Gold Nanoclusters-catalyzed Luminol Chemiluminescent Sensing Method for Sensitive and Selective Detection of Alkaline Phosphatase

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Abstract

The present study developed a sensitive sensing method for the determination of alkaline phosphatase (ALP) activity based on gold nanoclusters (Au NCs)-catalyzed luminol-H₂O₂ chemiluminescent (CL) reaction. The CL signal of luminol-H₂O₂-Au NCs can be quenched by ascorbic acid which was the product of magnesium ascorbyl phosphate (MAP) hydrolysis reaction catalyzed by ALP. The proposed sensing platform showed convenient, sensitive and selective detection of ALP in the range of 0.0027 - 1.3890 U L⁻¹, with the detection limit of 0.0026 U L⁻¹. The broad detection linear range and ultra-high sensitivity were inherited from the efficient free radical scavenging capability of ascorbic acid on the luminol-H₂O₂-Au NCs CL reaction. The CL sensing platform was applied to the detection of ALP activity in serum samples. We believe that this sensing platform is a universal CL strategy for ALP detection because ascorbic acid is an efficient CL quencher for many CL reactions.
**Introduction**

ALP, a metalloenzyme, is capable of catalyzing the hydrolysis and transphosphorylation of a variety of phosphoric acid monoesters under alkaline conditions and generates phosphate ion and a free hydroxyl group.\(^1\) ALP distributes widely in a variety of mammalian tissues, such as kidney, bone, liver, and intestine.\(^2\)

The abnormal increase of ALP level in serum commonly indicates the occurrence of various diseases such as bone diseases (osteomalacia and osteoblastic bone cancer),\(^3\) liver diseases (obstructive jaundice, hepatitis, and cancer),\(^4,5\) prostate,\(^6\) and bladder cancers.\(^7\)

Therefore, the development of simple and sensitive methods for the detection of ALP activity is of great importance. Over the past few years, a variety of methods including colorimetry,\(^8,9\) fluorescence,\(^10-12\) surface-enhanced Raman scattering,\(^13\) electrochemistry,\(^14,15\) photoelectrochemistry,\(^16\) and electrochemiluminescence,\(^17\) have been established for the detection of ALP activity. Although the reported colorimetric methods for ALP detection are simple, the detection sensitivity is poor. Also, the other methods commonly suffer from complex instruments, or tedious operation, which limits their practical applications.

Chemiluminescence analysis method, a trace analytic strategy based on the detection of optical radiation produced during a chemical reaction, is considered to be a superior method because of its simple instrument, high sensitivity, broad detection linear range, and easy operation.\(^18-20\)

Luminol-H\(_2\)O\(_2\) CL reaction is the most investigated CL system and is employed as a promising tool in clinical diagnosis,\(^21,22\) biological research,\(^23\) food safety,\(^24\) and environment monitoring field.\(^25\)

In recent years, various nanomaterials such as noble metal nanoparticles,\(^26\) carbon dots,\(^27\) graphene oxide,\(^28\) metal nanoclusters,\(^29\) MoS\(_2\) nanosheets,\(^30\) and so on were reported as catalysts to trigger the luminol-H\(_2\)O\(_2\) CL reaction and to
improve the detection sensitivity. Among these nanomaterials, gold nanoclusters (Au NCs), in particular, exhibited excellent catalytic activity, good stability, and biocompatibility.\textsuperscript{31-33}

The first Au NCs stabilized with bovine serum albumin (BSA) was prepared in 2009 by Xie group through a simple one-pot method.\textsuperscript{34} The BSA-Au NCs-catalyzed luminol-H\textsubscript{2}O\textsubscript{2} was reported by Deng group and applied to the detection of glucose.\textsuperscript{35} In addition, the luminol-H\textsubscript{2}O\textsubscript{2} CL reaction catalyzed by cationic BSA-Au NCs was also reported.\textsuperscript{36} Their mechanism studies showed that Au NCs can accelerate the generation of various oxygen-related free radicals such as hydroxyl radical (·OH) and superoxide anion free radical (O\textsubscript{2}·\textsuperscript{−}). Ascorbic acid, as a broad-spectrum radical scavenger, is commonly employed in CL researches to study the mechanism of a CL reaction.\textsuperscript{37,38} Ascorbic acid is capable of scavenging the oxygen-related free radicals and leads to a quenched CL signal. MAP is a derivative of ascorbic acid which can be hydrolyzed by ALP leading to the production of ascorbic acid.

