Self-assembled gold nanoclusters for fluorescence turn-on and colorimetric dual-readout detection of alkaline phosphatase activity via DCIP-mediated fluorescence resonance energy transfer

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ABSTRACT

We present a fluorescence turn-on and colorimetric dual-readout sensing system for the sensitive detection of alkaline phosphatase (ALP) activity via self-assembled gold nanoclusters (AuNCs) based on fluorescence resonance energy transfer (FRET). The positively charged polyallylamine hydrochloride (PAH)-crosslinked AuNCs (PAH-AuNCs) with aggregation-induced enhancement (AIE) characteristics can electrostatically adsorb the negatively charged 2, 6-dichlorophenolindophenol (DCIP). Thus, the fluorescence of PAH-AuNCs can be significantly quenched by the occurrence of FRET from PAH-AuNCs to DCIP. However, the reduction reaction of DCIP from blue to colourless by l-ascorbic acid (AA) which is generated by the ALP catalyse hydrolysis of 2-Phospho-L-ascorbic acid (APP) disturbs the FRET between PAH-AuNCs to DCIP. The quenched PAH-AuNCs fluorescence can be recovered efficiently. The strategy of first creating AIE-enhanced PAH-AuNCs, followed by the effective FRET manipulation, is an important contribution to the sensitive detection of ALP. More importantly, the distinct colorimetric signal change can be used to visually distinguish the presence of ALP. Good linear relationships of fluorescence and colorimetric sensing towards ALP were obtained in the range from 0.5 to 100 U/L, and the detection limits were 0.2 U/L and 0.5 U/L, respectively. In addition, the proposed FRET sensing system was applied to the detection of ALP in human serum samples with satisfactory results. The simple and efficient sensing approach proposed here has the potential to promote the development of chemo/biodetection methods using fluorescence and colorimetric dual-readout.

1. Introduction

Alkaline phosphatase (ALP) is an essential enzyme in phosphate metabolism which can catalyse the dephosphorylation process on various phosphorylated substrates containing phosphate esters [1]. It exists in various mammalian tissues, plays an important role in various biological processes, and has been regarded as an important biomarker in the diagnosis of many diseases such as diabetes [2], prostate cancer [3], bone disease [4], and liver dysfunction [5]. Therefore, the detection of ALP activity is of great significance in clinical diagnosis and therapy. Up to now, various methods have been developed for the detection of ALP activity, including electrochemistry [6–9], electrochemiluminescence [10], colorimetry [11–13], fluorescence [14–17], and surface-enhanced Raman scattering [18]. Among these methods, the fluorescence assay for ALP activity has been identified as a more promising method due to advantages that include high sensitivity, cost effectiveness, convenience in operation and easy readout [19–23]. Many fluorescent methods based on organic fluorophores [24–26], conjugated polyelectrolytes [13,27,28], and semiconductor quantum dots (QDs) [16,29,30] have been successfully developed for the detection of ALP activity. Although many of these approaches are well-established, some disadvantages such as cumbersome synthesis procedures, poor photostability and water-solubility, complicated purification and labelling processes, and high toxicity might limit their practical applications. Recently, some carbon fluorescence nanomaterials with good biocompatibility, such as carbon dots, have been used as a fluorescent signal output for the detection of ALP activity [31–34]. However, the turn-off operation mode of most of these fluorescence methods resulted in a limited scope of practical applications. Therefore, facile and effective measurement methods for fluorescence turn-on detection of ALP are still urgently desired.

Currently, metal nanoclusters (NCs) are expected to become...
potential replacements for fluorescent dye molecules due to their excellent fluorescence properties, good biocompatibility, and facile preparation methods [35,36]. They have attracted overwhelming attention and are widely applied for biosensing, bioimaging, and in therapy fields [37,38]. Among them, gold nanoclusters (AuNCs) are extremely light-emitting, have low toxicity, ultrasmall size and good biocompatibility and can be synthesized in a rather simple and economical way [39,40]. Considerable effort has been focused on the synthesis of water-soluble AuNCs and the improvement of their luminescent properties [41]. Recently, the phenomenon of aggregation-induced enhancement (AIE) of AuNCs has been reported, providing important information for understanding the growth mechanism of AuNCs and further broadening the application of AuNCs in biosensing [40,42]. Therefore, the application of AuNCs for designing a fluorescence assay for ALP could be considered as a very attractive and effective strategy [19].

