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ARTICLE TYPE

Enzyme-aided amplification strategy for sensitive detection of DNA utilizing graphene oxide (GO) as a fluorescence quencher

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A facile, sensitive and rapid method has been developed for detection of disease-related DNA based on lambda exonuclease-aided signal amplification by utilizing graphene oxide (GO) as a fluorescence quencher. The fluorescence of carboxyfluorescin–labeled DNA probe (F-DNA) was sharply quenched due to the electron or energy transfer between fluorescence dye and GO. While in the presence of target DNA, the formation of DNA hybrid released F-DNA from the surface of GO, leading to a fluorescence recovery. And then, the fluorescence enhancement was further amplified by using lambda exonuclease to liberate target DNA for cyclic hybridization. Fluorescence polarization and gel electrophoresis further verified the reliability of principle. Disease-related DNA can be sensitively detected based on the enzyme-aided amplification strategy. More importantly, single-base mismatch DNA can be effectively discriminated from complementary target DNA and random DNA. Therefore, it offered a universal, simple, sensitive and specific method for detection disease-related gene.

Introduction

The ability to sense and detect low concentrations of specific nucleic acid sequences plays a critical role in clinical diagnosis of genetic diseases, forensic analysis and environmental monitoring.[1-3] To date, various approaches have been developed for detection of DNA, including fluorescent, electrochemical and colorimetric methods and so on.[4-9] However, sensitivity is still a challenge for early diagnosis of genetic diseases since the amount of disease-related genes is extreme low. So, it is of great theoretical and practical importance to develop ultra-sensitive methods for detection of trace disease-related genes. Many DNA amplification techniques have been established, such as polymerase chain reaction (PCR),[10] rolling circle amplification (RCA),[11-13] as well as the ligase chain reaction (LCR).[14,15] Although a superior sensitivity can be achieved, these amplification protocols share the drawbacks of complex handling procedures, easy contamination and high costs.[16-18] Based on this, the application of enzyme-aided amplification has attracted more and more attention for sensitive detection of nucleic acid.[19-~~22~~ 21] For example, Xu et al and co-workers demonstrated a colorimetric DNA detection through the use of nicking endonuclease assisted amplification, which utilizes a combination of particle probes with a linker strand.[19] Zuo and co-workers have developed a Exo III-aided target recycling method for amplified DNA detection by using DNA molecular beacon (MB) as the signaling probe.[20] ~~Cui and co-workers constructed a DNase I amplification method for sensitive detection of miRNA by combining GO as a quencher.[21]~~

Here, a universal method was established for detection of disease-related DNAs based on λ exo-assisted amplification strategy by utilizing GO as a fluorescence quencher. Lambda exonuclease is a highly processive enzyme that acts in the 5′ to 3′ direction, catalyzing the removal of 5′–phosphorylated double stranded DNA. [22,23] So, 5′–phosphorylated single-stranded DNA was designed as a recognition probe with fluorescence dye tagged at 3′-terminus. The fluorescence of F-DNA was significantly quenched because of the strong binding between single-stranded DNA (ssDNA) and GO. However, the fluorescence restored when F-DNA hybridized with target DNA that is human immunodeficiency virus (HIV) gene, which may attribute to the fact that GO hardly interacted with rigid double-stranded DNA (dsDNA).[24,25] To further improve the sensitivity, Lambda exonuclease (λ exo) is introduced to specifically release target DNA from F-DNA/HIV duplex because λ exo selectively digests the 5’ phosphorylated strand of dsDNA. And then, the released target DNA rehybridized with another F-DNA to start cyclic hybridization, resulting in a dramatic increase in fluorescence due to λ exo-aided circular reaction. Therefore, it provided an ultra-sensitive and reliable approach for detection of disease-related genes.