Taken the above altogether, the present study developed a simple and convenient CL sensing method for the determination of ALP activity based on the Au NCs-catalyzed luminol-H\textsubscript{2}O\textsubscript{2} CL reaction. As depicted in Scheme 1, the hydrolysis of MAP by ALP leads to the generation of ascorbic acid which is capable of quenching the CL signal of the luminol-H\textsubscript{2}O\textsubscript{2} reaction catalyzed by Au NCs. In other words, the CL intensity of luminol-H\textsubscript{2}O\textsubscript{2}-Au NCs reaction is reversely proportional to the concentration of ALP in the sample. Based on the quenched CL signal, a quantitative method for the detection of ALP activity was developed as described in the following sections.
Experimental

Reagents and chemicals

All chemicals were of analytical reagent grade and were used as received. Distilled water (18.2 MΩ cm<sup>-1</sup>) was used throughout the current study. BSA was obtained from Sigma-Aldrich. Glycine, lysine and ALP were purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). The concentration of ALP was 6.7×10<sup>5</sup> U L<sup>-1</sup>. MAP was purchased from Dibai Bio-Technology Co., Ltd. (Shanghai, China). NaOH, NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, MgCl<sub>2</sub>·6H<sub>2</sub>O, KCl, NaCl, LiCl, NiCl, AgNO<sub>3</sub>, CaCl<sub>2</sub>·2H<sub>2</sub>O, BaCl<sub>2</sub>·2H<sub>2</sub>O, CuCl<sub>2</sub>·3H<sub>2</sub>O, CdCl<sub>2</sub>·H<sub>2</sub>O, FeCl<sub>2</sub>·4H<sub>2</sub>O, FeCl<sub>3</sub>·6H<sub>2</sub>O and CrCl<sub>3</sub>·6H<sub>2</sub>O were purchased from Guangfu Fine Chemical Research Institute (Tianjin, China). Luminol was purchased from Alfa Aesar. HAuCl<sub>4</sub>·4H<sub>2</sub>O, hydrogen peroxide (30%, v/v) and other chemical reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

The concentration of luminol stock solution was set at 10 mM, which was prepared by dissolving 0.0177 g of luminol in 10 mL of 0.1 M NaOH. The luminol stock solution should be stored in the refrigerator (4 °C) for at least one week before use. The working solution of luminol was made by gradually diluting the stock solution in 0.1 M NaOH. The stock solution of ALP (50 U L<sup>-1</sup>) was prepared by diluting ALP in Tris-HCl buffer (pH 8) containing 1 M KCl, 50 mM MgCl<sub>2</sub>, 0.2% (w v<sup>-1</sup>) TritonX-100 and 1% (w v<sup>-1</sup>) BSA.

Apparatus

The CL measurements were performed with a multifunctional microplate reader (Thermo Fluoroskan Ascent FL, USA) with dispensers.

Preparation of Au NCs
Au NCs stabilized by BSA was prepared according to a previously reported method. In a typical experiment, 5 mL of 10 mM HAuCl₄ was added to 5 mL of 50 mg mL⁻¹ BSA solution under vigorous stirring. After stirring at 37 °C for 2 min, 0.5 mL of 1 M NaOH solution was added into the mixture. The obtained solution was kept on stirring at 37 °C for 12 h. The color of the mixture turned to deep brown indicating the formation of Au NCs. The final product was dialyzed (membrane molecular weight cut-off: 3000) against 10 mM phosphate buffer (pH 7.4) for 48 h to remove the remaining NaOH and HAuCl₄.

Finally, the product was stored at 4 °C for further use. The UV-vis absorption spectrum and fluorescence spectrum of Au NCs were acquired using Molecular Devices (SpectraMax M2, USA). The Au NCs possessed a typical UV-vis absorbance peak at 300 nm (Fig. S1, Support Information), and a strong fluorescence emission peak centered at 660 nm being excited at 300 nm (Fig. S2, Support Information). The concentration of Au NCs was calculated to be 0.39 mM.

