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A label-free amplified fluorescence DNA detection based on isothermal circular strand-displacement polymerization reaction and graphene oxide†

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A label-free fluorescent DNA biosensor has been presented based on isothermal circular strand-displacement polymerization reaction (ICSDPR) combined with graphene oxide (GO) binding. The proposed method is simple and cost-effective with a low detection limit of 4 pM, which compares favorably with other GO-based homogenous DNA detection methods.

Nucleic acids are routinely used as biomarkers to help diagnose pathogenic infections and genetic disorders.[1] The development of different methods for rapid, economic, sensitive and selective DNA detection is of great importance for the clinical diagnosis, gene expression analysis and biomedical studies.[2,3] DNA amplification techniques are commonly used in the design of DNA biosensors due to the fact that the disease related nucleic acids are often found only in trace amounts in complex biological extracts. The amplified detection of nucleic acids has been achieved by means of various techniques, including polymerase chain reaction (PCR),[4,5] ligase chain reaction (LCR),[6] catalytic silver deposition or stripping assays,[7–9] and enzyme-linked electrochemical assays.[10,11] However, these methods all have some limitations. For example, PCR is time consuming and even the smallest contamination of DNA would lead to a false positive identification. The use of thermal cycling in both PCR and LCR also limits their applications. Other two methods either involve tedious procedures or require addition of many exogenous reagents. Therefore, the design of new approaches for sensitive DNA detection is in continuous demand.

Recently, isothermal circular strand-displacement polymerization reaction (ICSDPR) has emerged as a promising amplification technique in sensitive DNA detection. It often employs polymerase for strand displacement and is triggered by the target to continuously replicate one strand of the DNA duplex. After many ICSDPR cycles, large amounts of DNA products are produced and as a result the assay sensitivity is significantly improved. To date, several sensing systems have been constructed for sensitive DNA detection based on ICSDPR, such as hairpin fluorescence probe sensing platform,[12] immobilization-free electrochemical DNA sensor,[13] silver deposition amplified electrochemical sensor,[7] and hairpin probe-based amplified electrochemiluminescence.[14] However, those methods either employ fluorophore labels and modified gold nanoparticles as well as magnetic beads or require multiple electrochemical steps limiting their applications. Therefore, developing a new ICSDPR method for simple and economic DNA detection is of considerable interest.

To date, several optical DNA sensors based on graphene oxide (GO) have been reported.[15–17] These sensors all rely on the principle that GO shows a much lower binding affinity with double-stranded DNA (dsDNA) than single-stranded DNA (ssDNA) and GO has a super quenching capacity for a wide range of fluorophores.[18,19] Unfortunately, the sensitivity of these sensors is all limited to the nM range due to the hybridization property of DNA and lack of amplification. In addition, amplified DNA detection has also been proposed based on exonuclease III (Exo III)-induced target recycling.[20,21] But the fluorophore-labeled DNA probes in these methods require the careful control of the Exo III concentration and its digestion time, which might impose limitations on the convenience and simplicity for practical use. As far as we know, there is no amplified DNA detection proposed based on both the ICSDPR and the GO platform.

Herein, we present a label-free amplified fluorescence DNA detection based on the ICSDPR using a GO platform with SYBR Green I (SG) as a readout signal. This method relies on the hybridization of the target DNA with a hairpin probe as the prerequisite for ICSDPR and the preferential binding of GO to stained ssDNA over dsDNA. The use of SG and ICSDPR greatly improves the sensitivity of the DNA detection, and GO also acts as an efficient signal to background (S/B) ratio enhancer in this assay. The proposed method is simple in design and easy to use,