Fluorescence resonance energy transfer (FRET) is a well-studied physical process of the energy transfer between a donor fluorophore and an acceptor molecule. The occurrence of FRET generally depends on the extent of spectral overlap and the appropriate distance between the donor and acceptor molecules (usually in the range of 2–10 nm) [43]. In the past several decades, FRET-based sensing platforms that operate through the absorption of an acceptor with a donor have aroused widespread attention [44–47]. This type of FRET-based fluorescence approach does not require covalent linking between an acceptor and a donor, which is more flexible and straightforward to fabricate and implement. Therefore, the development of novel simple and sensitive FRET-based methods for the fluorescence turn-on detection of ALP is of great interest for clinical diagnosis and therapy.

2. Experimental section

2.1. Reagents and apparatus

Alkaline phosphatase (ALP), 2-phospho-1-ascorbic acid (AAP), 2,6-dichlorophenolindophenol sodium salt (DCIP), prostate specific antigen (PSA), glucose oxidase (GOx), human IgG and pepsin were purchased from Sigma-Aldrich Co. LLC. (St. Louis Missouri, USA). Chloroauric acid (HAuCl₄), glutathione (GSH), human serum albumin (HSA), bovine serum albumin (BSA), 1-ascorbic acid (AA) cysteine (Cys), glycine (Gly), lysine (Lys), phenylalanine (Phe), glutamic acid (Glu), tyrosine (Tyr), uric acid (UA), dopamine (DA), heparin, and hydrogen peroxide (H₂O₂) were obtained from Aladdin Industrial Inc. (Shanghai, China). Polycyclic aromatic hydrocarbon (PAH) was purchased from J&K Scientific Ltd. (Beijing, China). The buffer system used in this work was 10 mM Tris-HCl buffer (Tris-HCl, 150 mM NaCl, pH 8.0). The ultrapure water (resistivity > 18 MΩcm) used for the preparation of aqueous solutions was produced by a Millipore system.

Morphology and size characterizations of the synthesized AuNCs and PAH-AuNCs were performed using JEM-2100PLUS transmission electron microscope (TEM, Japan). UV–vis spectra were recorded on a Varian Cary-300 UV–vis spectrophotometer. The fluorescence measurements were carried out on an F-7000 spectrometer (Hitachi, Japan) at room temperature. The fluorescence emission photos were taken under a ZF-1B box-like ultraviolet analyzer (Shanghai, China).

2.2. Preparation of the PAH-AuNCs nanocomposites

The GSH-capped AuNCs were synthesized according to the protocol reported by Xie’s Group [39]. Typically, 0.84 mL of freshly prepared HAuCl₄ aqueous solution (24 mM) and 0.30 mL of GSH (100 mM) were mixed with 8.86 mL of ultrapure water in a brown sample bottle at 25 °C and then heated in a water bath to 70 °C under gentle stirring (500 rpm) for 24 h. The final light-yellow aqueous solution with a strong orange glow represents the formation of AuNCs. The PAH-AuNCs nanocomposites were prepared by adding PAH (Mₕ ~ 17,000, 50 μM, 20 μL) aqueous solution dropwise into 2 mL of AuNCs aqueous solution diluted 5-fold with stirring [40]. The mixture continued stirring at room temperature for 1 h. The formed PAH-AuNCs nanocomposites were stored in refrigerator at 4 °C.

2.3. Detection of ALP activity

Detection of ALP activity was conducted in 500 μL microcentrifuge tubes. The ALP and AAP solutions were prepared by Tris–HCl buffer. Typically, 20 μL of ALP solutions at different concentrations and 20 μL of AAP solution (6 mM) were mixed together and incubated at 37 °C for 20 min. Then, 40 μL of DCIP aqueous solution (800 μM) was added in the above solution and reacted at room temperature for 15 min. Finally, 100 μL of PAH-AuNCs solution and 20 μL of ultrapure water were added into the above solution and reacted at room temperature for 10 min. Subsequently, the fluorescence spectra were recorded. The absorbance spectra of the reacted mixture were recorded in the wavelength range of 450–800 nm. The human serum samples were diluted for the recovery experiments. All studies were approved by the Qufu Normal University Ethics Committee.
3. Results and discussion