Procedure of CL analysis

10 μL of 0.5 mM MAP solution was mixed with 90 μL of different concentrations of ALP solution. The mixtures were incubated at 37 °C for 30 min to ensure the hydrolysis of MAP by ALP. Then, 25 μL of the mixture was added into the well of a 96-well microplate and mixed with 25 μL of 0.049 mM Au NCs and 50 μL of 0.5 mM H₂O₂. Finally, 100 μL of 4 mM working solution of luminol was injected into each well through a dispenser, and the CL signal was recorded by the microplate reader simultaneously. Every sample was detected in parallelly three times.

Results and Discussion

Mechanism of the CL determination of ALP activity
It has been reported that Au NCs were capable of catalyzing the luminol-H_2O_2 reaction to emit light.\(^\text{35}\) Oxygen-related free radicals such as 'OH, O_2^\cdot are involved in the CL emission process. Ascorbic acid is generally used as a broad-spectrum radical scavenger. The addition of ascorbic acid to the luminol-H_2O_2 CL reaction would induce a decreased CL signal. In the present study, we found it interesting that MAP, a stable derivative of ascorbic acid, showed a negligible effect on the luminol-H_2O_2-Au NCs CL reaction. Base on this phenomenon, a simple CL method for the determination of ALP activity was developed. The mechanism for the CL detection of ALP activity is illustrated in Scheme 1. MAP has a negligible effect on the luminol-H_2O_2 CL reaction catalyzed by Au NCs. Therefore, the luminol-H_2O_2-Au NCs reaction exhibits a strong CL emission without the addition of ALP. The phosphoester bond of MAP can be hydrolyzed by ALP and trigger the release of ascorbic acid which was a strong inhibitor for the luminol-H_2O_2-Au NCs CL reaction. Thus, the addition of ALP in the reaction solution would induce a significant decrease in CL signal. The decreased CL signal was reversely proportional to the activity of ALP in the sample, which was a quantitative basis for the CL detection of ALP activity. Based on the quenching effect of ascorbic acid on the luminol-H_2O_2-Au NCs CL reaction, the present study developed a simple and convenient CL platform for the determination of ALP activity.

*Feasibility of the CL method for the detection of ALP activity*

In order to verify the hypothesis, we did feasibility experiments. The CL kinetic curves of luminol-H_2O_2 reaction are shown in Fig. 1 respectively. In the absence of a catalyst, the oxidation of luminol by H_2O_2 is a slow process. Therefore, a weak CL signal was observed when luminol was mixed with H_2O_2 directly (curve a). Au NCs, as a mimetic peroxidase, can catalyze the luminol-H_2O_2 CL reaction. As shown in curve b,
a fast and intense CL signal was observed when Au NCs was added into the luminol-H$_2$O$_2$ CL reaction. Ascorbic acid is a broad-spectrum free radical scavenger and can quench the CL signal of luminol-H$_2$O$_2$-Au NCs CL reaction efficiently. 95.5 % of the CL signal could be quenched by 0.05 mM ascorbic acid (curve c). MAP is a stable derivative of ascorbic acid. As shown in curve d, the CL signal of luminol-H$_2$O$_2$-Au NCs in the presence of 0.05 mM MAP was only slightly affected as that in the absence of MAP indicating that MAP had no quenching effect on the luminol-H$_2$O$_2$ CL reaction catalyzed by Au NCs. Then, we incubated the 0.05 mM MAP with 0.14 U L$^{-1}$ ALP at 37 °C for 30 min. During this process, the MAP could be hydrolyzed by ALP to produce ascorbic acid which inhibits the luminol-H$_2$O$_2$-Au NCs CL reaction efficiently. Curve f shows that 90.3% of the CL signal could be inhibited when MAP was incubated with 0.14 U L$^{-1}$ ALP. The control experiment exhibited that ALP itself had no quenching effect on the luminol-H$_2$O$_2$-Au NCs CL reaction (curve e). Therefore, the quenched CL signal should be attributed to the ascorbic acid produced by the hydrolysis of MAP by ALP. Based on the decreased CL signal, the activity of ALP can be determined quantitatively.

