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$_2$O$_2$ 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$_3$VO$_4$ (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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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 H2O2 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 H2O2 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, a specific volume of the solution of H2O2, HRP, and GQDs was added continuously. All of the solutions were mixed evenly before being measured.

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-H2O2 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 H2O2.39 Therefore, the assay of phenol can be achieved based on the GQDs/H2O2/HRP system. The PL intensity of GQDs decreases gradually upon the addition of an increasing concentration of phenol. The relationship between I/I0 and the phenol concentration is linear from 0.05 to 1 μM. The linear calibration curve is: I/I0 = 0.980 - 0.0671 × Cphenol (μM) with a correlation coefficient of R2 = 0.999 and a detection limit (LOD) of 15 nM. Here, “I” and “I0” refer to the emission intensity of the GQDs in the presence or absence of phenol, and “C” refers to the concentrations of phenol.
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, H₂O₂ 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, Na3VO4, was introduced into GQDs/HRP/ALP system. The degree of decreased emission intensity recovered gradually with increasing Na3VO4 concentrations (Fig. 5b). This result indicates that the rate of ALP hydrolysis gradually decreases with increasing Na3VO4 concentrations. The IC50 value of Na3VO4 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, Fe3+

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**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                         | —    |

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**Fig. 6** Effect of interfering substances on the PL intensity of the detecting system.
(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 H₂O₂ 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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**References**

1. H. M. Wang, L. X. Mu, G. W. She, H. T. Xu, and W. S. Shi, *Sens. Actuators, B*, 2014, 203, 774.

2. Q. H. Hu, F. Zeng, C. M. Yu, and S. Z. Wu, *Sens. Actuators, B*, 2015, 220, 720.

3. W. J. Kang, Y. Y. Ding, H. Zhou, Q. Y. Liao, X. Yang, Y. G. Yang, J. S. Jiang, and M. H. Yang, *Microchim. Acta*, 2015, 182, 1161.

4. C. T. Wai, J. K. Greenson, R. J. Fontana, J. A. Marrero, H. S. Conjeevara, and A. S. F. Lok, *Hepatology*, 2003, 38, 518.

5. F. H. Luyckx, C. Desaive, A. Thiry, W. Dewé, A. J. Scheen, J. E. Gielen, and P. J. Lefèbvre, *Int. J. Obes.*, 1998, 22, 222.

6. M. Nannipieri, C. Gonzales, S. Baldi, R. Posadas, K. Williams, S. M. Haffner, M. P. Stern, and E. Ferrannini, *Diabetes Care*, 2005, 28, 1757.

7. K. Ooi, K. Shiraki, Y. Morishita, and T. Nobori, *J. Clin. Lab. Anal.*, 2007, 21, 133.

8. P. Garnero and P. D. Delmas, *J. Clin. Endocrinol. Metab.*, 1993, 77, 1046.

9. J. E. Brown, R. J. Cook, P. Major, A. Lipton, F. Saad, M. Smith, K. A. Lee, M. Zheng, Y. J. Hei, and R. E. Coleman, *J. Natl. Cancer Inst.*, 2005, 97, 59.

10. Z. S. Qian, L. J. Chai, Y. Y. Huang, C. Tang, J. J. Shen, J. R. Chen, and H. Feng, *Biosens. Bioelectron.*, 2015, 68, 675.

11. K. S. Park, C. Y. Lee, and H. G. Park, *Analyst*, 2014, 134, 4691.

12. L. Yu, Z. Z. Shi, C. Fang, Y. Y. Zhang, Y. S. Liu, and C. M. Li, *Biosens. Bioelectron.*, 2015, 69, 307.

13. L. Dumitrascu, N. Stănciuc, I. Aprodu, M. A. Ciuciu, P. Alexe, and C. E. Bahrim, *J. Food Sci. Technol.*, 2015, 52, 6290.

14. V. Román-Pizarro, J. M. Fernández-Romero, and A. Gómez-Hents, *J. Agric. Food Chem.*, 2014, 62, 1819.

15. J. J. Deng, P. Yu, Y. X. Wang, and L. Q. Mao, *Anal. Chem.*, 2015, 87, 3080.

16. H. Wei, C. G. Chen, B. Y. Han, and E. K. Wang, *Anal. Chem.*, 2008, 80, 7051.

17. T. Hasegawa, M. Sugita, L. Takatani, H. Matsusara, T. Umemura, and H. Haraguchi, *Bull. Chem. Soc. Jpn.*, 2006, 79, 1211.

18. S. Goggins, C. Naz, B. J. Marsh, and C. G. Frost, *Chem. Commun.*, 2015, 51, 561.

19. Y. Kanno, K. Ino, K. Y. Inoue, A. Suda, R. Kunikata, M. Matsudaira, H. Shiku, and T. Matsue, *Anal. Sci.*, 2015, 31, 715.

