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A Graphene Quantum Dots-Enzyme Hybrid System for the Fluorescence Assay of Alkaline Phosphatase Activity and Inhibitor Screening

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A graphene quantum dots (GQDs) and horse radish peroxidase (HRP) hybrid system was designed for the sensing of alkaline phosphatase (ALP) activity and inhibitor screening. We found that the photoluminescence (PL) intensity of GQDs could be quenched efficiently in the presence of phenol, H_2O_2 and HRP. Moreover, ALP could hydrolyze disodium phenyl phosphate (DPP) to produce phenol, and also could result in the photoluminescence quenching of GQDs. The decrease in the PL intensity was linear to the activity of ALP in the concentration range of 0.02 – 0.8 U/L, with a detection limit of 0.008 U/L. The proposed GQDs/HRP hybrid system was successfully applied to ALP determination in human serum samples. The inhibition study was further analyzed, and Na_3VO_4 (as an ALP inhibitor) showed a clear inhibition effect. The results suggest that the GQDs/HRP hybrid system has good potential applications for the assay of ALP activity and inhibitors screening in related biochemical fields.

Keywords Graphene quantum dots, fluorescent probe, alkaline phosphatase, enzyme hybrid biosensors

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Introduction

Alkaline phosphatase (ALP), one of the membrane-bound enzymes existing in all tissues throughout the body, but mainly concentrated in the liver, bone and kidney, is a phosphomonoester hydrolase enzyme that can catalyze the dephosphorylation process to remove the phosphate groups from proteins, nucleic acids, as well as small molecules.[1-3] Owing to the abnormal level of ALP in serum always being closely connected with various diseases, such as hepatitis,[4] diabetes,[5,6] liver dysfunction,[7] bone disease,[8] and prostate cancer,[9] it is often considered to be a most commonly assayed enzyme and biomarker in practical clinical diagnosis.[10,11] In addition, because of the fact that most pathogenic bacteria are present at lower thermal temperature, the level or activity of ALP is employed to confirm the degree of milk or other drinks pasteurization in dairy manufacturing.[12,13] Therefore, wide attention has been made to find out a facile, sensitive and selective detection method for ALP based on its great significance.

Until recently, a number of methods for detecting the activity of ALP have been reported, including spectrofluorometry,[14,15] colorimetry,[16] chromatographic,[17] electrochemical,[18,19] chemiluminescence,[20] and surface enhanced resonance Raman scattering.[21] Among those, spectrofluorometry has become the most critical method because of the advantages of higher sensitivity, more convenience, less sophisticated instrument requirements, and a rapider response.[3,15] However, some fluorescent detection technologies have utilized polyelectrolytes, organic dyes, and DNA template metal nanoparticles as fluorescent probes. They showed inescapable shortcomings, like complex synthetic procedure, poor photostability and water-solubility, complicated labelling process, huge toxicity, high cost for expensive reagents. They have thus been suffered from serious limitations.[22,23] Thus, the development of quantum dots (QDs) has opened up a new avenue for the fluorescence method of detecting ALP.

Up to now, fluorescent semiconductor QDs have been drawn tremendous attention due to their superiorities, such as optical, electronic, thermal, magnetic, and chemical properties, especially in biology and medicine. Unfortunately, most traditional QDs contain heavy metals, which are known to display high toxicity at low concentration. Further, they are not suitable for diagnosis or bioimaging. Hence, after GQDs were reported in 2004,[24] they have increasingly become hot points in terms of biosensors, bioimaging, disease detection and drug delivery because of their rapid synthesis routine, low toxicity, great biocompatibility, excellent photostability, good water solubility as well as high fluorescence yield.[25-37]