Experimental Section

Chemicals

Graphene oxide (0.5 mg/mL) was purchased from Xianfeng

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nanomaterials Co., Ltd. (Nan-jing, China). Lambda exonuclease (5 units/μL) and its 10× reaction buffer (containing 670 mM glycine-KOH (pH 9.4), 25 mM $MgCl_2$, 0.1% (v/v) Triton X-100) were purchased from New England Biolabs (NEB, U.K.). DNA oligonucleotides used in this work are listed as follows: the carboxyfluorescein (FAM)-labeled DNA probe (5'-PHOS-AGTC AGTGTGGAAAATCTCTAGC-FAM-3', F-DNA), target DNA (5'-GCTAGAGATTTTCCACACTGACT-3', T1), single-base mismatched target (5′-GCTAGAGATTTCCCACACTGACT-3′, T2), Random DNA (5'-AAAAAAAAAAAAAAAAAA AAAAA -3', T3), and non-phosphorylated probe (5'- AGTCAGTGTGGA AAATCTCTAGC-FAM-3', FDNA2) were synthesized by Shanghai Sangon Biotechnology Co. (Shanghai, China). They were all purified by reverse-phase high-performance liquid chromatography (HPLC). The oligonucleotide stock solutions were prepared with Tris-HCl buffer (70 mM Tris-HCl, pH 8.0) and stored at 4℃. Millipore Milli-Q (18.2 MΩ. cm) water was used in all experiments.

Measurements

All fluorescence measurements were performed on Hitachi F-7000 fluorescence spectrophotometer (Kyoto, Japan). The vertical electrophoresis system was purchased from Bio-Rad Laboratories, Inc. The Molecular Imager system was purchased from Shanghai Peiqing science & Technology.Co., Ltd (Shanghai, China).

Fluorescence measurement

First, the fluorescence spectra of F-DNA were recorded on a fluorometer (F-7000, Hitachi) with excitation at 480 nm and an emission range from 500 to 600 nm. Then, GO was added to the above solution and incubated for 10 min before measurement. For the typical DNA assay, appropriate concentrations of target DNA were added into the above system, and the mixture was incubated for 10 min, and then the fluorescence intensity was measured. In the amplified strategy, λ exo was added to the above solution and the mixture was incubated at room temperature for 20 min. Finally, the fluorescence intensity was measured.

Fluorescence polarization measurement

Fluorescence anisotropy is sensitive to the rotational change of fluorescent molecules. The fluorescence polarization (P) can be expressed by the following correction factor:

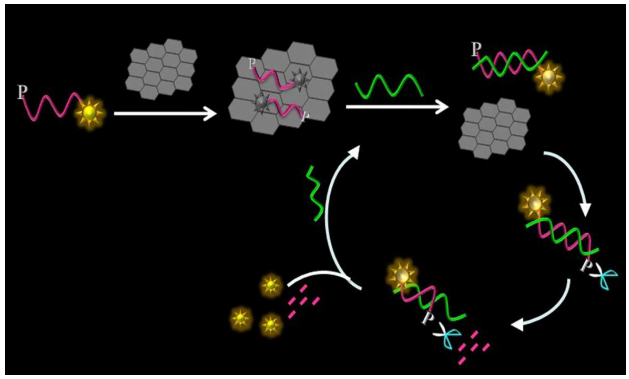
$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensity parallel and perpendicular to the excitation plane, respectively.

Gel electrophoresis analysis

A 12.5% polyacrylamide gel was prepared using 1×TBE (100 mM Tris-HCl, 83 mM boric acid, 1 mM EDTA, pH 8.0). 20 μL of the reaction product along with 2 μL loading buffer was loaded onto a 20% polyacrylamide gel and then run in 1×TBE buffer at 100 V for 30 min. The gels were stained with silver for 30 min. The visualization and photography were performed using Molecular Imager with Gel Doc system.

Results and discussion



Scheme1 Schematic illustration of the enzyme-aided amplification strategy for sensitive detection of DNA

Sensing mechanism

Herein, a λ exo-aided amplification method has been proposed to detect disease-related DNA by utilizing GO as a fluorescence quencher. As illustrated in Scheme 1, the ssDNA probe which was modified with a FAM at its 3′- terminus and a phosphate group at its 5′- terminus can be adsorbed onto the surface of GO because of the π-π stacking interaction. As a result, the fluorescence intensity of F-DNA was effectively quenched by GO due to the electron or energy transfer GO and F-DNA[26,27]. However, the quenching efficiency reduced when F-DNA hybridized with complementary target DNA, which mainly attributed to the weaker affinity of rigid dsDNA with GO. So the fluorescence intensity of F-DNA increased due to the formation of DNA duplexes. Furthermore, a significant increase in fluorescence was obtained when λ exo was introduced to specifically digest phosphorylated F-DNA for triggering cyclic hybridization. Therefore, disease-related gene can be sensitively detected by enzyme-assisted amplification.