**Optimization of assay conditions for the CL detection of ALP**

In order to achieve the detection of ALP with high sensitivity and selectivity, we optimized the experimental conditions including the concentration of MAP, pH of the hydrolysis reaction, the concentration of Au NCs, H$_2$O$_2$ and luminol, and the dilution solution of luminol. The quenching efficiency, (CL$_0$-CL)/CL$_0$ in which CL$_0$ and CL referred to the CL intensity in the absence and in the presence of ALP respectively, was employed to evaluate the effects of assay conditions on the CL detection of ALP. ALP catalyzes the hydrolysis of MAP to produce ascorbic acid which inhibits the luminol-H$_2$O$_2$-Au NCs CL reaction. MAP plays an important role in the CL detection of
ALP activity. We investigated the effect of the concentration of MAP on the CL detection of ALP firstly. As shown in Fig. 2a, the quenching efficiency increased quickly with the increase of the concentration of MAP from 0 to 0.1 mM, and increased slightly when the concentration of MAP ranged from 0.1 to 0.5 mM. When the MAP concentration was higher than 0.5 mM, the quenching efficiency maintained almost the same. Thus, the concentration of MAP was set at 0.5 mM in the following studies. ALP can remove the phosphate group on the substrate molecule, MAP, by hydrolyzing the phosphate monooester bond. ALP is most active in alkaline environments. We investigated the effect of reaction pH ranged from 6 to 10 (Fig. 2b). When the pH of the enzyme reaction was above 7, the quenching efficiency maintained almost the same. In our work, pH 8 was selected for the following studies.

The concentrations of Au NCs, H$_2$O$_2$ and luminol, and the pH of the dilution solution of luminol would affect the CL intensity of the luminol-H$_2$O$_2$-Au NCs reaction. So we investigated the influence of the above reaction conditions on the CL detection of ALP activity. Both of the sample signal (CL intensity with the addition of ALP) and the blank signal (CL intensity without the addition of ALP) increased with the increasing concentration of Au NCs. However, the results showed that the quenching efficiency was slightly dependent on the concentration of Au NCs (Fig. S3a, Support Information). Considering the measured CL value and the consumption of Au NCs, 0.049 mM Au NCs was selected for the following studies. Both of the sample signal and the blank signal enhanced monotonously with the increase of H$_2$O$_2$ concentration (Fig. S3b, Support Information). The quenching efficiency maintained almost the same when the concentration of H$_2$O$_2$ ranged from 0 to 0.5 mM and decreased when the concentration of H$_2$O$_2$ was higher than 0.5 mM. Thus, the concentration of H$_2$O$_2$ was set at 0.5 mM. As shown in Fig. S3c (Support Information), higher pH value was a benefit to the
detection of ALP, and 0.1 M NaOH was used as a dilution solution to prepare the working solution of luminol. From Fig. S3d (Support Information), the results indicated that the concentration of luminol had a negligible effect on the CL detection of ALP activity, and 4 mM luminol was employed in the following studies.

Analytical performance for the CL detection of ALP activity

Under the optimal conditions, the quantitative ability of the proposed CL method for the detection of ALP was evaluated by detecting the CL response with different concentrations of ALP. A strong CL signal was observed in the control group in which MAP didn’t incubate with ALP, and the CL signal decreased with increasing concentration of ALP in the range of 0.0027 - 1.3890 U L$^{-1}$ (Fig. 3a). A good linear relationship between the quenching efficiency ($Q$) and the logarithm of ALP concentration in the range of 0.0027 - 1.3890 U L$^{-1}$ was obtained. The equation of linear regression was represented by $Q = 0.3439\log C + 0.8964$ with a regression coefficient of 0.9970. The LOD was calculated based on the concentration of ALP which gave a 3-fold signal of the standard deviation of a blank signal (which was detected seven times parallely). The LOD was calculated to be 0.0026 U L$^{-1}$. The reproducibility of this CL method for the detection of ALP was evaluated by a series of 8 repetitive measurements of 0.0054, 0.0217 and 0.1736 U L$^{-1}$ ALP. The relative standard deviation of 0.0054, 0.0217 and 0.1736 U L$^{-1}$ ALP was 3.48%, 4.59% and 3.56%, respectively.