In this work, a GQDs and HRP hybrid system was designed

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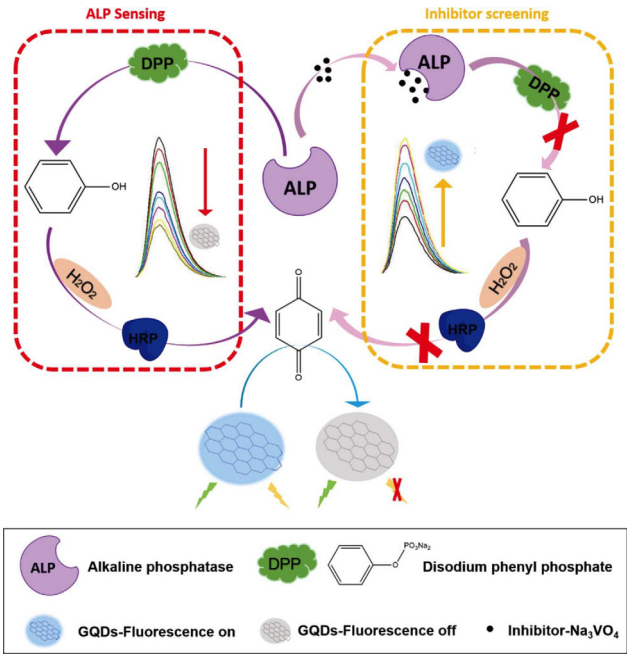

Scheme 1 Schematic illustration of the assay of ALP and inhibitor screening.

for ALP sensing and inhibitor screening. As shown in Scheme 1, disodium phenyl phosphate (DPP) was hydrolyzed by ALP to give phenol. Phenol was oxidized by H_2O_2 and HRP to give benzoquinone. Further benzoquinone is an efficient quencher which can cause a large fluorescence decrease of GQDs. Then a fluorescence method was thus proposed for the sensing of ALP activity. Also, ALP inhibitor screening was also proposed.

Experimental

Chemicals and instrumentation

ALP was obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). All other chemicals were of analytical grade and used without further purification. The water used for solution preparation was purified water purchased from Hangzhou Wahaha Group Co., Ltd. (China). Emission spectra were recorded using a Shimadzu RF-5301 spectrometer with an excitation wavelength of 360 nm; the PL intensity referred to the maximum emission of GQDs at 466 nm. GQDs were synthesized according to our previous paper.[38]

ALP detection and inhibitor screening

For ALP sensing, the solution of H_2O_2 for 1 mM, the solution of HRP for 100 μg/mL, and the solution of GQDs diluted 25 times were prepared in a phosphate buffer solution (pH 7.40). The solution of DPP for 10 mM and the solution of ALP were prepared in phosphate buffer (pH 10.00). After 50 μL of DPP was mixed with different concentrations of ALP, all of the mixture solutions were heated in water at a temperature of 37°C, pH 10.00 for 60 min. Finally, specific volumes of the solution of H_2O_2, HRP, and GQDs were added in order. All of the solution were mixed evenly before being measured.

For inhibitor screening, the solution of H_2O_2 for 1 mM, the solution of HRP for 100 μg/mL, and the solution of GQDs diluted 25 times were prepared in phosphate buffer (pH 7.40), and 10 mM DPP, 1 M Na_3VO_4 and ALP solution were prepared in a phosphate buffer solution (pH 10.00). And after different concentrations of the Na_3VO_4 solution were added into the mixture solution, mixed a certain volume of ALP with 50 μL DPP solution, the mixture solutions were heated in the water at a temperature of 37°C, pH 10.00 for 60 min. Finally, a specific volume of the solution of H_2O_2, HRP, and GQDs was added continuously. All of the solutions were mixed evenly before being measured.