The fluorescence spectra of FDNA under different condition were measured to verify enzyme-aided amplification strategy. It is clear from curve a to b in Fig. 1A that the fluorescence of FDNA was significantly quenched by GO. Upon the addition of target DNA, the fluorescence of the FDNA recovered (curve c). While a tremendous increase in fluorescence was observed after incubation with λ exo (curve d). When the λ exo was inactivated, from the curve e, it can be seen that the fluorescence intensity is almost identical to that in the presence of HIV (curve c), indicating that the fluorescence amplification is due to the specifically digest FDNA by λ exo and release target DNA to trigger cyclic hybridization. Thus, trace target DNA can hybridize with a large number of FDNA and leads to a significant amplification of the fluorescence signal. By monitoring the increase in fluorescence intensity, target DNA can be ultra-sensitively detected.

To further verify the reliability of this protocol, more control experiments have been performed. First, the influence of λ exo on the adsorption of ssDNA to the GO surface should be investigated. It can be seen that the fluorescence intensity almost never change form curve b to curve c (Fig. 1B), indicating that λ exo would not affect the adsorption of ssDNA to the GO surface. Then, a random DNA was chosen as a negative control. It is clear from the curve a to b in Fig. S 1A that the fluorescence of probe DNA has been quenched by GO. However, the fluorescence of

the FDNA did not recover in the presence of random DNA (curve c). Meanwhile, the fluorescence hardly changed (curve d) after

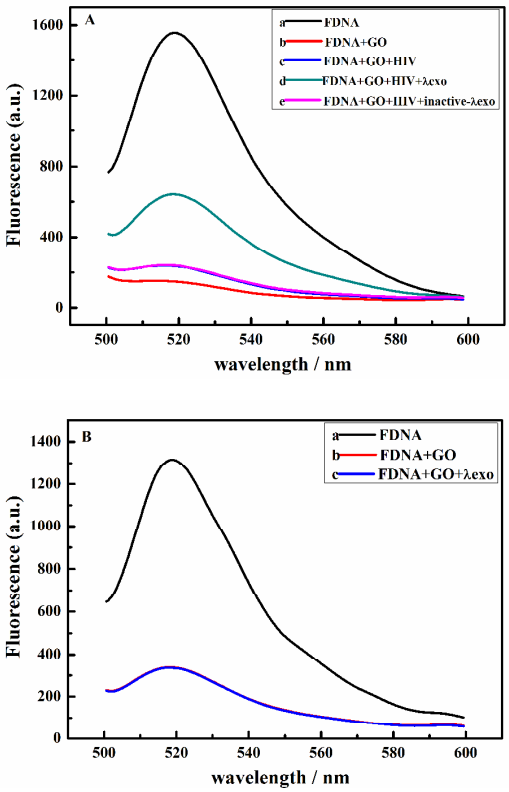


Fig.1 Fluorescence emission spectra of FDNA at different conditions: (A) (a) 20 nM FDNA; (b) FDNA+ GO; (c) FDNA + GO + 100 nM T1; (d) FDNA + GO + 100 nM T1 + 0.005U μL^{-1} λ exo; (e) FDNA + GO + 100 nM T1 +inactive- 0.005U μL^{-1} λ exo. (B) (a) 20 nM FDNA; (b) FDNA+ GO; (c) FDNA + GO +0.005U μL^{-1} λ exo

incubation with λ exo. Finally, we choose a non-phosphorylated FDNA2 probe as a control. As shown in Fig. S 1B, the fluorescence intensity of FDNA2 enhanced in some degree as a result of the formation of complement DNA duplexes. However, the fluorescence intensity hardly changed when the mixture incubated with λ exo. These results demonstrate that the molecular recognition is specific and the amplification strategy is feasible.

Verification of amplification strategy

Fluorescence polarization (FP) is a universal technique that is widely introduced for the analysis of molecular interactions in biological/chemical ~~systems~~[27] systems[28,~~28~~29]. So fluorescence polarization under different circumstances was recorded to verify the principle. As shown in Fig. 2, the FP value of FDNA alone is low. As we all known, FP of a fluorophore is highly related to its rotational relaxation time which is relevant to the viscosity of solvent, temperature, molecular volume or fluorescence ~~lifetime~~[29] lifetime[30,~~30~~31] ~~its molecular volume or molecular weight.~~[28] So, FP of FDNA is low when free FDNA rotated fast in solution. However, a significant increase in FP value was observed upon addition of GO, which may attribute to the ~~non-specific adsorption of FDNA by GO that enlarged the molecular volume and slowed down the rotation. In turn, an increase in FP revealed that~~ FDNA had been adsorbed onto the surface of GO. However, a decrease in FP was observed when FDNA hybridized with complementary target DNA, indicating that the rigid double-stranded FDNA/cDNA released from GO surface and speeded up DNA rotation. Finally, a further decrease in FP value was obtained when λ exo was introduced to trigger cyclic hybridization. That is, the specific digestion of phosphorylated FDNA continuously liberates free dye which can freely rotate and reduce fluorescence

