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Graphene oxide-based biosensor for sensitive fluorescence detection of DNA based on exonuclease III-aided signal amplification

Xu-Hua Zhao[a,b], Qiu-Juan Ma[a,*], Xiang-Xiang Wu[a], Xin Zhu[a]

[a] School of Pharmacology, Henan University of Traditional Chinese Medicine, Zhengzhou 450008, PR China
[b] State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, PR China

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ABSTRACT

Based on the super fluorescence quenching efficiency of graphene oxide and exonuclease III aided signal amplification, we develop a facile, sensitive, rapid and cost-effective method for DNA detection. In the presence of target DNA, the target-probe hybridization forms a double-stranded structure and exonuclease III catalyzes the stepwise removal of mononucleotides from the blunt 3′ termini of probe, resulting in the recycling of the target DNA and signal amplification. Therefore, our proposed sensor exhibits a high sensitivity towards target DNA with a detection limit of 20 pM, which was even lower than previously reported GO-based DNA sensors without enzymatic amplification, and provides a universal sensing platform for sensitive detection of DNA.

1. Introduction

In recent years, interest in nanomaterials has increased rapidly due to their unique optical, electronic, magnetic and catalytic properties. Significantly, many nanomaterials including gold nanoparticles, quantum dots, carbon nanotubes and graphene are being integrated with biomolecules and used extensively for development of biosensors [1–6]. Graphene has a unique planar structure, as well as novel electronic properties, its and its derivates have been developed to detect small molecule, DNA, proteins and cells [7]. Moreover, graphene has also been reported as an advanced transparenter for drug delivery, as well as bioimaging in living cells [7]. Tang and co-workers report for the first time the use of DNA hybridization for the controllable assembly of a graphene nanosheet, and exploit the DNA-graphene dispersed sheets as highly ultrasensitive detection [8]. As one of graphene derivatives, graphene oxide (GO) has received more attention due to its remarkable properties such as good water dispersibility and biocompatibility, large surface area, facile surface modification and low manufacturing cost [9–11]. Furthermore, GO is an efficient signal-to-background enhancer and can selectively bind and quench a dye-labeled single stranded DNA (ssDNA) probe, which is induced by hydrophobic and π-stacking interactions between the nucleobases and GO [12]. More recently, GO has been employed to develop sensing systems for detection of DNA, proteins and other small molecules by using complementary oligonucleotides or aptamers as recognition units [13–16]. For example, Lu and co-workers present a novel and highly selective molecular beacon biosensor using GO as the nanoquencher to detect DNA. The graphene oxide-quenched MB needs only one labeled fluorophore. This design provides a more convenient one-step synthesis/purification protocol compared to conventional molecular beacons [17]. He and co-workers develop a rapid and versatile strategy for multicolor fluorescent DNA analysis in homogeneous solution by exploiting interactions between GO and DNA molecules and explore the mechanism underlining GO–DNA interactions via both theoretical and experimental studies [18]. However, in these assays of DNA detection, each probe DNA binds to only a target DNA. This 1:1 hybridization ratio limits signal enhancement and thus its sensitivity.

Method for sensitive and selective detection of nucleic acids is of great importance in mutation identification, clinical diagnostics, gene therapy [19,20]. Various analytical approaches have been developed for DNA detection, including fluorescent, electrochemical and colorimetric methods and so on [21–24]. Recently, in order to improve the sensitivity of DNA detection, the strategies based on enzyme-aided signal amplification have been employed, such as polymerase chain reaction (PCR) and Nicking endonucleases signal amplification, etc. [25,26]. However, these methods demonstrate many significant flaws. For example, PCR is a

* Corresponding author. Tel.: +86 371 65962746; fax: +86 371 65962546.
E-mail address: maqiujuan104@yahoo.com.cn (Q.-J. Ma).

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Table 1
Synthesized oligonucleotide sequences (5′ → 3′) used in the experiments.