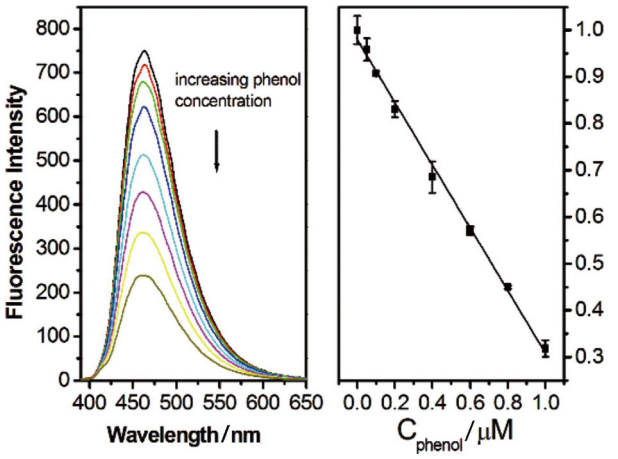

Fig. 1 Effect of phenol concentration on the PL intensity of GQDs/HRP system (H_2O_2 50 μM, HRP 10 μg/mL) at pH 7.40.

ALP detection in serum samples

Blood samples were obtained from two volunteers at the Hospital of Changchun China, Japan Union Hospital. The blood samples were centrifuged at 12000 rpm for 5 min, and left standing for 3 h at room temperature. Then, the serum was separated and stored frozen. The resulting serum samples were added with different concentrations of the GQDs-DPP-HRP-H_2O_2 system separately to prepare spiked samples. The fluorescence spectra of the serum samples and the spiked serum samples were measured, respectively, and the concentrations of ALP in the samples could be calculated *via* the regression equation between the PL intensity and the concentration of ALP. The results from three individual experiments were averaged.

Results and Discussion

Phenol induced fluorescence quenching of GQDs in the present of HRP

Phenol-induced fluorescence quenching of GQDs is analyzed in Fig. 1. According to former literature,[37] benzoquinone is an excellent quencher for GQDs. Phenol can be oxidized to benzoquinone in the presence of HRP and H_2O_2.[39] Therefore, the assay of phenol can be achieved based on the GQDs/H_2O_2/HRP system. The PL intensity of GQDs decreases gradually upon the addition of an increasing concentration of phenol. The relationship between I_0/I and the phenol concentration is linear from 0.05 to 1 μM. The linear calibration curve is: $I/I_0 = 0.980 - 0.0671 \times C_{\text{phonel}}$ (μM) with a correlation coefficient of $R^2 = 0.999$ and a detection limit (LOD) of 15 nM. Here, "I" and "I_0" refer to the emission intensity of the GQDs in the presence or absence of phenol, and "C" refers to the concentrations of phenol.

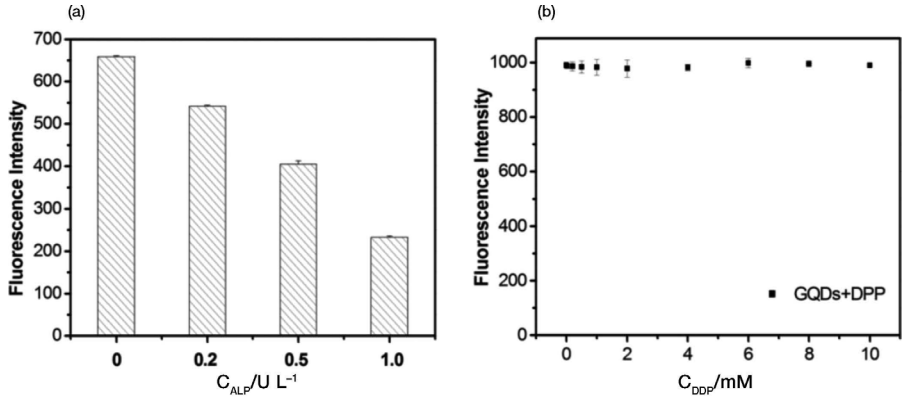

Fig. 2 (a) Effect of the ALP (pH 10.0) on the PL intensity of GQDs in the presence of 50 μM H_2O_2 and 10 μg/mL HRP; (b) effect of DPP on the PL intensity of GQDs.

