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ARTICLE TYPE

Amplified fluorescence detection of T4 polynucleotide kinase activity based on coupled Exonuclease III reaction and graphene oxide platform

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A novel amplified fluorescence graphene oxide (GO) sensing platform for sensitive detection of T4 polynucleotide kinase (PNK) activity and inhibition was developed based on the Exonuclease III (ExoIII) reaction. The efficient digestion capacity of ExoIII and the super quenching ability of GO both contribute to the detection sensitivity.

Highly sensitive and selective detection of enzyme activities plays important roles in many areas, such as biological/biochemical research, new drug screening, clinical diagnosis, and environmental monitoring. [1-3] Polynucleotide kinase (PNK) is one of the most important end-processing enzymes, and has been extensively studied in recent years. [4,5] PNK can catalyze the phosphorylation of the 5′-hydroxyl terminus via transferring the γ-phosphate of ATP to nucleic acid molecules. The phosphorylation process plays a critical role in numerous nucleic acid-related bio-events, such as DNA recombination, DNA replication, and DNA repairing during strand interruption. [6-9] Accordingly, PNK has been also widely used as an efficient tool in the field of molecular biology research, [10,11] and the development of assays for the information of activity profile of PNK is therefore of fundamental importance.

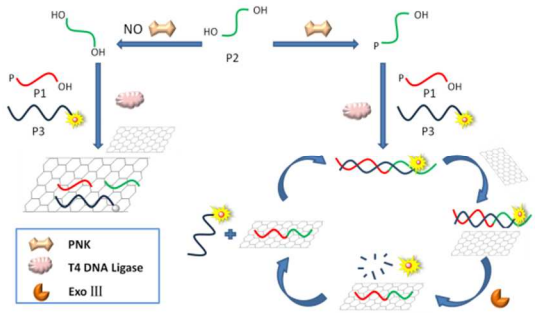
To date, various bioassays to achieve the reliable detection of PNK activity have been developed. The traditional assay methods for PNK detection are mainly based on radioisotope ^{32}P-labeling, polyacrylamide gel electrophoresis and autoradiography. [12-16] However, these methods have the shortcomings of time-consuming, high cost and radio-labelling of substrates which are harmful to human health.

To overcome these drawbacks, convenient and high sensitive PNK assays have been reported in recent years, including fluorescent assays, [9,17-26] colorimetric assays, [27]nanochannel biosensors, [28] electrochemical methods, [29,30] and bioluminescent sensor. [31]Among these methods, fluorescence-based strategies attracted much attention due to their high sensitivity and convenient manipulation. For example, Li and co-workers recently developed a novel strategy for PNK activity and inhibition assay by coupling the exonuclease enzyme reaction and bimolecular beacon based signal amplification, which provides a sensitive, facile and universal tool for the research of DNA phosphorylation related process and the detection of many other nucleic acid enzymes. [24]Although all these fluorophore-labelled methods have been proved to have more advantages than traditional ones for monitoring PNK activity, there are still some problems need to be resolved. First, design of specific dye-labelled DNA molecular beacon or sequences with restriction sites is complicate and time-consuming.[18,25] Second, sometimes design is simple but it's the lack of an amplification mechanism that leads to low sensitivity.[22,26] Thus, it is still highly desirable to develop more convenient and sensitive methods to monitor the PNK activity.

Exonuclease III (ExoIII) is a double-strand-specific exodeoxyribonuclease which can catalyze the stepwise removal of mononucleotides from 3′ to 5′ and has very low activity on single-stranded DNA or 3′-overhang termini of double-stranded DNA. [32,33] In contrast to the sequence-specific nicking endonuclease, the enzymatic reaction of ExoIII does not require the specific recognition sequence. Therefore, ExoIII can directly digest any double-stranded DNA at the blunt or recessed 3' end, which is anobvious advantage in the development of universal signal amplification strategies for various bioassays. [34,35]

Graphene oxide (GO), is a two-dimensional (2D) carbon nanomaterial with only one atom thickness, which was reported to be a fluorescence superquencher with the long-range nanoscale energy transfer property and has been successfully employed to develop fluorescent sensing systems for various targets. [36] Herein, we describe a simple but effective GO sensing platform, using T4 PNK as a model analyte, for nucleotide kinase activity and inhibition analysis based on ExoIII-aided amplification in the presence of ligase as well as the FRET between dye labelled DNA and GO. To the best of our knowledge, this is the first time to realize fluorescence signal amplification by ExoIII-aided enzymatic reaction in the presence of ligase as well as the FRET between dye labelled DNA and GO for the detection of PNK activity and inhibition.

