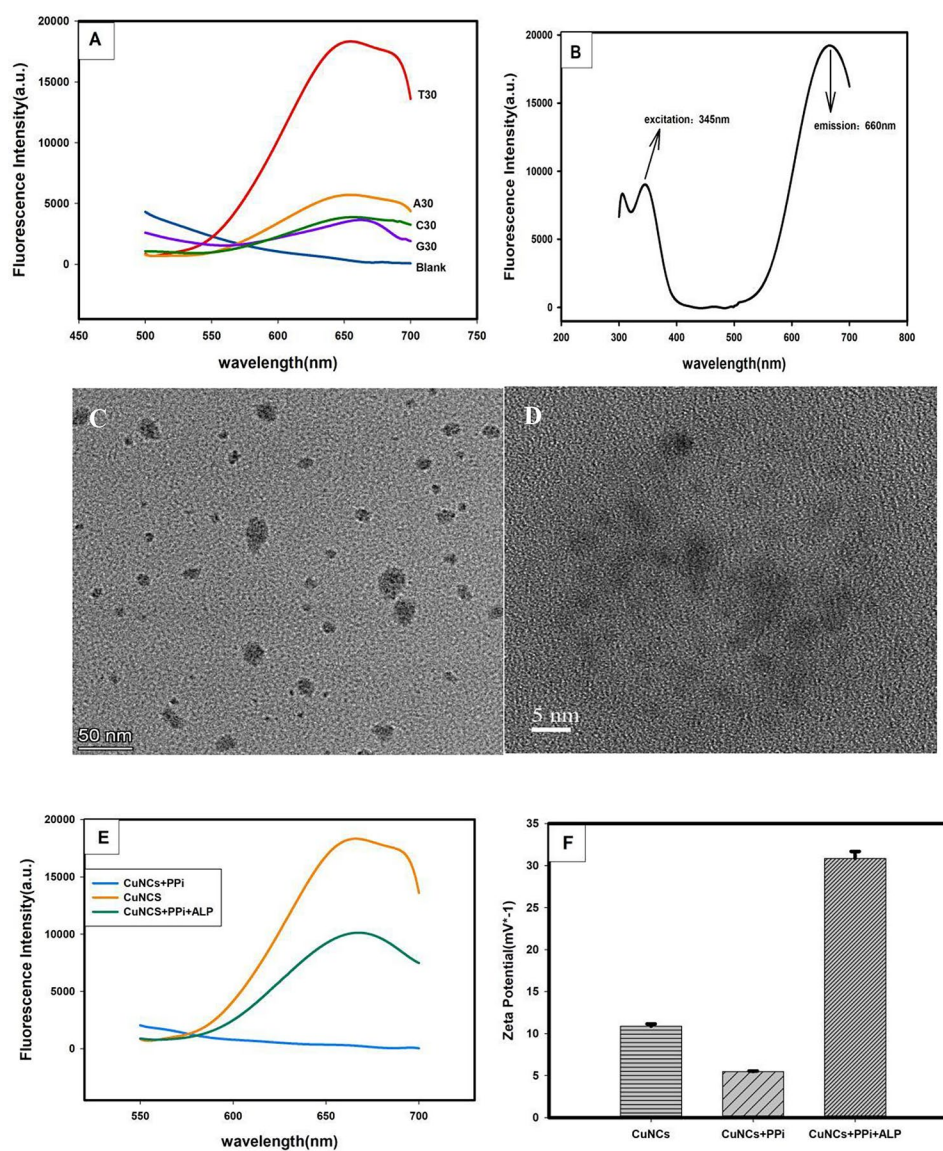
(Fig. 2 A). It could be proved that T base had the strongest affinity to Cu^{2+} , preparation of copper nanoclusters based on polyT ssDNA was feasible.

To prove the formation of DNA-CuNCs, the UV-Visible absorption spectrum and fluorescence spectrometry were firstly investigated. DNA-CuNCs possessed a typical absorption peak at ~ 345 nm and a maximum fluorescence peak at 660 nm. Moreover, the strongest fluorescence appears at 660 nm under 345 nm excitation light through detecting emission spectra and when setting 660 nm emission, the strongest fluorescence appears at 345 nm (Fig. 2B). As a consequence, we can draw a conclusion that the optimal excitation wavelength and optimal fluorescence emission wavelength of DNA-CuNCs are 345 and 660 nm, respectively. In addition, the morphology of DNA-CuNCs was studied by higher solution transmission electron

microscopy (HRTEM). According to the results presented in Fig. 2 C (50 nm), it could be found that the DNA-CuNCs exhibited spherical shape, and their average diameters were 4.5 nm in Fig. 2D (5 nm).