20. J. S. Blum, R. H. Li, A. G. Mikos, and M. A. Barry, *J. Cell Biochem.*, 2001, 80, 532.

21. A. Ingram, B. D. Moore, and D. Graham, *Bioorg. Med. Chem. Lett.*, 2009, 19, 1569.

22. Z. S. Qian, L. J. Chai, C. Tang, Y. Y. Huang, J. R. Chen, and H. Feng, *Anal. Chem.*, 2015, 87, 2966.

23. Y. H. Zhu, G. F. Wang, H. Jiang, L. Chen, and X. J. Zhang, *Chem. Commun.*, 2015, 51, 948.

24. X. Y. Xu, R. Ray, Y. L. Gu, H. J. Ploehn, L. Gearheart, K. Raker, and W. A. Scrivens, *J. Am. Chem. Soc.*, 2004, 126, 12736.

25. X. T. Zheng, A. Ananthanarayana, K. Q. Luo, and P. Chen, *Small*, 2015, 11, 1620.

26. S. Y. Lim, W. Shen, and Z. Q. Gao, *Chem. Soc. Rev.*, 2015, 44, 362.

27. Y. Q. Dong, R. X. Wang, G. L. Li, C. Q. Chen, Y. W. Chi, and G. N. Chen, *Anal. Chem.*, 2012, 84, 6220.

28. Q. Liu, B. D. Guo, Z. Y. Yao, B. H. Zhang, and J. R. Gong, *Nano Lett.*, 2013, 13, 2436.

29. S. J. Zhu, J. H. Zhang, C. Y. Qiao, S. J. Tang, Y. F. Li, W. J. Yuan, B. Li, L. Tian, F. Liu, R. Hu, H. N. Gao, H. T. Wei, H. Zhang, H. C. Sun, and B. Yang, *Chem. Commun.*, 2011, 47, 6858.
30. W. B Shi, Q. L. Wang, Y. J. Long, Z. L. Cheng, S. H. Chen, 
   H. Z. Zheng, and Y. M. Huang, Chem. Commun., 2011, 47, 
   6695.
31. L. Zhou, Y. H. Lin, Z. Z. Huang, J. H. Ren, and X. G. Qu, 
   Chem. Commun., 2012, 48, 1147.
32. J. H. Shen, Y. H. Zhu, C. Chen, X. L. Yang, and C. Z. Li, 
   Chem. Commun., 2011, 47, 2580.
33. Y. P. Sun, B. Zhou, Y. Lin, W. Wang, K. A. S. Fernando, P. 
   Pathak, M. J. Meziani, B. A. Harruff, X. Wang, H. F. Wang, 
   P. G. Luo, H. Yang, M. E. Kose, B. Chen, L. M. Veca, and 
   S. Y. Xie, J. Am. Chem. Soc., 2006, 128, 7756.
34. H. T. Li, X. D. He, Z. H. Kang, H. Huang, Y. Liu, J. L. Liu, 
   S. Y. Lian, C. H. A. Tsang, X. B. Yang, and S. T. Lee, 
   Angew. Chem., Int. Ed., 2010, 49, 4430.
35. H. Zhu, X. L. Wang, Y. L. Li, Z. J. Wang, F. Yang, and X. 
   R. Yang, Chem. Commun., 2009, 103, 5118.
36. M. Howarth, K. Takao, Y. Hayashi, and A. Y. Ting, Proc. 
   Natl. Acad. Sci. U. S. A., 2005, 102, 7583.
37. S. J. Zhu, Q. N. Meng, L. Wang, Z. H. Zhang, Y. B. Song, 
   H. Jin, K. Zhang, H. C. Sun, H. Y. Wang, and B. Yang, 
   Angew. Chem., Int. Ed., 2013, 52, 3953.
38. Y. X. Li, H. Huang, Y. H. Ma, and J. Tong, Sens. Actuators, 
   B, 2014, 205, 227.
39. J. P. Yuan, W. W. Guo, and E. K. Wang, Biosens. 
   Bioelectron., 2008, 23, 1567.
40. L. Dong, Q. Q. Miao, Z. J. Hai, Y. Yuan, and G. L. Liang, 
   Anal. Chem., 2015, 87, 6475.
41. Z. G. Song, R. T. K. Kwork, E. G. Zhao, Z. K. He, Y. N. 
   Hong, J. W. Y. Lam, B. Liu, and B. Z. Tang, Appl. Mater. 
   Interfaces, 2014, 6, 17245.
42. L. F. Zhang, T. Hou, H. Y. Li, and F. Li, Analyst, 2015, 140, 
   4030.
43. J. Peng, X. X. Han, Q. C. Zhang, H. Q. Yao, and Z. N. Gao, 
   Anal. Chim. Acta, 2015, 878, 87.