Scheme 1 illustrates the general principle of the current sensing platform for amplified fluorescence detection of T4 PNK activity. In the absence of T4 PNK, the 5′-hydroxyl terminus of P2 cannot be phosphorylated, and the subsequent ligation process mediated by T4 DNA ligase will not proceed. Due to the low melting temperature (Tm) values (estimated to be less than 25℃) for the hybrids of P1 and P2 with P3, it is very hard to form the nicked DNA duplex at the reaction temperature of 37℃.[37] Therefore, the fluorescence of free P3 was quenched by GO, resulting in low fluorescence intensity. However, in the presence of T4 PNK, the phosphorylation process of P2 can be catalyzed and triggered the subsequent ligation process to form the stable dsDNA, as well as form the 3′-blunt termini recognition sequence for ExoIII. Due



Scheme 1 Schematic illustrating the principle mechanism of the ExoIII-aided amplified fluorescence assay for T4 PNK.

to the different interaction intensity of ssDNA and dsDNA with GO, dye-labelled dsDNA exhibits strong fluorescence. Then, ExoIII can catalyze the stepwise removal of mononucleotides from the 3′-blunt terminus to 5′ of signal probe P3 in the DNA duplex, resulting in the release of the ligated ssDNA and the fluorophore. The released ligated ssDNA can then hybridize with another signal probe P3 and trigger the second cycle of digestion. Therefore, the ExoIII induces multiple enzymatic recycling reaction of dsDNA resulting in the further improvement of fluorescence signal, providing a high sensitivity for T4 PNK.

To estimate the optimum temperature of the ligation reaction, the experiment about the effect of temperature on the fluorescent response of the probe was measured. The result indicated that there was no significant difference between 25℃ and 37℃ (Fig. S1). To estimate the signal amplification function of the proposed sensing system for screening T4 PNK activity, the target-triggered fluorescence enhancement was measured in the presence and absence of ExoIII, respectively. As shown in Fig. 1, the intensity without ExoIII was very low for the background fluorescence (curve e), and there was a negligible fluorescence enhancement in the presence of ExoIII (curve d), which was attributed to the unstable duplex of the short P1 and P2 with P3 at the reaction temperature of 37 ℃. However, in the presence of 0.2 U mL^{-1} T4 PNK, a 785 ± 18% increase in the fluorescence signal was readily achieved by introducing ExoIII signal amplification technology (curve a). In contrast, in the absence of ExoIII, only a 318 ± 7% fluorescence signal enhancement was observed under the same condition (curve b). In the same time, in order to prove T4 DNA Ligase has no effect on the sensing system for screening T4 PNK activity, the target-triggered fluorescence enhancement was measured in the presence and absence of T4 DNA Ligase. Both the fluorescence signal

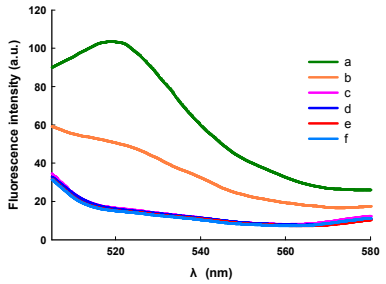


Fig. 1 Fluorescence responses of the sensing system to 0.2 U mL^{-1} target T4 PNK in the presence (a), absence (b) of 0.1 U μL^{-1} of ExoIII, and absence of 1 U μL^{-1} T4 DNA Ligase (c) with corresponding backgrounds (d, e and f), respectively.

and background fluorescence in the absent of T4 DNA Ligase were very low (curve c and f). Therefore, the sensing resultsdemonstrated that the proposed ExoIII-aided signal amplification sensing system could be applied in the amplifying detection of T4PNK activity.