DNA	Sequence (5′ → 3′)
Probe	FAM-CAG CAC CAG TAA CTC
Target	GAG TTA CTG GTG CTG AATG
Single base mismatch 1	GAA TTA CTG GTG CTG AATG
Single base mismatch 2	GAG TTA CTT GTG CTG AATG
Two bases mismatch	GAG TCA CTG GTT CTG AATG

relatively complex handling procedures and prone to false positives arising from artifactual amplification. Nicking endonucleases signal amplification requires target DNA with a specific sequence for enzyme recognition and thus is limited in general applications. To develop a versatile nuclease-amplified DNA detection method, exonuclease III (Exo III) was used in our sensing system. Exo III is sequence-independent and can catalyze the stepwise removal of mononucleotides from 3′-hydroxyl termini of double-stranded DNA, while it is unable to catalyze the removal of bases from a ssDNA substrate [27,28]. The function has been used to develop different Exo III-based amplified detection assays [29–32]. For example, Zuo and co-workers designed a DNA detection scheme employing a stem-loop DNA molecular beacon (MB) as the signaling probe and Exo III as an amplifying biocatalyst [29]. Therefore, remarkable signal amplification was achieved. However, MB probes have some limitations, such as high-cost synthesis, insufficient sensitivity and difficult selection of dye-quencher pair in certain cases.

In order to overcome these limitations, we use the GO as super fluorescence quencher and the Exo III as an amplifying biocatalyst to develop a facile, sensitive, rapid and cost-effective method for DNA detection. Our proposed design offers several significant advantages. First of all, due to the superquenching ability of GO, the GO-based DNA sensor has better sensitivity as compared to the conventional dually labeled fluorescent molecular beacon sensors. Secondly, GO can be prepared in large quantities at very low cost and in contrast to double-labeled MBs, the probe is only labeled with a single dye, which reduces the cost for DNA detection. Thirdly, using Exo III as an amplifying biocatalyst can improve the versatility and sensitivity of the DNA assays. For these advantages, we expect that this strategy could be a generalized platform for DNA detection.

2. Experimental

2.1. Materials

DNA oligonucleotides used in this work were synthesized and purified by Takara (Dalian, China). Thermodynamic parameters and secondary structures of all oligonucleotides were calculated using bioinformatics software (http://mfold.rna.albany.edu/) and the sequences of oligonucleotides are given in Table 1. Graphene oxide was purchased from Xianfeng nanomaterials Co., Ltd. (Nanjing, China). Exonuclease III and NEB buffer 1 were purchased from New England Biolabs (Ipswich, MA) and used without further purification. Milli-Q water (resistance >18 MΩ cm) was used in all runs.

2.2. Apparatus

All fluorescence measurements were made on a Hitachi F-4500 Fluorescence Spectrometer (Tokyo, Japan). The instrument settings were chosen as follows: λ_{ex} = 494 nm, λ_{em} = 518 nm, ex slit = 5 nm, em slit = 10 nm, PMT detector voltage = 950 V.

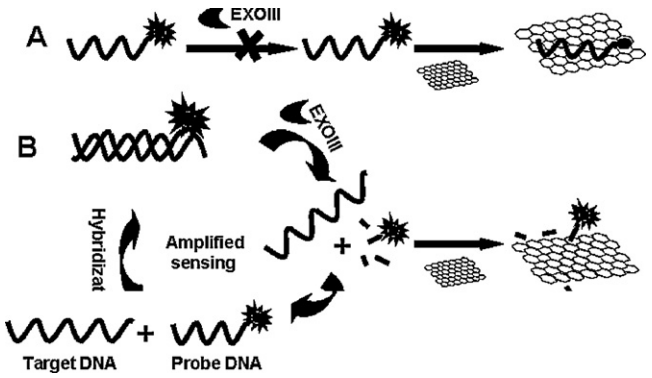

Fig. 1. Schematics of GO-based sensing system for the detection of target DNA by Exo III signal amplification strategy.

2.3. Preparation of GO solution

Before being used, GO (10 mg) was dissolved in 10 mL deionized water and then strong sonicated for 1 h (60 W). Subsequently, large GO layers were removed by centrifugation (6000 rpm, 10 min) and stored in 4 °C.

2.4. Procedure for fluorescence detection

The hybridization between probe DNA (50 nM) and varying concentrations DNA target was carried out in 1× NEB buffer 1 (10 mM Bis Tris Propane–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), pH 7.0) for 10 min. Then, exonuclease III (1 U) was added and the mixture was incubated at 37 °C for 30 min. Upon the addition of the GO, the fluorescence of the mixture was measured.