3.1. Characterization of the synthesized AuNCs and PAH-AuNCs

First, TEM images were obtained to characterize the size and morphology of the synthesized AuNCs and PAH-AuNCs. As shown in Fig. 1A, the synthesized AuNCs have uniform particle size and good dispersion in water. The estimated average size of the AuNCs is approximately 1.95 nm in diameter (Fig. 1B). After the addition of PAH into the GSH-capped AuNCs solution, self-assembled PAH-AuNCs nanocomposites are formed via the electrostatic interaction between the protonated amino groups of PAH and the carboxyl group of GSH (Scheme 1). The formation of PAH-AuNCs nanocomposites was also confirmed by TEM (Fig. 1C and D), which clearly shows that the AuNCs are effectively and uniformly crosslinked by PAH, and the PAH-AuNCs have a spherical shape with a size of approximately 80 nm.

The excitation-related emission characteristics of the AuNCs were investigated by varying the excitation wavelength from 350 nm to

![Scheme 1. Schematic illustration of the detection strategy for ALP activity based on FRET.](image)

Fig. 1. TEM images of (A) AuNCs, (C) PAH-AuNCs, and (D) PAH-AuNCs at high magnification. (B) The size distribution histogram of AuNCs.
390 nm (Fig. 2A). The results indicate that the highest fluorescence intensity of the AuNCs appears at 570 nm under the excitation wavelength at 365 nm. To verify the occurrence of AIE in the PAH-AuNCs, the fluorescence excitation and emission spectra of the AuNCs and PAH-AuNCs were recorded. Fig. 2B shows that both samples exhibit broad emission bands centred around 570 nm with the excitation peaks at 365 nm, and obvious fluorescence intensity enhancement can be observed compared to the AuNCs. The inset of Fig. 2B shows that a more bright orange emission is observed in the PAH-AuNCs when illuminated under an ultraviolet lamp (λex = 365 nm).

3.2. The feasibility of the sensing system

The proposed fluorescence turn-on and colorimetric dual-readout assay for facile and sensitive sensing of ALP is based on FRET from PAH-AuNCs to DCIP (Scheme 1). The PAH-AuNCs with high fluorescence emission are directly employed as the fluorometric donor, and DCIP is chosen as an acceptor. Herein, the zeta potentials were first measured to confirm the electrostatic interactions between PAH-AuNCs and DCIP. As shown in Fig. S1, the GSH-capped AuNCs and the DCIP exhibit negative zeta potentials at about −18.2 mV and −11.3 mV, respectively, while the PAH-AuNCs exhibit a distinct positive zeta potential at about 21.6 mV due to the large amount of protonated amino groups of PAH. The mixture of DCIP and PAH-AuNCs shows a little positive zeta potential at about 3.9 mV, indicating that there is a strong electrostatic interaction between the DCIP and PAH-AuNCs. Therefore, the zeta potential results indicate the feasibility of the distance dependence of FRET between PAH-AuNCs donors and DCIP acceptors.

The UV-Vis absorbance and fluorescence emission spectra were further investigated to prove the occurrence of FRET and the feasibility for ALP detection (Fig. 3). As shown in Fig. 3A, the emission peak of the PAH-AuNCs is 570 nm (curve a), and the DCIP absorption spectrum is located in the range from 450 to 700 nm (curve b). The good spectral overlap between the PAH-AuNCs fluorophore and the DCIP absorber illustrates the theoretical spectral feasibility of the FRET. The absorption peak of the PAH-AuNCs is located at 375 nm (Fig. 3A, curve c). It is worth noting that the addition of DCIP into the PAH-AuNCs solution results in the disappearance of the absorption peak of the PAH-AuNCs, while it has no effect on the absorption of DCIP (Fig. 3A, curve d). The disappearance of the absorption of PAH-AuNCs indicates the effective fluorescence quenching of PAH-AuNCs by DCIP, which is confirmed by the fluorescence emission spectrum of the PAH-AuNCs upon the addition of DCIP (Fig. 3B, curve c). The above results demonstrate the generation of an observed complex between PAH-AuNCs and DCIP via the adsorption of DCIP onto the PAH-AuNCs surface with the fluorescence effective quenching of this system [48]. In addition, the quenching profile of negatively charged GSH-capped AuNCs by DCIP was investigated (Fig. 3B, curve a and d), and a much lower quenching efficiency than that of the PAH-AuNCs was observed. The result indicates that the fluorescence quenching of the PAH-AuNCs may also result from the inner filter effect, photoinduced electron transfer process, and ground-state complex formation, but it is mainly caused by FRET [48]. However, in the presence of ALP and AAP, their catalytic hydrolysate of AA can reduce DCIP from blue to colourless (Fig. 3C), and the absorption peak of DCIP at 600 nm disappears while the absorption peak of the PAH-AuNCs at 375 nm appears again (Fig. 3A, curve e). As a result, the FRET process is inhibited, and the fluorescence of the PAH-AuNCs is significantly recovered (Fig. 3B, curve f). Therefore, the proposed method for fluorescence turn-on and colorimetric dual-readout sensing of ALP is feasible.