Fluorescence emission spectra were used to investigate the fluorescence quenching of dye resulting from the interaction between FAM- labelled P3 and GO. As shown in Fig. S2, the fluorescence intensity gradually decreased with the increase of GO concentration. Up to 95% fluorescence emission was quenched upon the addition of 20 μg mL^{-1} of GO within 5 min. The result is attributed to the strong adsorption of the ssDNA on the GO surface and the super fluorescence quenching ability of GO originated from the effective FRET between dye and GO. Therefore, 20 μg mL^{-1} of GO was selected as the quencher for the further T4 PNK assay.

In order to achieve the best sensing performance, the concentrations of T4 DNA ligase and ExoIII were optimized, respectively. Firstly, we investigated the effect of T4 DNA ligase concentration on the performance of the sensing system, the experiment was performed by setting the T4 PNK and ExoIII concentrations at 0.2 U mL^{-1} and 0.1 U μL^{-1}, respectively. The result was shown in Fig. S3(A), as the T4 DNA ligase concentration increased, the fluorescence response of signal-to-background ratio (SBR) at 518 nm increased rapidly and reached a maximum at the T4 DNA ligase concentration of 1 U μL^{-1}. Therefore, 1 U μL^{-1} was chosen for the following experiment. The effect of ExoIII concentration was also investigated, and the result indicated that when the concentration of ExoIII reached 0.1 U μL^{-1}, the fluorescence performance of the sensing system reached the maximum SBR (Fig. S3(B)). Therefore, 0.1 U μL^{-1} of ExoIII was selected as the optimized concentration for the sensing system.

To evaluate the sensitivity of the proposed method, we measured the T4 PNK activity at various concentrations under the optimized conditions. As shown in Fig. 2(A), the fluorescence increased with the increase of T4 PNK concentration and reached a plateau when the T4 PNK concentration increased to 5 U mL^{-1}. Fig. 2(B) illustrates the relationship between the T4 PNK concentration and the fluorescence intensity at 518 nm. In the concentration range from 0 to 0.2 U mL^{-1}, the fluorescence intensity exhibits a linear correlation to T4 PNK concentration. The detection limit was estimated to be 0.003 U mL^{-1} in terms of the rule of three times standard deviation over the blank response, which is superior or comparable to that of the previously reported

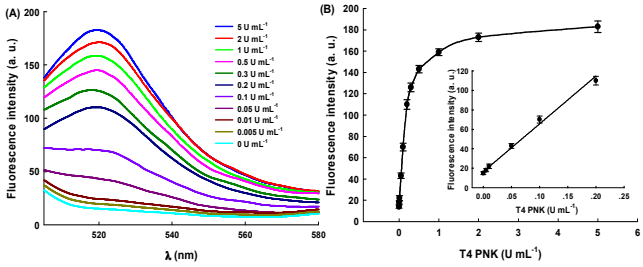


Fig. 2(A) Fluorescence spectra responses of the sensing system in the presence of different concentrations of T4 PNK. (B) The relationship of the fluorescence enhancement with the T4 PNK concentration. Inset: The responses of the sensing system to T4 PNK at low concentrations. Error bars were estimated from three replicate measurements.