3. Results and discussion

3.1. Probe design and analytical principle

The design strategy of our method is depicted schematically in Fig. 1. The 5′ end of the probe DNA is labeled with a fluorophore carboxyfluorescein (FAM). In the absence of target DNA (Fig. 1A), Exo III is unable to catalyze the removal of bases from the probe DNA. Upon the addition of GO, the fluorescence of FAM was significantly quenched because of the strong binding between ssDNA and GO. In the presence of target DNA (Fig. 1B), the target-probe hybridization forms a double-stranded structure that contains an exonuclease III-resistant 3′ protruding termini and a blunt 3′ termini. Exo III can catalyze the stepwise removal of mononucleotides from the blunt 3′ termini, which resulting in the releasing of the target and fluorophore. The released target DNA can hybridize with another probe DNA and then initiate a next round of cleavage. The numbers of releasing fluorophore are positively related to the target DNA concentration. The introduction of GO into the sensing solution will induce weak quenching of the fluorophore because of the weak affinity of the fluorophore and GO, and the fluorescence intensity gradually increases with increasing target concentration.

3.2. Signal amplification of the sensing system

In order to evaluate the amplification function of the proposed sensing system, the target-induced fluorescence increase in the presence and absence of Exo III were recorded, respectively. As shown in Fig. 2, the Exo III also can degrade some ssDNA probe, which increases the background fluorescence of the sensing system. Nevertheless, upon the addition of the target DNA, a 235.2 ± 21% signal enhancement was observed due to Exo III catalyzed target DNA recycling. In contrast, only a 73.2 ± 9%

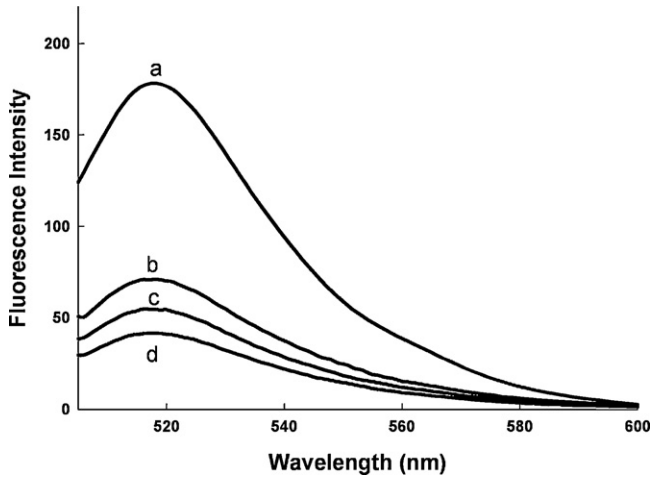

Fig. 2. Fluorescence emission spectra of the sensing system under different conditions. (a) Probe + Exo III + target (10 nM) + GO; (b) probe + target (10 nM) + GO; (c) probe + Exo III + GO; (d) probe + GO.

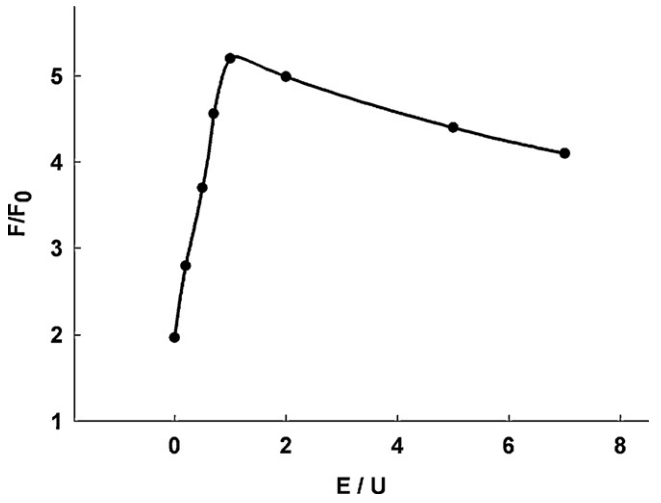

Fig. 4. The effect of Exo III concentration. F and F_0 are the fluorescence intensity of the sensing system in the presence and absence of 50 nM of target DNA, respectively. GO concentration is 100 μg mL^{-1}.

fluorescence increase was observed in the absence of Exo III. These results confirm that this assay can enable a significant signal amplification for DNA detection.

3.3. Optimization of assay conditions

In order to obtain an optimal assay condition and achieve a high signal-to-noise ratio, the effects of GO and Exo III concentrations have been investigated by fixing the 50 nM probe and 50 nM target DNA, respectively.