3.3. Optimization of assay conditions

The sensitive fluorescence turn-on and colorimetric dual-readout detection of ALP using the proposed method is because the DCIP absorber can be reduced by AA, which is the hydrolysate of AAP by ALP. Therefore, several experimental conditions were investigated to obtain the optimized analytical performance, including the concentrations of DCIP and AAP, the pH values, and the reaction time (Fig. S2-S5). As the fluorescence acceptor, the concentration of the DCIP can greatly affect the fluorescence of the PAH-AuNCs donor and thus was optimized first. As shown in Fig. 52, the fluorescence of the PAH-AuNCs decreases during the increase of the concentrations of DCIP and AAP, the pH values, and the reaction time (Fig. S2-S5). As the fluorescence acceptor, the concentration of the DCIP can greatly affect the fluorescence of the PAH-AuNCs donor and thus was optimized first. As shown in Fig. S2, the fluorescence of the PAH-AuNCs decreases during the increase of the concentrations of DCIP in the range from 0 to 250 μM. In the presence of 200 μM DCIP, the emission of PAH-AuNCs is almost quenched to the baseline level with a 97% quenching efficiency, revealing the robust FRET from PAH-AuNCs to DCIP. Considering that higher concentrations of DCIP may affect the fluorescence recovery efficiency of PAH-AuNCs induced by the presence of ALP, 200 μM DCIP is used for the following experiments.

Similarly, the concentration of the substrate AAP affects the generated amount of AA and thus influences the fluorescence recovery efficiency of the PAH-AuNCs. Therefore, an investigation into the concentration of AAP was conducted by varying the AAP concentration under fixed concentrations of 200 μM DCIP and 80 U/L ALP and a fixed volume of 100 μL of the PAH-AuNCs solution. As shown in Fig. S3, along with the increase in AAP concentration, the fluorescence intensity first increases due to the fact that the amount of AA being generated also increases accordingly. However, concentrations of AAP over
600 μM will result in a slight decrease of the system fluorescence. Thus, 600 μM is chosen as the optimal concentration of AAP in the following experiments. Since the enzyme activity and reaction rate are greatly influenced by the pH of the enzymatic reaction, we then investigated the pH value of the Tris–HCl buffer. As shown in Fig. S4, the maximum fluorescence intensity can be achieved when the pH value of the enzymatic reaction buffer is around 8.0. Therefore, the ideal pH value is 8.0 in Tris–HCl buffer. Moreover, the reaction time investigation results indicate that the enzymatic reaction between ALP and AAP can be completed within 20 min (Fig. S5A), and their catalytic hydrolysis of AA reducing DCIP can be achieved in 15 min (Fig. S5B), suggesting fast reaction rates of the enzymatic reaction and that the subsequent reduction of DCIP can be expected.

3.4. The performance for ALP detection

To demonstrate the analytical capability of this assay, the responses for different concentrations of ALP were investigated under optimal conditions. To ensure the accuracy and reliability of the experiment, 3 parallel tests were performed for each determination. As shown in Fig. 4A, the fluorescence signal of PAH-AuNCs is gradually recovered when the ALP concentration increases in the range from 0 to 200 U/L. When the ALP concentration increases to 80 U/L, the fluorescence signal almost reaches the plateau. As shown in Fig. 4B, a good linear relationship between the fluorescence intensity at 570 nm and the concentration of ALP can be achieved in a wide range of 0.5–40 U/L and 40–80 U/L can be found. The two linear ranges for ALP with two different slopes may be due to the difference of the reaction rate between the two concentration ranges. In the low concentration range of ALP, small amount of AA can be generated, resulting in a low reduction reaction rate of DCIP by AA, and thus only a low fluorescence recovery rate can be obtained. However, the adequate AA can be generated by ALP at high concentration catalytic hydrolysis of AAP, and the resulting quick rate of reduction of DCIP by AA can produce a high fluorescence recovery rate.