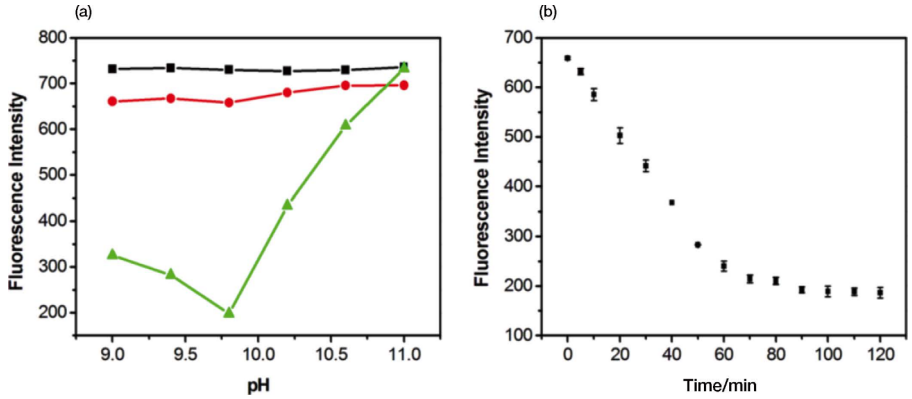

Fig. 3 (a) Effect of environment pH on the PL intensity of GQDs. The black plots (square), GQDs only; the red plots (ball), GQDs with HRP, H_2O_2 and DPP; the green plots (triangle), GQDs with HRP, H_2O_2, DPP, and ALP. (b) The reaction dynamics of the detecting system after the addition of ALP.

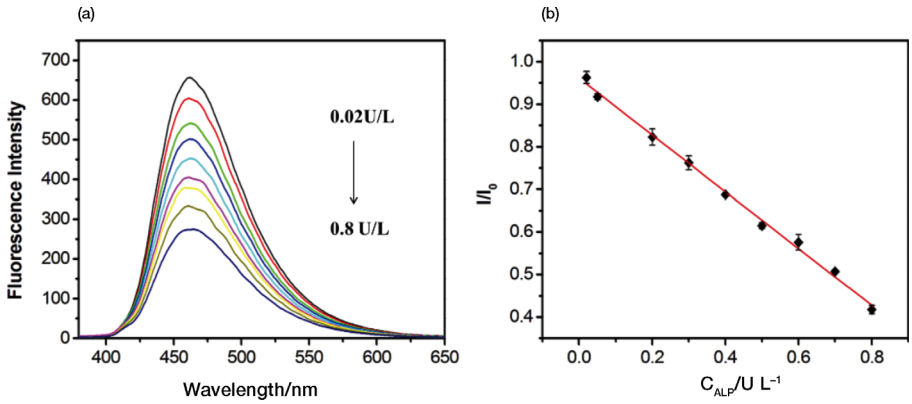

Fig. 4 (a) Fluorescence spectral changes of the GQDs upon the addition of increasing ALP concentration (0.02, 0.05, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 U/L, respectively) in the presence of 50 μM H_2O_2 and 10 μg/mL HRP. (b) PL intensity was plotted against ALP concentration.

The strategy for ALP sensing

A feasibility study of ALP detection is shown in Fig. 2a. Higher concentrations of ALP can cause a larger decrease in the PL intensity, which indicates that the quenching degree of GQDs could be used in ALP quantification. Moreover, the result of DPP's effect on the GQDs (Fig. 2b) suggests that DPP has a negligible effect on the PL intensity of GQDs, which means that the signal is led by both ALP and DPP.

Effect of the buffer pH and incubation time

The effect of the buffer pH on the PL intensity of GQDs is analyzed in Fig. 3a. The PL intensity of GQDs is basically unchanged if only GQDs exits. Also, the PL intensity of the GQDs also shows no obviously fluorescence quenching with HRP, H_2O_2 and DPP at different pH values. However, with the addition of ALP into the system, the PL intensity of the GQDs changes obviously with the lowest point, which indicates that

the best pH values for the detection system is pH 9.80.