In order to evaluate the specificity of the proposed CL method for the detection of ALP, we investigated the influence of the same interferences which may exist in serum including some common ions, amino acids, and proteins. As shown in Fig. 3b, 98.6% CL signal was quenching with 1.39 U L$^{-1}$ ALP. However, no quenching effect was observed when Na$^+$ (1 mM), Ca$^{2+}$ (1 mM), H$_2$PO$_4^-$ (0.01 mM), HPO$_4^{2-}$ (0.01 mM), glycine (2.5 mM), lysine (5 mM), calmodulin (0.5 mg mL$^{-1}$), hIgG (0.5 mg mL$^{-1}$),
streptavidin (0.5 mg mL\(^{-1}\)) and hemoglobin (0.1 mg mL\(^{-1}\)) was added in the detection system. The above results indicated that the proposed method had good specificity for the CL detection of ALP activity.

Detection of ALP in serum samples

The good specificity of the proposed method for the CL detection of ALP indicated that the applicability of the method to detect ALP activity in real samples. In order to evaluate the applicability of the proposed method in real sample detection, a standard addition method was employed. The human serum was incubated at 75 °C for 5 min to deactivate the native ALP. Three concentrations of ALP (0.0111, 0.1111 and 0.5556 U L\(^{-1}\)) were spiked into the human serum which was diluted 100-fold with PBS buffer (pH 7.4) and then incubated with 0.5 mM MAP.

The CL signals of the resulting mixtures were detected by using the proposed CL method. The results are summarized in Table S1 (Support Information). The recovery of 0.0111, 0.1111 and 0.5556 U L\(^{-1}\) ALP was calculated to be 104.40% ± 5.96%, 113.61% ± 3.20%, and 83.02% ± 2.11%, respectively. The above results demonstrated that the developed method can be used to detect ALP in real serum samples with good accuracy. Moreover, compared with other analytical methods for the detection of ALP activity which were reported previously, the present method showed a good detection dynamic range and the highest detection sensitivity (Table S2, Support Information).

Conclusions

In summary, the present study found that the product of the hydrolysis reaction between the MAP and ALP, ascorbic acid, showed a significant inhibition effect on the luminol-H\(_2\)O\(_2\)-Au NCs CL reaction, while the substrate
MAP has a negligible effect on this CL reaction. This result suggested that MAP can be used as a substrate for the CL detection of ALP activity. Therefore, a simple CL method for the determination of ALP activity was established by using MAP as a substrate. In the absence of ALP, a strong CL signal was obtained. The CL signal decreased with an increasing concentration of ALP in the range of 0.0027 - 1.3890 U L\(^{-1}\). The LOD for the CL detection of ALP was calculated to be 0.0026 U L\(^{-1}\). The established method is simple and can detect the ALP activity within 30 min. In addition, the method also demonstrated good specificity for the detection of ALP and applicability in real sample detection.

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**Supporting Information**

UV-vis spectrum (Fig. S1), FL spectrum of Au NCs (Fig. S2), recovery of ALP spiked in serum samples (Table S1), and comparison of the analytical performance of this CL method with other methods (Table S2). This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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Figure captions

Fig. 1 The CL kinetic curves of the luminol-H$_2$O$_2$ reaction under different conditions. (a) luminol + H$_2$O$_2$; (b) luminol + H$_2$O$_2$ + Au NCs; (c) luminol + H$_2$O$_2$ + Au NCs + ascorbic acid; (d) luminol + H$_2$O$_2$ + Au NCs + MAP; (e) luminol + H$_2$O$_2$ + Au NCs + ALP; (f) luminol + H$_2$O$_2$ + Au NCs + MAP + ALP. Experimental conditions for enzymatic reaction: 0.5 mM MAP, 10 μL; 0.14 U L$^{-1}$ ALP, 90 μL; reaction time, 30 min. Experimental conditions for CL reaction: 4 mM luminol, 100 μL; 0.5 mM H$_2$O$_2$, 50 μL; 0.049 mM Au NCs, 25 μL; product of enzymatic reaction, 25 μL.

Fig. 2 Optimization of experimental conditions for enzymatic hydrolysis reaction. (a) The effect of MAP concentration on CL intensity. (b) The effect of pH of hydrolysis reaction on CL intensity. Experimental conditions were the same as Fig. 1.

Fig. 3 (a) The calibration plot of this CL system for the detection of ALP activity. (b) The specificity of this CL system for the detection of ALP activity. Experimental conditions were the same as Fig. 1.

Scheme 1 Schematic mechanisms of the CL method for the detection of ALP.
Fig. 1
Fig. 2
Fig. 3
Scheme 1