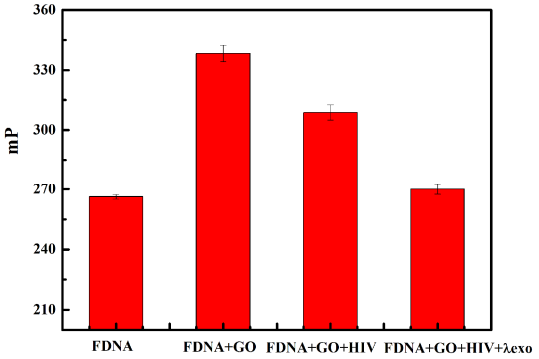


Fig.2 Change of fluorescence polarization of FDNA at different conditions

polarization. All changes in FP under different stages demonstrated that the λ exo-aid amplification really worked as we designed.

To further confirm λ exo-induced circle amplification, we utilized gel electrophoresis to monitor digestion reaction. It can be seen from lane 4 in Fig. 3 that dsDNA formed when FDNA incubated with complementary target DNA (T1). So the new band of dsDNA revealed a slower migration of dsDNA than ssDNA (lane 2) because molecular weight of dsDNA is larger than that of ssDNA. Two bands appeared when dsDNA incubated with lambda exonuclease, including a shallow dsDNA band and a new band corresponding to the released target DNA. It is mainly ascribed to the specific digestion of FDNA by λ exo which can processive phosphorylated DNA of dsDNA in 5′ to 3′ direction. Consequently, the new band in lane 5 revealed that dsDNA has been effectively digested by λ exo. On the contrary, no new band appeared in lane 6 when λ exo was replaced by heat-inactivated λ exo, which demonstrated once again enzymatic cleavage really occurred. Results of FP and gel electrophoresis indicated that λ exo-aided amplification is feasible and reasonable.

Optimization of assay conditions

To achieve a better performance, factors that influence efficiency of amplification should be optimized. First, the effect of GO

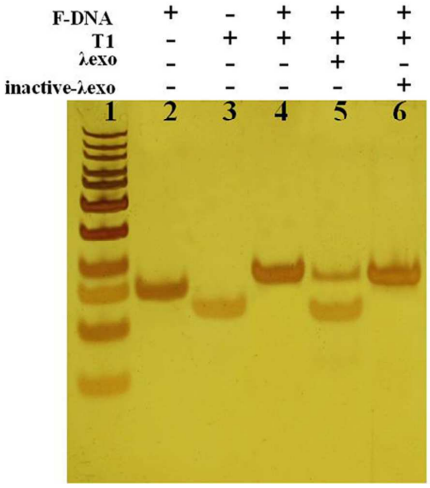


Fig.3 Gel electrophoretic analysis of the products by the λ exo assisted amplification method. Lanes 1–6: (1) Marker; (2) FDNA; (3) T1; (4) F-DNA +T1; (5) FDNA+T1+λ exo ; (6) FDNA+T1+ inactive-λ exo.

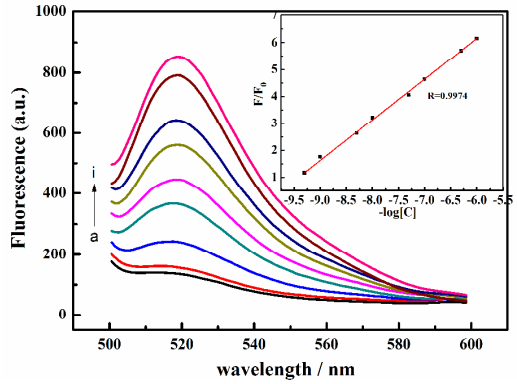


Fig.4 Fluorescence emission spectra of FDNA in the presence of T1 and. λ exo From curve a to i the concentration of T1 is 0, 0.5, 1, 5, 10, 50, 100, 500 and 1000 nM. (Inset) Linear correlation of the fluorescence change with logarithmic concentrations of T1. F_0 and F are the fluorescence intensity of FDNA/GO/λ exo mixture in the absence and presence of target DNA, respectively.