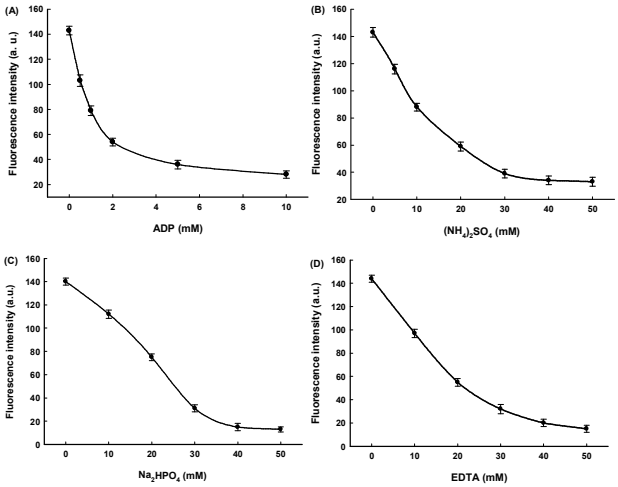


Fig. 3(A) The inhibitory effect of ADP on T4 PNK activity. (B) The inhibitory effect of $(NH_4)_2SO_4$ on T4 PNK activity. (C) The inhibitory effect of Na_2HPO_4 on T4 PNK activity. (D) The inhibitory effect of EDTA on T4 PNK activity. The concentration of T4 PNK was 0.5 U mL^{-1}. Error bars were estimated from three replicate measurements.

methods (Table S1). [9,21,24] The results demonstrated that the proposed sensing system can be successfully applied to detect the T4 PNK activity.

Additionally, the inhibition effect on PNK was also investigated by using adenosine diphosphate (ADP), ammonium sulfate ($(NH_4)_2SO_4$), disodium phosphate (Na_2HPO_4) and ethylenediaminetetraacetic acid sodium (EDTA) as four model inhibitors, which have been confirmed that they could inhibit the PNK activity by changing the affinity of the enzyme for its substrates. To eliminate the possible disturbances on the ligation reaction and enzymatic reaction of ExoIII, the effects of the four used inhibitors on the two reactions were also examined by using P4, P5 and P3, respectively. The corresponding control experiments suggested that their disturbances were almost negligible when concentrations of ADP, $(NH_4)_2SO_4$, Na_2HPO_4 and EDTA, reached 10 mM, 30 mM, 40 mM and 40 mM respectively, which ensured accurate assay of T4 PNK inhibition (data not shown). As shown in Fig. 3(A), the fluorescence intensity of samples dramatically decreased with the increasing concentration of ADP in the phosphorylation reaction, and the 1 mM ADP caused a 50% fluorescence decrease. The inhibition effect is probably due to that the phosphorylation reaction tended to run in reverse in the presence of high concentrations of ADP and 5′-phosphate DNA simultaneously. In addition, $(NH_4)_2SO_4$, EDTA and Na_2HPO_4 was further used to evaluate the inhibition effect on T4 PNK activity, and the result was depicted in Fig. 3(B)(C)(D). As expected, the increasing concentration of $(NH_4)_2SO_4$, EDTA and Na_2HPO_4 resulted in the decrease of fluorescence intensity, and a 50% fluorescence intensity decrease could be observed when the inhibiting concentration of $(NH_4)_2SO_4$, EDTA and Na_2HPO_4 increased to about 13 mM, 15 mM and 20 mM which is consistent with the reported results. [9,17] These results indicate that the developed sensing system could be extended to evaluate of the effects of kinase inhibitors.

To investigate the selectivity of the fluorometric T4 PNK assay, a number of proteins including methylase (Dam), alkaline phosphatise (ALP), bovine serum albumin (BSA) and human serum albumin (HSA) were tested. The concentration of each enzyme was kept at 10 μM or 10 U mL^{-1}. As shown in Fig. 4, none of these proteins could induce the distinct increase in fluorescence except T4 PNK. The result further confirmed that

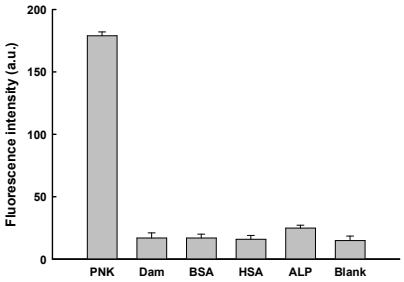


Fig. 4 The selectivity of the biosensor for T4 PNK compared with other enzyme. The concentration of other enzyme was 10 μM, Dam was 10 U mL^{-1} and the T4 PNK was 5 U mL^{-1}.

the proposed biosensor exhibited excellent selectivity towards T4 PNK.