PPI and ALP addition feasibility analysis: We added PPI on the basis of existing fluorescence and further added ALP in the system of PPI quenching fluorescence. As shown in Fig. 2E, the inhibition effect of PPI and the recovery effect of ALP on fluorescence intensity were determined. Meanwhile we compared the Zeta potential of DNA-CuNCs alone with the one after the addition of pyrophosphate and alkaline phosphatase to improve the characterization of the fluorescent DNA-CuNCs. As shown in the Fig. 2 F, we can see that the Zeta potential decreases after the addition of PPI and recovers again after the addition of ALP. This is because the interaction between PPI and Cu^{2+} prevents the potential

Fig. 2 (A) fluorescence effect of DNA-CuNCs with different DNA substrates (T30, A30, C30, G30, blank in order from top to bottom); (B) The excitation spectra versus emission spectra; (C) TEM images of polyT DNA-CuNCs (50 nm); (D) TEM images of polyT DNA-CuNCs (5 nm); (E) Addition of PPI as well as addition of ALP to the DNA-CuNCs; (F) The zeta potential of DNA-CuNCs alone with the addition of pyrophosphate and alkaline phosphatase



signal, and the surface potential signal can be restored again after ALP hydrolyzes PPi and released Cu^{2+} .

Design optimization analysis

The effect of different DNA template strands

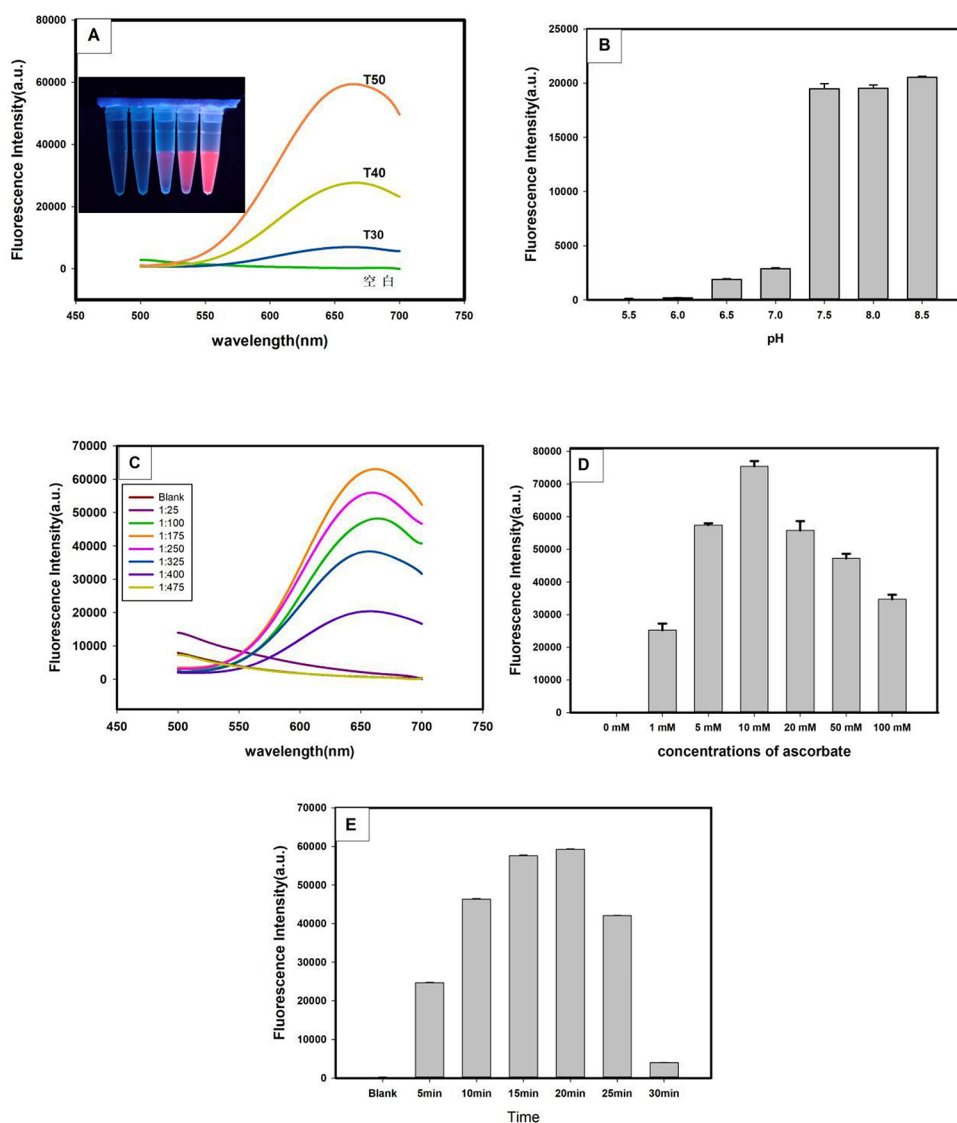
In order to obtain the best sensitivity of this sensor, this experiment systematically estimated the effect of various factors on fluorescence intensity. First of all, the effect of different DNA template strand lengths on the fluorescence intensity were explored. We performed experiments following the above protocol, with different length polyT ssDNA solutions as templates. The fluorescence curve as shown in Fig. 3 A was obtained, and the results proved that the longer polyT ssDNA was, the more conducive to the formation