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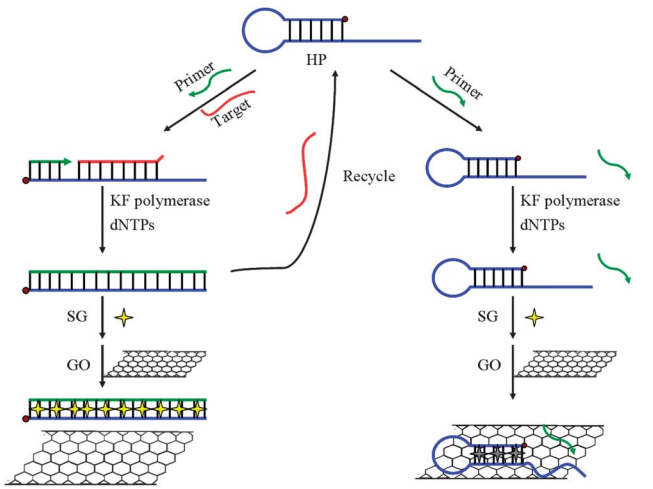
which may provide a promising method of choice for sensitive and convenient DNA detection. Schematic illustration of the amplified fluorescence DNA detection is shown in Scheme 1. As shown in Scheme 1, three DNA oligonucleotides were used in the sensing system including a HP template probe, a short primer and a target DNA. The template probe contains two short single stranded self-complementary sequences which can form a hairpin structure with 3′-phosphoryl end under appropriate hybridizing conditions. The hairpin probe (HP) formed in such a way possesses a stem-loop structure with a stem of 11 bp long and a 5′ protruding 15-nt sequences long, and the loop is complementary to the target DNA. Besides, HP could not induce the self-polymerization reaction due to its 3′-phosphoryl end (alternative design with a single-stranded overhang at the 3′ end may also be used through careful optimization). The primer is designed to be 8-nt sequences long and is complementary to the stem region of the HP at the 3′ end. The target DNA is partially complementary to the whole loop of the HP as well as complementary to 6-nt sequences of the stem with a 3-nt thymine mismatch at its 3′ end. In the absence of the target DNA, the stem-loop conformational HP cannot anneal with the primer to initiate the ICSDPR. After being stained by SG and addition of GO, HP and primer would be absorbed onto the GO surface, leading to a great quenching of the fluorescence. However, in the presence of the target DNA, HP hybridizes with the target DNA and undergoes conformational changes, resulting in the stem separation. Subsequently, the primer anneals with the open stem and induces a polymerization reaction in the presence of KF polymerase and dNTPs. During the polymerization reaction, the target is displaced and released, then the released target DNA will hybridize with another HP and initiate a new cycle of triggering, priming, and displacement. After many ICSDPR cycles, a large quantity of HP–cDNA duplex is formed. Upon treatment with SG and addition of GO, the stained DNA duplex products would not significantly be absorbed by GO and as a result one could obtain a strong fluorescence signal. The concentration of the target DNA is directly proportional to the fluorescence intensity generated. Quantitative detection of the target DNA could be realized by monitoring the fluorescence changes of the sensing system.

A proof of principle experiment was performed to verify the feasibility of the proposed DNA detection. Fig. 1A displays the fluorescence emission spectra under different conditions. As for the reaction mixture containing only the HP and primer, one could obtain a rather small fluorescence signal (curve D, Fig. 1A) due to the strong affinity between the ssDNA and GO. When only target DNA was added, the ICSDPR could not be initiated, and the stained HP and primer would be absorbed by GO, leading to a similar small fluorescence signal (curve C, Fig. 1A). However, in the presence of the target DNA, KF polymerase and dNTPs, HP underwent a conformational change and induced many ICSDPR cycles to produce a large quantity of HP–cDNA duplex. After being stained by SG, a significantly strong fluorescence signal (curve A, Fig. 1A) with the highest fluorescence intensity was obtained. Upon addition of GO, the above formed HP–cDNA duplex would not be greatly absorbed onto the GO surface and as a result one could still observe an obviously strong fluorescence signal (curve B, Fig. 1A). Whereas in the absence of target DNA, HP still kept the stem-loop structure and no ICSDPR was triggered. Selective staining of the stem region of HP with SG, a relatively significant fluorescence signal was observed (curve A_0, Fig. 1A). After addition of GO, the stained HP and primer would be absorbed by GO, resulting in a small fluorescence signal (curve B_0, Fig. 1A).