From Fig. 3, we can see that the S N^{-1} ratio of the sensing system increased significantly when the concentrations of GO ranging from 60 to 100 μg mL^{-1}. However, when the concentration of GO was higher than 100 μg mL^{-1}, the S N^{-1} ratio decreased with a further increasing concentration of GO, which might be ascribed to the excessive quenching effect of the GO at a high concentration on the cleavage-produced fluorophore. Therefore, we chose 100 μg mL^{-1} GO as the optimum assay condition.

Next, the effect of concentration of Exo III was further studied and the results were shown in Fig. 4. It is clear that the S N^{-1} ratio of the sensing system increased with increasing Exo III concentration until the concentration reached 1 U. However, when the concentration exceeded 1 U, the S N^{-1} ratio of the sensing system decreased. Taking into account the response sensitivity, 1 U Exo III was used in the final solution.

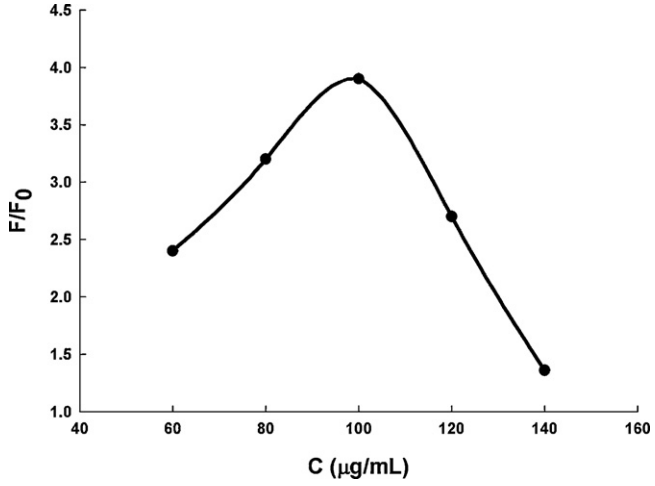

Fig. 3. The effect of GO concentration. F_0 and F are the fluorescence intensity of the sensing system in the absence and presence of 50 nM target DNA, respectively. Exo III concentration is 5 U.

3.4. The sensitivity of the sensing system

Fig. 5 illustrates the fluorescence spectra of the sensing system in the optimum assay condition upon addition of different concentrations of target DNA. As can be seen from Fig. 5A, the fluorescence intensity increased obviously as the concentration of the target DNA increased and an approximately 18-fold fluorescence enhancement was observed when the target concentration reached 300 nM. The large fluorescence increase of the sensing system might arise from the super quenching efficiency of GO to lower the background fluorescence and Exo III aided target recycling. Fig. 5B describes the relationship between the fluorescence intensity and the different concentrations of target DNA. A dynamic range from 50 pM to 300 nM for target was achieved with a linear relationship range until 100 nM. The detection limit (taken to be 3 times the standard deviation in the blank solution) was 20 pM, which was even lower than previously reported GO-based DNA sensors without enzymatic amplification [17,18]. These results suggested that this sensing system was appropriate for quantitative determination of target DNA concentration.

3.5. The specificity of the sensing system

To investigate the specificity of the sensing system, we compared the fluorescence response induced by DNA strands containing single-base and two-base-mismatched oligonucleotides with that of target DNA. All results are displayed in Fig. 6. It was found that the target DNA with the perfectly matched sequence induced 8.9-fold fluorescence enhancement. However, 4-fold and 2-fold fluorescence increase was observed when the DNA targets containing single-base-mismatched and two-base-mismatched oligonucleotides, respectively. So these results demonstrated that this DNA sensing system can be used to discriminate perfectly matched and mismatched DNA targets. The DNA probe and the single base mismatch DNA could hybridize to form double-stranded DNA (dsDNA) structure under 37 °C. In the present of Exo III, the dsDNA containing the single base mismatch DNA 1 would be digested partly and a short dsDNA with 12 base pairs was left finally; meanwhile, the dsDNA containing the single base mismatch