of fluorescence. So, T50 was chosen as the template to be “ON” in the experiment.

The effect of different pH conditions

Exploring the synthesis of DNA-CuNCs under different pH conditions, the results showed (Fig. 3B) that under relatively acidic conditions, Cu^{2+} could not bind to template DNA, while under relatively basic circumstances, different pH values had no or few effect on the fluorescence. Therefore, considering the application of ALP, to simulate the relatively stable environment of the internal environment better, the PBS buffer with pH 8.0 was choose in the experiment.

Fig. 3 (A). the effect of different length of poly thymine single stranded DNA solution (T50, T40, T30 from top to bottom in turn as well as blank control) on the fluorescence intensity of the synthesized DNA-CuNCs, the inset is the fluorescence effect of the synthesized DNA-CuNCs with different number of T bases (T50, T40, T30 in order from left to right in PBS solution, ssDNA, T30, T40, T50); (B). From left to right is the effect of PBS buffer at pH 5.5, 6.0, 6.5, 7.5, 8.0, 8.5 on the fluorescence intensity of DNA-CuNCs in turn; (C); Effect of DNA: Cu^{2+} at 1:25, 1:100, 1:175, 1:250, 1:325, 1:400, 1:475 on the fluorescence intensity of DNA-CuNCs, respectively; (D). The effect of different concentrations of ascorbate on fluorescence intensity, 0 mM, 1 mM, 5 mM, 10 mM, 20 mM, 50 mM, 100 mM in order from left to right; (E). T50 -- change of fluorescence intensity of DNA-CuNCs with time



The effect of different DNA: Cu²⁺ ratio

Controlling the usage of different amounts of template and reducing agent also affected the formation of fluorescence intensity, accordingly, a series of DNA: Cu²⁺ ratio (1:25, 1:100, 1:175, 1:250, 1:325, 1:400, 1:475) were used to explore its effect on the fluorescence intensity. The detection results are shown in Fig. 3 C. In a certain range, the fluorescence become stronger as the molar ratio of Cu²⁺ to polyT ssDNA rises, while beyond a certain amount, the excessive Cu²⁺ will agglomerate to interfere the binding with DNA to form fluorescence. Ultimately, it was revealed that the fluorescence intensity was highest at a DNA: Cu²⁺ of 1:175, this concentration of DNA: Cu²⁺ was choose in further experiments.

The effect of different concentrations of ascorbate

Ascorbic acid was used as reducing agent to synthesize DNA-CuNCs, and it was of equal significance to explore the different concentrations of ascorbic acid for the fluorescence intensity. As shown in Fig. 3D, excessive ascorbic acid would react with Cu²⁺ to produce hydrogen oxygen radicals, which caused the degradation of T-DNA and interfered in the yielding of fluorescence. While low concentration of ascorbic acid couldn't realize the preparation of fluorescence. Thus, it was determined that the optimal ascorbic acid concentration for the synthesis of DNA-CuNCs was 10 mM.

Explore the change trend of T50 fluorescence intensity over time

Combined with the optimization of all the conditions mentioned above, using T50 DNA as the template, controlling the DNA: Cu²⁺ as 1:175, and 10 mM ascorbic acid as the reducing agent, DNA-CuNCs were prepared to determine the time of strongest fluorescence, which was prepared for subsequent measurements. The detection results demonstrated that (Fig. 3E), after mixed with the addition of

ascorbic acid, the fluorescence soon appeared, and the fluorescence reached the strongest at around 20 min. Therefore, the reaction time selected for subsequent experiments is 20 min.