Fig. 1B shows the S/B ratio under different conditions. As can be seen from Fig. 1B, in the absence of GO, the S/B ratio obtained after ICSDPR cycles was 4.879. Moreover, this ratio increased from 4.879 to 17.83 upon addition of GO, which indicated that GO really acting as an efficient S/B enhancer in this assay. In addition, in the presence of GO, the S/B ratio exhibited a 17-fold amplification after ICSDPR cycles. These data demonstrated that the proposed fluorescent DNA biosensor could be used for sensitive DNA detection through CSDPR.



Scheme 1 Schematic illustrating the principle mechanism involved in the label-free amplified fluorescence DNA detection on the GO platform.

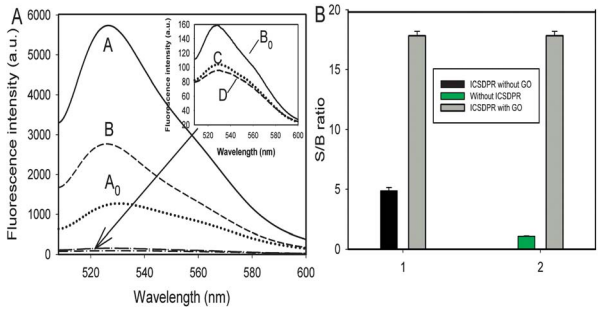


Fig. 1 (A) Fluorescence emission spectra under different conditions. Curve A: HP + primer + target + KF + dNTPs + SG; curve A_0: HP + primer + KF + dNTPs + SG; curve B: HP + primer + target + KF + dNTPs + SG + GO; curve B_0: HP + primer + KF + dNTPs + SG + GO; curve C: HP + primer + target + SG + GO; curve D: HP + primer + SG + GO. (B) Bar graphic of the comparison of S/B ratios under different conditions. Black bar and grey bar represent the S/B ratio obtained after ICSDPR cycles in the absence or presence of GO, respectively; green bar stands for the S/B ratio obtained without ICSDPR in the presence of GO. The S/B ratio is defined as the ratio of fluorescence peak intensity from the target (10 nM) to that from the blank.

In order to achieve the best assay performance, a series of experimental conditions including the amounts of GO and SG, concentrations of KF polymerase and dNTPs as well as the primer length were investigated. Fig. 2 depicts the investigation results in relation to these conditions.

The amount of GO is an important parameter highly related to the fluorescence intensity and S/B ratio. To obtain the best assay performance, we firstly optimized the GO amount. As shown in Fig. 2A, in the presence of the target DNA, the generated fluorescence intensities gradually decreased with the increasing GO amount. A similar trend of fluorescence intensity decrease was also observed in the absence of target DNA. The maximum of S/B ratio was obtained when using 12 $\mu g\ mL^{-1}$ GO, thus this GO amount was chosen to be the optimal and used throughout subsequent experiments. To further optimize the assay conditions, SG of different amounts was used to study the effects of the SG amount on the S/B ratio. As shown in Fig. 2B, the fluorescence signal ratio increased with the increase of the SG amount at first till its maximum then decreased gradually after that, and an optimal signal ratio was achieved using 2 μL SG (100×). Therefore, this SG amount was used throughout subsequent experiments.