As shown in Fig. 3b, when ALP is added to the system, the PL intensity of the GQDs decreases gradually at first hour. When the incubation time is longer than one hour, approximately, the PL intensity reaches a plain. The result proves that the process of the ALP and HRP catalyzing oxidation reaction is almost completed in 1 h. In following research, an incubation time of 60 min was adopted.

Fluorescence detection of ALP

The fluorescence detection of ALP was carried out under the optimized condition (pH 9.80, 60 min) based on the GQDs/HRP-hybrid system. Figure 4 shows the PL spectra of the GQDs with a series of concentrations of ALP added (0.02, 0.05, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 U/L, respectively). The PL intensity decreases linearly with the ALP concentration in the range of 0.02 – 0.8 U/L. The detection limit for ALP was calculated to be 0.008 U/L by 3σ according to the IUPAC standard ("σ" is the standard deviation of 20-times detection of blank samples). The linear calibration curve is: $I/I_0 = 632.12 - 436.12 \times C_{ALP}$ (U/L) with a correlation coefficient of $R^2 = 0.997$. A comparison between other reported methods and our proposed methods for ALP sensing on linear ranges and detection limits are listed in Table 1. It can be seen that compared with most sensors, the detection limit in this paper is comparable of the best methods. Also the detection ranges of this work are sufficiently wide. The above results revealed that our fluorescence system is available, sensitive, facile preparation, nontoxic and low cost, which has great potential applications.

Effect of an inhibitor

The inhibitors effect is also studied in Fig. 5. When an inhibitor of ALP was added into the detection system, the ALP enzymatic activity could be affected. Then, the quenching was weak and the increase emission intensity would be expected. A common ALP inhibitor, Na_3VO_4, was introduced into GQDs/HRP/ALP system. The degree of decreased emission intensity recovered gradually with increasing Na_3VO_4 concentration (Fig. 5b). This result indicates that the rate of ALP hydrolysis gradually decreases with increasing Na_3VO_4 concentrations. The IC_{50} value of Na_3VO_4 is estimated to be 0.436 mM. The phenomena clearly suggest that our assay could be used for potential ALP inhibitor screening.

Selectivity study

The selectivity of the proposed detection method was studied, and a number of potentially interfering substances, including glucose oxidase, urate oxidase, trypsin, pepsin, BSA, Fe^{3+}

Table 1 Comparison of different ALP methods

System	Linear range/U L^{-1}	Detection limit/U L^{-1}	Ref.
Real-time fluorescent assay	16.7 - 782.6	1.1	10
Disposable lateral flow-through strip	0.1 - 150	0.1	12
Stimulus responsive infinite coordination polymer nanoparticles	25 - 200	10	15
Recyclable real-time fluorescent assay	4.6 - 383.3	1.4	22
Enzymatic hydrogelation-induced fluorescence turn-off	0 - 2800	60	40
Ratiometric fluorescent probe based on ESIPT and AIE	0 - 150	0.15	41
Electrochemical assay	0 - 10	0.1	42
Nanoparticle-decorated graphene	0.1 - 100	0.02	43
This work	0.02 - 0.9	0.008	—

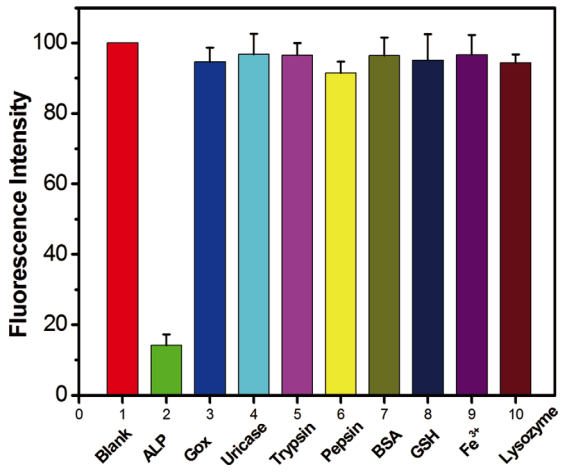

Fig. 6 Effect of interfering substances on the PL intensity of the detecting system.