The detection of pyrophosphate

Under the optimal conditions, the fluorescence spectra were obtained from DNA-CuNCs upon the action of different concentrations of PPi (Fig. 4 A). It can be seen from the picture that the initial growth rate of fluorescence slows down with the increase of PPi concentration, suggesting that the more PPi was added, the more Cu²⁺ was chelated, and the slower the formation rate of CuNCs, and this is the “OFF”. As shown in Fig. 4B, the fluorescence intensity was quasi-linearly correlated with PPi concentration in the range of 0 to 4 μM, with a correlation coefficient of $R^2 = 0.9906$. The detection limit of PPi was 0.29 μM, which was lower than the previously reported detection limit. The peak intensity tends to be constant when the concentration of PPi is greater than 4 μM. According to the design principle, the increase in the sensitivity of ALP detection is dependent on the decrease in the concentration of PPi used. And compared to others, the results were satisfactory. Therefore, 4 μM PPi was choose to obtain sufficient suppression of fluorescence intensity for ALP assays.

The detection of alkaline phosphatase activity

Finally, ALP were used to build “ON” to restore fluorescence. As shown in Fig. 5 A, observing the fluorescence spectra measured under different ALP concentration conditions under the optimal conditions, when the content of ALP was between 10⁻⁷ and 10⁻³ U/ml, the fluorescence intensity at 660 nm decreased with the ALP concentration decreasing, and the curve between peak intensity and ALP concentration shows a quasi-linear correlation over the range of 10⁻⁷ to 10⁻⁴ U/ml, $R^2 = 0.9708$ (Fig. 5B). The lowest limit actually detected was 10⁻⁷ U/mL, which corresponds to the activity unit from 0.1 mU/L. Compared with other detection

Fig. 4 (A) Effect of different concentrations of PPi on fluorescence intensity with sequential 0, 0.5, 1, 1.5, 2, 3, 4, 5 μM, (B) the linearity of peak intensity from 0~4 μM PPi solution concentration. Error bars were estimated from three replicate measurements. ($R^2 = 0.9906$)

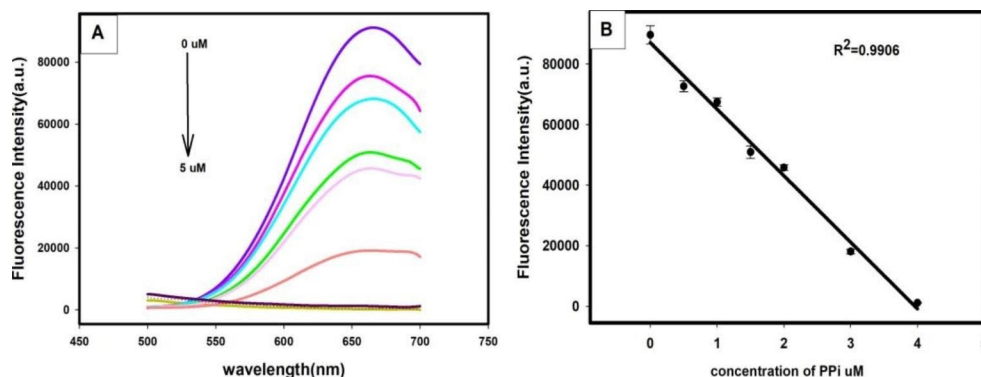
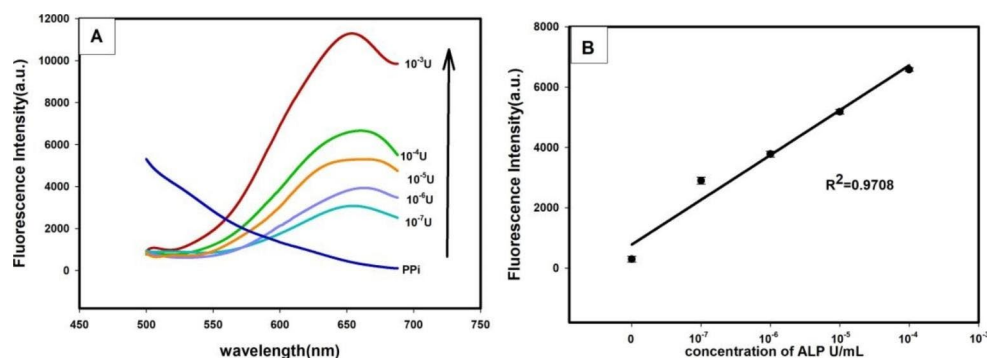


Fig. 5 (A). Effects of different concentrations of ALP on fluorescence recovery, the ALP concentrations from top to bottom were 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 0 U/ml; (B) the linearity of peak intensity with the content of ALP was between 10^{-7} and 10^{-4} U/ml. Error bars were estimated from three replicate measurements ($R^2=0.9708$)



methods (as shown in Table 2), this fluorescence method could detect ALP sensitively and has good reproducibility, which can effectively meet the requirements of sensitivity for ALP activity detection in clinical medical research.