In order to investigate the possibility of the sensing platform in complex biological samples, A549 cell extracts were added in the buffer to simulate the intracellular environment for the detection of T4 PNK activity. As shown in Fig. S4, in the diluted cell extracts, the fluorescence intensity increased gradually with the addition of T4 PNK from 0.02 to 5 U mL^{-1}. These results demonstrate that the proposed sensing system works well in complex mixtures with other possible coexisting interfering species, indicating that this method holds a potential application for real sample analysis.

In summary, we have developed a novel amplified fluorescence GO sensing system for sensitive detection of T4 PNK activity and inhibition based on ExoIII reaction. Contribute to the efficient digestion capacity of ExoIII and the super quenching ability of GO, the proposed method provides a linear range from 0 to 0.2 U mL^{-1} and a low detection limit of 0.003 U mL^{-1}. In addition, this strategy is simpler than the nicking endonucleases-based molecular beacon probe due to that the ExoIII does not require specific enzymatic recognition sequence. Moreover, the proposed method can be used to screen the inhibitors of PNK. The proposed method may provide a new platform for monitoring the DNA phosphorylation related process, drug discovery and clinical diagnostics.

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Notes and references

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1 L. E. Edgington, M. Verdoes and M. Bogyo, *Curr. Opin. Chem. Biol.*, 2011, **15**, 798.
2 G. Das, P. Talukdar and S. Matile, *Science*, 2002, **298**, 1600.
3 T. Chauvin, P. Durand and M. Bernier, *Angew. Chem. Int. Ed.*, 2008, **47**, 4370.

4 C. Chappell, L. A. Hanakahi, F. Karimi-Busheri, M. Weinfeld and S. C. West, *EMBO J.*, 2002, **21**, 2827.

5 M. J. Scheiienberg and R. S. Williams, *Proc. Natl. Acad. Sci. USA*, 2011, **108**, 20855.

6 L. K. Wang, C. D. Lima and S. Shuman, *EMBO J.*, 2002, **21**, 3873.

7 C. J. Whitehouse, R. M. Taylor, A. Thistlethwaite, H. Zhang, F. Karimi-Busheri, D. D. Lasko, M. Weinfeld and K. W. Caldecott, *Cell*, 2001, **104**, 107.

8 C. B. Ma and E. S. Yeung, *Anal. Bioanal.Chem.*, 2010, **397**, 2279.

9 Z. W. Tang, K. M. Wang, W. H. Tan, C. B. Ma, J. Li, L. F. Liu, Q. P. Guo and X. X. Meng, *Nucleic Acids Res.*, 2005, **33**, e97.

10 C.Frauendorf, F.Hausch, I.R¨ohl, A.Lichte, S.Vonhoff and S.Klussmann, *Nucleic Acids Res.*, 2003,**31**, e34.

11 D.H.Phillips and V.M.Arlt, *Nat.Protoc.*, 2007,**2**, 2772.

12 C. B.Ma and E. S.Yeung,*Anal. Bioanal. Chem*., 2010, **397**, 2279.

13 C. C.Richards, *Proc. Natl. Acad. Sci. USA,* 1965, **54**, 158.

14 N. K.Bernstein,R.S.Williams, M.L.Rakovszky, D.Cui,R.Green, F.Karimi-Busheri, R. S. Mani, S. Galicia, C. A. Koch, C. E. Cass, D. Durocher, M.Weinfeld and J.N. M. Glover, *Mol. Cell*, 2005, **17**, 657.

15 F.Karimi-Busheri, G.Daly, P.Robins, B.Canas,D. J. C.Pappin, J.Sgouros, G. G.Miller, H.Fakhrai, E. M.Davis, M. M.Le Beau and M.Weinfeld, *J.Biol. Chem*.,1999, **274**, 24187.