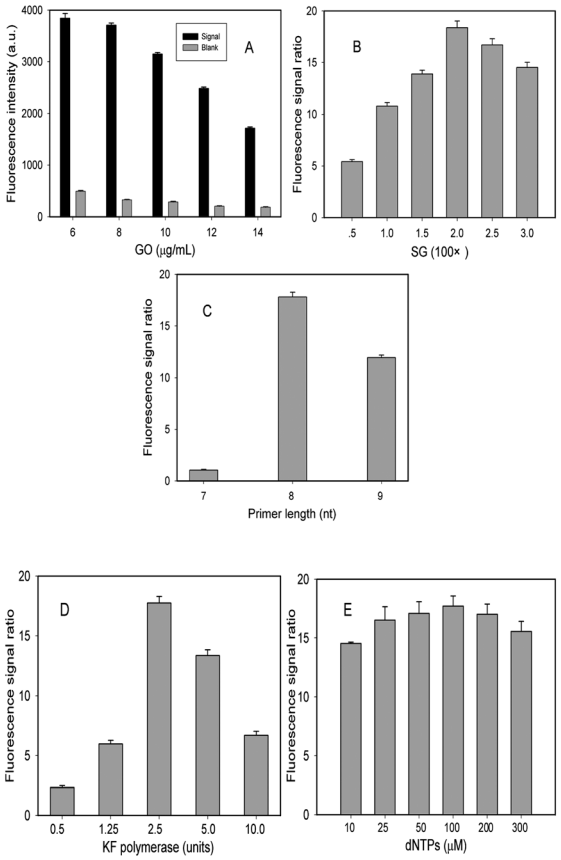


Fig. 2 (A) Dependency of the fluorescence signal ratio on the amount of GO; (B) dependency of the fluorescence signal ratio on the amount of SG; (C) dependency of the fluorescence signal ratio on the primer length; (D) dependency of the fluorescence signal ratio on the concentration of KF polymerase; (E) dependency of the fluorescence signal ratio on the concentration of dNTPs. Fluorescence signal ratio is defined as the ratio of fluorescence peak intensity at 525 nm from 10 nM target DNA to that from the blank.

Fig. 2C depicts the dependency of the fluorescence signal ratio on the primer length. As shown in Fig. 2C, three different lengths of primers containing 7, 8 and 9-nt sequences long were used to study the primer length effects on the assay performance. An optimal fluorescence signal ratio was obtained from the sensor adopting the primer of 8-nt sequences long (primer 1). No further increase in the fluorescence signal ratio was observed when extending the primer length, and a dramatic decrease in the ratio was obtained using the primer of 7-nt sequences long. Therefore, the optimal primer length was chosen to be 8-nt sequences long and this primer was used throughout subsequent experiments.

Other experimental conditions including the concentrations of KF polymerase and dNTPs were also investigated. Fig. 2D and E depict the dependency of the fluorescence signal ratio on the concentrations of KF polymerase and dNTPs, respectively. Experimental results revealed that the optimal concentrations for KF polymerase and dNTPs were 2.5 units and 100 μM, respectively, and the two optimal concentrations were used throughout subsequent experiments.

Under the optimal experimental conditions, the sensitivity of the label-free fluorescent DNA biosensor was investigated. Fig. 3A depicts the typical fluorescence responses of the DNA biosensor towards target DNA with various concentrations.

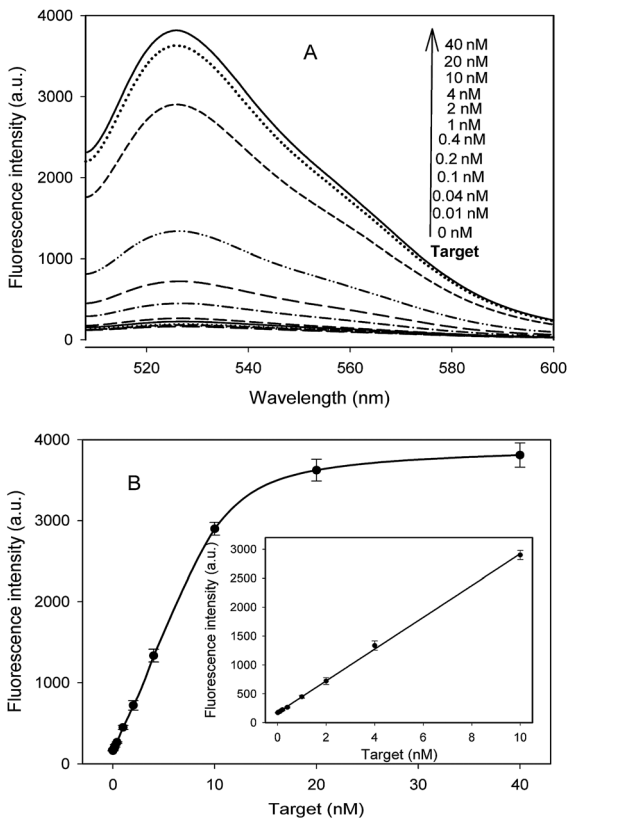


Fig. 3 (A) Typical fluorescence responses of the label free amplified fluorescence assay towards target DNA of varying concentrations. (B) Corresponding fluorescence peak intensity *versus* the concentrations of target DNA. The error bars represent standard errors from three independent experiments.