Selectivity detection

Selectivity is not only a key parameters to evaluate the ability of a sensor to specifically identify target molecules in complex biological samples and but also an important indicator of the sensor's application. Therefore, the effect of phosphate compounds (HPO_4^{2-} , H_2PO_4^-) and the following common amino acids: arginine, threonine, glycine and glutamic acid on the specificity of the detection system were investigated. The results in Fig. 6 A showed that adding the same amount of above substances as PPI to the DNA-CuNCs synthesis system had no significant effect on the fluorescence intensity. But only adding PPI was able to quench the fluorescence of the system, indicating that the

analytical method developed in this experiment had good selectivity for the determination of PPI.

The biological enzymes such as bovine serum albumin (BSA), trypsin and lipase were used to investigate the selectivity of ALP. As shown in Fig. 6B, the fluorescence intensity of the system remained almost constant (less than 5% change in fluorescence intensity) after adding other biological enzymes. Only after adding ALP that the fluorescence intensity of the sensing system recovered significantly, leading to the conclusion that the sensing system was highly selective for ALP.

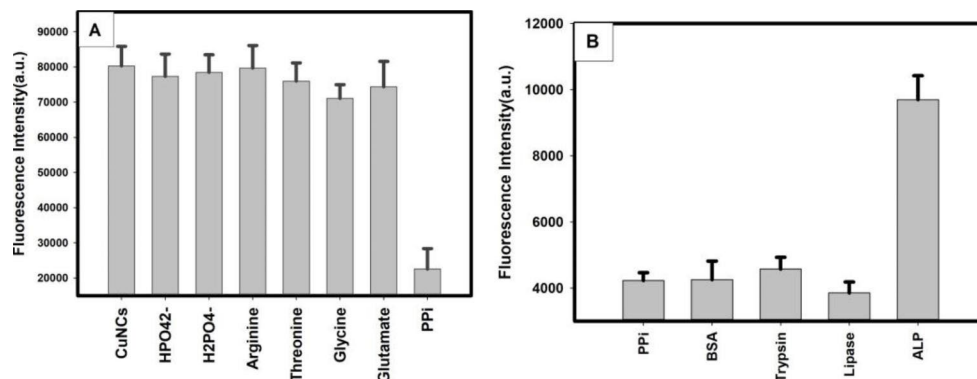
Conclusions

In this paper, based on polyT ssDNA, a DNA-CuNCs fluorescent sensor was constructed to develop a simple sensitive, efficient and feasible label free fluorescence method, which uses PPI as its natural substrate, and follows an "ON-OFF-ON" strategy to analyze ALP. According to the decrease amount of DNA-CuNCs fluorescence after adding PPI, rapid detection of PPI could be achieved. On this basis, detection of ALP activity was realized according to the recovery of DNA-CuNCs fluorescence after adding ALP. Compared to traditional detection, this protocol was directly performed in homogeneous solution, requiring short time and low cost. Moreover, it could detect experimental data simply and quickly, and its optimal detection conditions are consistent with physiological conditions, which could

Table 2 Comparison of different ALP activity detection methods

Detection method	LOD	Reference
Electrochemical method	0.2 U/L	[37]
Colorimetric method	5.4 mU/L	[38]
Fluorescence	0.05 U/L	[39]
Chemiluminescence	0.27 mU/L	[40]
Surface enhanced Raman scattering method	0.05 U/L	[41]
Fluorescent DNA-CuNCs	0.1 mU/L	This work

Fig. 6 (A)The selectivity of the CuNCs-based method for PPI; (B)The selectivity of the CuNCs-based method for ALP assay. Error bars were estimated from three replicate measurements



simulate the actual activity level of ALP under physiological conditions. Thus, this method could assess the function of ALP in biological systems more accurately, and it may hold promising application prospects in practical sample detection.

Authors' contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Liu Yao, Xin Li, Hong Li, Zhibin Liao, Chuchu Xie, Ge Ning, Yaohui Wu, Yonghong Wang. The first draft of the manuscript was written by Liu Yao and all authors commented on previous versions of the manuscript. Writing - review and editing by Yonghong Wang, Ge Ning. All authors read and approved the final manuscript. Liu Yao and Xin Li are co-first authors.

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Data Availability All data generated or analysed during this study are included in this published article.

Declarations

Competing interests 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.

Ethics approval and consent to participate This is an observational study. The XYZ Research Ethics Committee has confirmed that no ethical approval is required.

Consent to Participate Not applicable.

Consent for publication Not applicable.

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