16 L. K.Wang and S.Shuman,*J. Biol. Chem.*, 2001, **276**, 26868.

17 C. Song and M. Zhao,*Anal. Chem.*, 2009,**81**, 1383.

18 F. Chen, Y. Zhao, L. Qi and C. Fan,*Biosens.Bioelectron.*,2013, **47**, 218.

19 Y. Huang, J. Chen, M. Shi, S, Zhao, Z.F. Chen and H. Liang,*J. Mater. Chem. B.*, 2013, **1**, 2018.

20 H. Jiao, B. Wang, J. Chen, D. Liao, W. Li and C. Yu,*Chem.Commun.*, 2012,**48**,7862.

21 L. Lin, Y. Liu, X. Zhao and J. Li, *Anal. Chem.*, 2011, **83**, 8396.

22 L. Zhang, J. Zhao, H. Zhang, J. H. Jiang and R. Q. Yu,*Biosens.Bioelectron.*, 2013,**44**, 6.

23 W. Wu, H. Hu, F. Li, L. Wang, J.Gao, J. Lu and C. Fan, *Chem.Commun.*, 2011, **47**, 1201.

24 T.Hou, X. Z. Wang, X. J. Liu, T. T. Lu, S. F. Liu and F. Li,*Anal. Chem.*, 2014,**86**, 884.

25 T.Hou, X. Z. Wang, T. T. Lu, X. J. Liu and F. Li,*Anal.Bioanal. Chem.*, 2014,**406**, 2943.

26 J.Ge, L. J. Tang, Q. Xi, X. P. Li, R. Q. Yu,J. H. Jiang and X. Chu, *Nanoscale*, 2014,**6**, 6866.

27 C. Jang, C. Yan, J. Jiang and R. Yu,*Anal.Chim.Acta.*, 2013,**766**, 88.

28 L. Lin, Y. Liu, J. Yan, X. Wang and J. Li, *Anal. Chem.*, 2013, **85**, 334.

29 G. Wang, X. He, G.Xu, L. Chen, Y. Zhu, X. Zhang and L. Wang,*Biosens.Bioelectron.*, 2013,**43**, 125.

30 Y. Wang, X. He, K. Wang, X. Ni, J. Su and Z. Chen, *Biosens.Bioelectron.*, 2012, **32**, 213.

31 J. Du, Q.Xu, X. Lu,and C. Zhang,*Anal. Chem.*, 2014, **86**, 8481.

32 C.C. Richardson, I.R. Lehman and A.Kornberg, *J.Chem. Biol.*, 1964, **239**, 251.

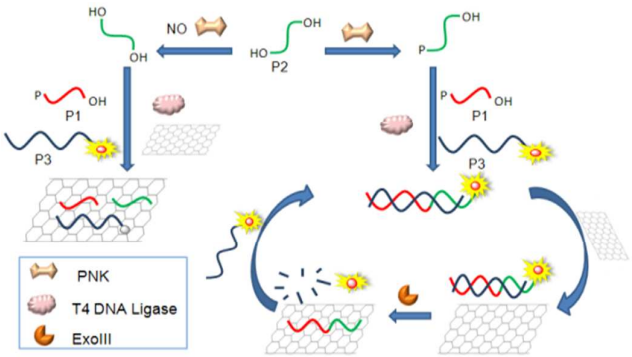
33 J.Wang, T.Li, X.Guo and Z.Lu, *Nucleic Acids Res.*, 2005,**33**, 23.

34 D.Wu, B.C.Yin and B.C.Ye,*Biosens.Bioelectron.*, 2011, **28**, 232.

35 E.Ju, X.Yang, Y.Lin, F.Pu, J.Ren and X.Qu, *Chem.Commun.*, 2012,**48**, 11662.

36 Q. Zheng, H. Wu, N. Wang, R. Yan, Y. H. Ma, W. J. Guang, J. Z. Wang and K. J. Ding,*Curr. Nanosci.*, 2014, **10**, 627.

37 Y. C. Zhang, W. B. Qu, Y. M. Lu, Y. Zhou, C. G. Zhang, *Mil Med Sci*, 2011, **35**, 3.



A novel amplified fluorescence graphene oxide (GO) sensing system for sensitive detection of T4 polynucleotide kinase (PNK) activity and inhibition was developed based on the coupled ligation reaction and Exonuclease III (ExoIII)-aided fluorescence signal amplification.