Dynamically increased fluorescence peaks in response to target DNA with the concentrations that ranged from 0–40 nM was observed and is displayed in Fig. 3B. There is a good linear relationship between the fluorescence peak intensity and the concentration of target DNA in a four-decade concentration range from 0.01–10 nM with a readily achieved detection limit of 4 pM. The detection limit of the proposed DNA biosensor was much lower than that of previous GO-based DNA biosensors,[22,23] and compared favorably with several amplified fluorescence DNA detection methods.[24–26] The detailed comparison is given in Table 2 (see ESI†). The experimental results demonstrated that the proposed DNA biosensor could be used successfully for sensitive DNA detection.

Finally, the selectivity of this DNA biosensor was also investigated. The proposed DNA biosensor was challenged with single-base mismatched target T1-m, four-base mismatched target T4-m and non-complementary target T-n. Fig. 4 displays the fluorescence intensity of the DNA biosensor upon incubation with T1-m, T4-m and T-n in comparison with that of the target DNA.

As shown in Fig. 4, the addition of T1-m and T4-m led to two obvious weaker fluorescence signals compared with the strong fluorescence signal from incubation with the target DNA. Besides, the addition of T-n only caused negligible fluorescence change compared with the blank. The data indicated that the proposed DNA biosensor could differentiate single mismatches and as a result might hold great potentials for single-nucleotide polymorphism (SNP) analysis. Note that in this assay the hairpin probe is designed to have a relatively large loop, which is beneficial for ICSDPR amplification and sensitivity enhancement but may decrease the specificity of the probe. Further improvement in the selectivity can be obtained by optimization of the loop sequence and the stem sequence.

In summary, we have presented a label-free, amplified fluorescence DNA detection based on the ICSDPR combined with GO binding. In this paper, the combination of SG, GO adsorption with ICSDPR greatly improves the sensitivity of this DNA biosensor. The proposed method is simple, label free and rapid, the estimated detection limit is among the best reported to date for GO-based homogenous DNA detection. In addition, through the introduction of hairpin probe, this DNA biosensor can differentiate single mismatches and may find applications in SNP analysis. Given the cost-effectiveness, simplicity, sensitivity and selectivity of this method, the proposed strategy might open the door for the development of new assays for DNA or other biomolecules based on the GO platform.

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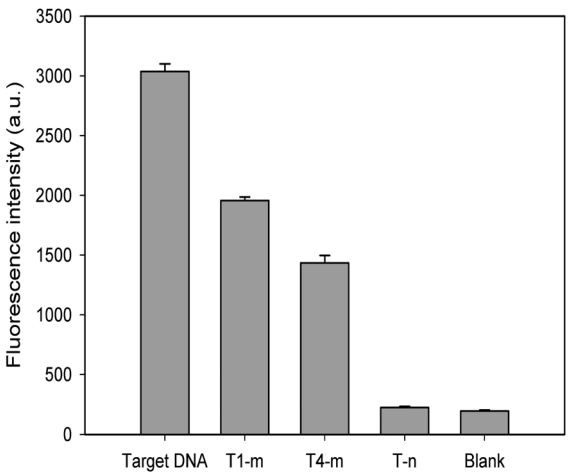

Fig. 4 Specificity of the amplified fluorescence DNA assay for different targets. T1-m: single-base mismatched target; T4-m: four-base mismatched target; T-n: non-complementary target. The concentrations of target DNA, T1-m, T4-m and T-n are all 10 nM.

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