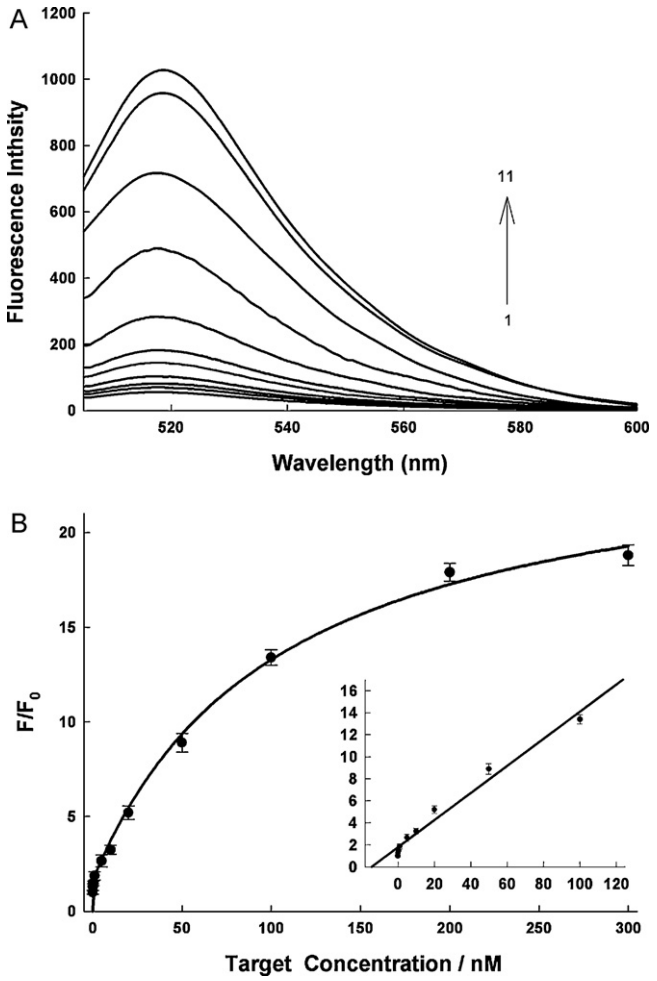

Fig. 5. (A) Fluorescence emission spectra of the sensing system upon the addition of different concentrations target DNA and then mixed with GO: (1) 0; (2) 50 pM; (3) 300 pM; (4) 1 nM; (5) 5 nM; (6) 10 nM; (7) 20 nM; (8) 50 nM; (9) 100 nM; (10) 200 nM; (11) 300 nM. (B) Fluorescence increase over background fluorescence at varying target DNA concentrations. (Inset) Probe responses to low concentrations of target DNA. F and F_0 are the fluorescence intensity in the presence and absence of target DNA.

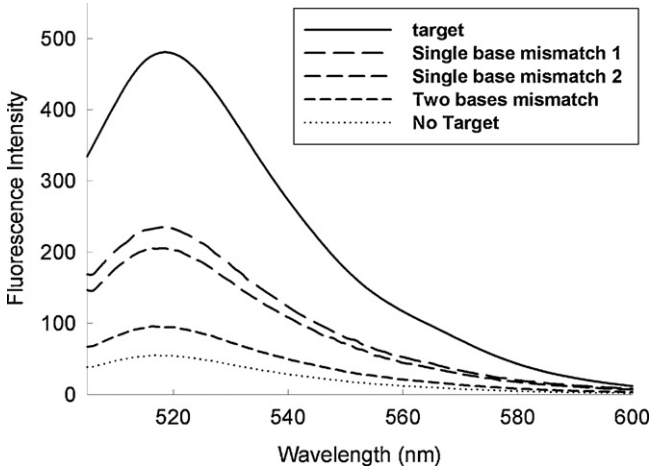

Fig. 6. Fluorescence response of the sensing system toward perfectly matched and mismatched DNA targets. The concentrations for probe and target were fixed at 50 nM.

DNA 2 would also be digested partly and a FAM-linked ssDNA with 7 bases was released. The introduction of GO into the sensing solution will induce weak quenching of the fluorophore due to the weak affinity of the short dsDNA and the short FAM-linked oligonuleotide fragment to GO. These reasons proposed above would explain the fluorescence intensities of single base mismatch 1 and 2 in Fig. 6.

4. Conclusions

In summary, we have developed a novel GO-based biosensor for enzymatic amplified DNA detection. By taking advantage of the super fluorescence quenching efficiency of GO and exonuclease III aided signal amplification, our proposed sensor exhibits a high sensitivity towards target DNA with a detection limit of 20 pM, which was even lower than previously reported GO-based DNA sensors without enzymatic amplification. Moreover, the sensing system is facile, rapid and cost-effective and can provide a universal sensing platform for sensitive detection of various DNA.

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