The absorbance of the sensing system for different concentrations of ALP was also recorded to verify the colorimetric detection performance. As shown in Fig. 4C, with the increase of ALP concentration, the maximum absorption peak of DCIP gradually disappears due to the increasing amount of AA produced by the decomposition of AAP. Fig. 4D shows that the absorption intensity at 600 nm exhibits the same 2-stage linear relationship with the ALP concentration as that of the fluorescence detection, indicating that the absorbance decreases at two different speeds which are caused by the same reasons as those of fluorescence detection, and the regression equations are $A = 3.38-0.0268C_{ALP}$ (U/L) with the $R^2$ of 0.9920 and $F = 2.23–0.0563C_{ALP}$ (U/L) with the $R^2$ of 0.987, respectively. The LOD is estimated to be 0.5 U/L by a signal to noise ratio of 3. At the same time, the colour of the solution gradually turns from initial blue to colourless. The obvious colour variation under natural light is depicted in Fig. 4E, and the colour changes caused by ALP activity with a concentration of approximately 20 U/L can be easily distinguished by the naked eye. Meanwhile, visual readout for the fluorescent colour change under the ultraviolet lamp can also be achieved.

In addition, the analysis of the performance was compared with previously reported fluorescence methods (Table S1). Compared with most other fluorescent methods, the present method exhibits better detection sensitivity. Moreover, one can easily observe the colorimetric signal change, thus achieving the naked-eye detection of ALP. The good performance indicates the success of the novel sensing design for fluorescence turn-on and colorimetric dual-readout detection of ALP.

3.5. Selectivity study

To evaluate the specificity of the developed sensing system for ALP, a wide range of interfering substances, including common metals (Na⁺, K⁺, Mg²⁺, Zn²⁺, Ca²⁺, Fe²⁺, Fe³⁺), amino acids (Cys, Gly, l-Lys, l-Phe, Glu, Tys), redox chemicals (GSH, UA, DA, H₂O₂), and other non-specific substances (BSA, HSA, GOx, HRP, IgG, pepsin, trysin and heparin) were investigated under the same conditions. As shown in Fig. 5, while the addition of heparin can induce a weak fluorescence increase, the other interfering substances induce negligible fluorescence changes in comparison with the significant fluorescence increase induced by
The weak fluorescence increase induced by negatively charged heparin may be due to the electrostatic interaction with PAH-AuNCs which disturbs the FRET from the PAH-AuNCs to DCIP. Fortunately, the fluorescence increase induced by heparin is still much lower than that of ALP. These results indicate the high selectivity of the PAH-AuNCs/DCIP sensing system for the detection of ALP.

3.6. Detection of ALP activity in human serum samples

To assess the practical applicability of the method, the measurement of ALP in diluted human serum samples (2%) was performed by standard addition method. As shown in Table 1, using the proposed PAH-AuNCs/DCIP sensing system, the detected concentrations of ALP in the two spiked serum samples are highly consistent with those of added ALP. The recoveries in the range from 96.4% to 105.5% with the relative standard deviations (RSD) lower than 4.9% indicate the reliability and accuracy of this method in complicated biological environments.

4. Conclusion

In this work, a fluorescence turn-on and colorimetric dual-readout sensing system for the sensitive detection of ALP activity was developed based on the FRET from PAH-AuNCs to DCIP. The fluorescence of PAH-AuNCs was efficiently quenched by DCIP due to the good spectral overlap and the electrostatic adsorption between DCIP and PAH-AuNCs. However, the presence of ALP can catalyse the hydrolysis of ALP to AA, which triggers the reduction reaction of DCIP from blue to colourless, resulting in the turn-on fluorescence signal of PAH-AuNCs. As a consequence, the simple and efficient detection of ALP can be realized by the PAH-AuNC/DCIP sensing system. In addition, visual detection of ALP can also be realized due to the obvious observed colour change of
the solution. This method exhibits satisfactory results towards ALP in human serum samples, indicating the promising potential application in real biological systems. Based on the electrostatic interaction induced efficient FRET strategy, the proposed method can be expanded to detect various targets by using AuNCs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2018.09.108.

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Table 1

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