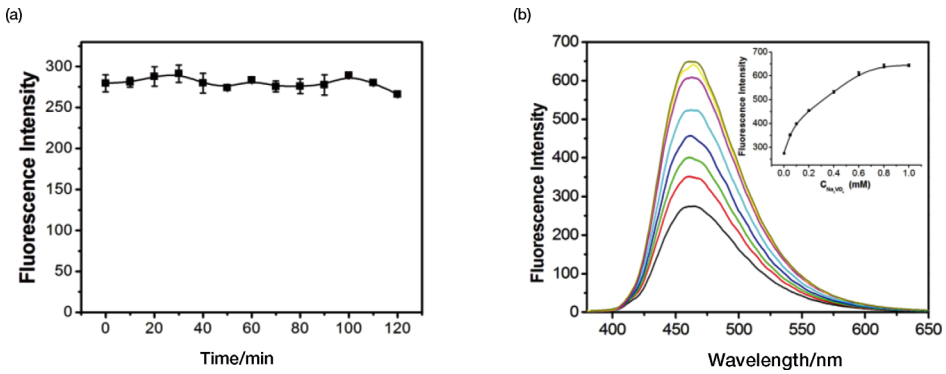

Fig. 5 (a) The relationship between the PL intensity of GQDs and the incubation time in the presence of ALP 0.8 U/L, DPP 100 µM, Na_3VO_4 1 mM, H_2O_2 50 µM, HRP 10 µg/mL in pH 10.00 PBS. (b) Effect of the inhibitor Na_3VO_4 on the PL intensity of our system.

Table 2 ALP determination in dilute serum samples (n = 3)

Sample	Added/ U L^{-1}	Measured/ U L^{-1}	Recovery, %	RSD, %
Serum 1	0.2	0.205	102.5	4.1
	0.5	0.518	103.6	1.7
Serum 2	0.2	0.196	98.0	3.2
	0.5	0.502	100.4	2.0

(10 μM), lysozyme, were studied. Figure 6 show that none of these bimolecular or ions produce a significant fluorescence decrease, which clearly indicates that these substances do not show any noticeable interference on the proposed ALP detection method.

Serum sample detection

To evaluate the feasibility of the GQDs-enzyme hybrid system for ALP detection in serum samples, the proposed technique was used in ALP detecting in two different human serum samples by the standard addition method as listed in Table 2. The recoveries of the two serum samples in the range 98.0 to 103.6% were obtained, and the RSDs were no more than 5%. The above results reveal that this fluorescence detection based on the GQDs-enzyme is an applicable detection technique for ALP activity analysis in practical samples with extensive potential applications.

Conclusions

A facile fluorescence method based on the GQDs-enzyme hybrid system has been designed for the assay of ALP activity and inhibitor screening. The PL intensity of GQDs could be efficiently quenched by phenol in the present of H_2O_2 and HRP. Moreover, DPP was hydrolyzed by ALP to give phenol, which also resulted in the PL quenching of GQDs. The decrease in the fluorescence is linear to the activity of ALP in the concentration ranges of 0.02 – 0.8 U/L. Also, the detection limit is 0.008 U/L. The proposed method is easy, nontoxic, available, sensitive, facile preparation, nontoxic and low cost, simple, which displays good sensitivity and selectivity. What is more, the method could be used to detect ALP in serum samples that display good potential application prospects. The inhibitor study indicates that the proposed method could be utilized for the screening of ALP inhibitors. The results show that our GQDs fluorescent probe could be used for ALP activity sensing in various biochemical applications.

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