concentration was studied by ranging GO concentrations from 30 to 50 μg mL^{-1}. Fig. S2 depicted that quenching efficiency increased as the GO concentration increased in the range of 30 to 40 μg mL^{-1}. The higher GO concentration is beneficial to reduce background fluorescence. However, the excessive GO is adverse to the fluorescence restoration. So, the optimal concentration of GO was 40 μg mL^{-1}. Then, the influence of λ exo concentration was investigated. As shown in Fig. S3, fluorescence amplification enhanced as the λ exo concentration increased from 0.001 to 0.005 U μL^{-1} and then reached to a plateau. That is, higher λ exo concentration is beneficial to improve amplification efficiency Hence, 0.005U μL^{-1} λ exo was used throughout the experiment. Finally, the influence of reaction time was considered. The changes in fluorescence intensity over time were recorded in the presence of 0.005U μL^{-1} λ exo. It has been found from Fig. S4 fluorescence intensity enhanced as reaction time prolonged and reached its plateau around 20 min. Therefore, enzymatic reaction time was set as 20 minutes.

Specificity and sensitivity for DNA detection

The ultrasensitive detection of disease-related genes is of great theoretical and practical importance in early diagnosis of genetic diseases because the amount of disease-related genes is extreme

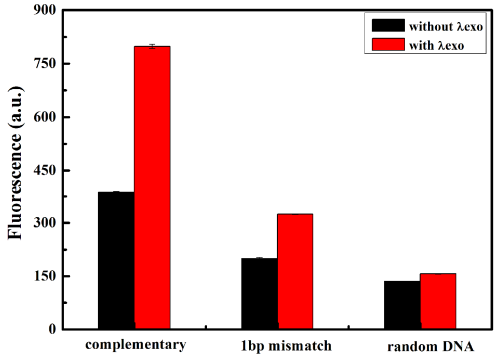


Fig. 5 Investigation of the selectivity of the proposed method. Experiments were performed in 20 nM FDNA in with 100 mM complementary DNA (T1), 1 bp mismatched DNA (T2) and random DNA (T3), in the absence and presence of 0.005 unit λ exo.

low. To evaluate the sensitivity of this assay, the relationship between DNA concentrations and fluorescence response has been studied. It can be observed from Fig.4 that fluorescence intensity continually enhanced when target concentration increased from 0.5 nM to 1000 nM. As shown in the inset of Fig. 4, the proposed method exhibited a good linear response (R= 0.9974) when target DNA concentration ranged from 0.5 to 1000 nM and the detection limit is 0.2 nM (three times the standard deviation of the blank solution). To verify λ exo-aided amplification, the sensitivity of GO-based fluorescence assay without λ exo was futher investigated. It is clear from Fig. S5 that fluorescence intensity linearly related to the logarithm of DNA concentration in the range of 5 nM to 100 nM, and the detection limit is 3 nM. Therefore, sensitivity improved by λ exo-aided amplification strategy.

Selectivity is a key issue should be considered for detection DNA in biological media. So the fluorescence responses of different DNAs were investigated, including complementary DNA (T1), single-base mismatched DNA (T2), and random DNA (T3). As shown in Fig. 5, a significant fluorescence enhancement was obtained in the presence of complementary DNA. However, T2 induced only about 30.35% enhancement of fluorescence compared with T1 at the same concentration. Moreover, the fluorescence intensity hardly changed in the presence of random DNA. The difference in fluorescence response is attributed to the fact that T1 can effectively hybridize with F-DNA to form dsDNA and release from the GO surface, whereas T2 and T3 have much lower hybridization efficiency. So it is a highly selective method that can discriminate perfectly matched DNA from mismatched targets.

Conclusions

In summary, a facile, sensitive and rapid method for DNA detection had been developed by combining GO as a fluorescence quencher with λ exo-triggered cyclic hybridization. Compared with previously reported approaches, it offered several unique advantages for detection of disease-related gene. Firstly, DNA probe is just single-labeled by using GO as a fluorescence

quencher, which is beneficial to cost-effective analysis. Secondly，in addition to a fluorescence quencher, GO also to contribute to eliminate the influence of non-specific interaction, which make it possible to discriminate perfectly matched DNA from mismatched targets. Furthermore, it offered a sensitive assay for detection of disease-related gene by introducing λ exo-aided amplification strategy. Therefore, it provides a fast, sensitive and selective strategy for detection of DNA

Acknowledgements

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