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A DNA machine for sensitive and homogeneous DNA detection via lambda exonuclease assisted amplification

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ABSTRACT

This work designs a DNA machine with three assistant DNAs and lambda exonuclease (Exo-λ) for sensitive and homogeneous fluorescent detection of DNA. The selective digestion of Exo-λ to blunt or recessed 5′-phosphorylated strand of probe 1–probe 2 duplex results in the release of target DNA and probe 2 to produce the fluorescence restoring of fluorophore labeled to probe 1. The released target DNA could hybridize with another probe 1–probe 2 duplex to trigger the target recycling for signal amplification, while the released probe 2 hybridized with molecular beacon to restore its fluorescence for signal enhancement. This DNA machine showed a fast response to target DNA with a linear concentration range from 0.4 pM to 4 nM. The limit of detection was 68 fM at a signal-to-noise ratio of 3. The high selectivity of the method may result from the Exo-λ's specific recognition-site of double-stranded DNA and the specific hybridization of target DNA with probe 1–probe 2 duplex. This DNA machine with the homogenous detection, rapid response as well as simplicity provides a new approach for sensitive detection of DNA.

1. Introduction

Sensitive and simple detection of DNA is of vital significance in medical diagnosis, monitoring of environmental pollutants, food safety and homeland security [1–5]. The detection can take place both in the homogeneous solution and on the heterogeneous interface [6–11]. The latter has been more extensively applied since it is convenient to combine with other signal amplification techniques, and thus can achieve high sensitivity. For example, an enzyme-based electrochemical DNA sensor with a femtomolar detection limit has been designed via the biotin–avidin recognition at an avidin-modified electrode surface [12]. By the hybridization chain reaction to form a long-range DNA nanostructure at a gold electrode, a DNA biosensor can detect target DNA as low as 5 aM [13]. These methods on heterogeneous interface usually involve separation and washing steps, which leads to both complicated and time-consuming procedure and the loss of specificity and practicality of the methods. On the other hand, homogenous detection method has many advantages of rapid response, high specificity as well as simplicity in DNA detection due to absence of undesired interactions with the surface. For example, based on Förster resonance energy transfer between graphene oxide and fluorophore, a homogeneous fluorescence method was designed for multiplexed detection of DNA with detection limit of 5 pM [7]. More importantly, homogenous detection method can be employed to set up a DNA machine for fast and convenient DNA analysis.

DNA machine consisted of some sequence-designed nucleic acids that can automatically produce a signal for readout through specific recognition when the “fuel”, that is the target molecule, is introduced [14–19]. Owing to the simplicity, DNA machine has extensively been utilized for the detection of DNA in complex biological samples. Typically, a turn-on fluorescent DNA machine has been prepared using a DNA molecular beacon (MB) labeled with fluorophore and quencher [20–22]. In order to realize the ultrasensitive detection, some amplification strategies such as molecular biological [23–27] and nanomaterials-based [28–32] amplification techniques have been introduced into DNA machine. For example, an endonuclease assisted target recycling has been combined with a surface-enhanced Raman scattering-based DNA machine for specific detection of DNA in cancer cells [33]. Here an exonuclease (Exo) was introduced into DNA machine for signal amplifications in the detection of DNA.

Different from endonuclease that attacks nucleic acids inside the polynucleotide chain, Exo can catalyze the stepwise removal of mononucleotides from an end of the polynucleotide. An Exo, Exo-III that can selectively digest double-stranded DNA (dsDNA) from 3′-terminus when the 3′-terminus is blunt or recessed, has been used to arouse target recycling for amplifying the detectable signals [34,35], which leads to a detection limit of 0.3 nM for

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fluorescent analysis of DNA [35]. As a complementation of Exo-III, lambda exonuclease (Exo-λ) selectively digests the blunt or recessed 5′-phosphorylated strand of dsDNA [36,37]. This work introduced the selective digestion of Exo-λ into fluorescent detection of DNA and designed a sensitive homogeneous analytical method by coupling the Exo-λ assisted amplification with MB-based fluorescent DNA machine (Fig. 1). The DNA machine coupling with molecular biological amplification technique provides a proof-of-concept for ultrasensitive detection of DNA in clinic analysis.

2. Experimental

2.1. Materials and reagents

Lambda exonuclease along with enzymatic reaction buffer was purchased from Fermentas (Ipswich, MA, USA). Tris-(hydroxymethyl) aminomethane (Tris) was purchased from Sigma-Aldrich Inc. The stock solution of DNA (10 μM) was prepared in the TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) and diluted to a desired concentration using the TE buffer. All solutions were prepared in deionized water. The other reagents were of analytical reagent grade and used as purchased without further purification. The oligonucleotides were synthesized by TaKaRa Bio Inc. (Dalian, China) and HPLC-purified. The oligonucleotide sequences are listed in Table 1.

2.2. Apparatus

The fluorescence spectra were recorded from 505 to 610 nm with a step of 1 nm at an excited wavelength of 495 nm on a RF-5301PC spectrofluorometer (Japan) equipped with a xenon lamp. The slit width was 5 nm.

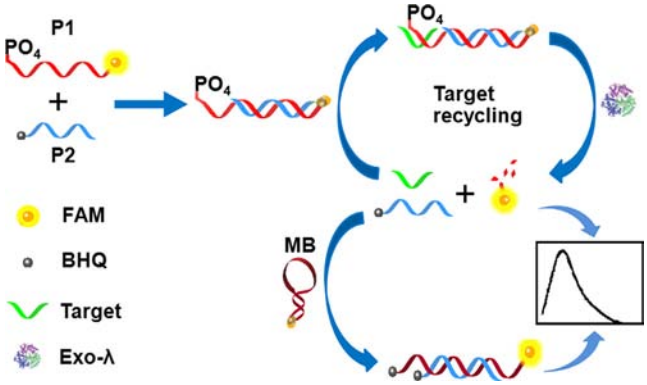

Fig. 1. Schematic illustration of amplification strategy in Exo-λ assisted DNA machine for DNA detection.

2.3. Reaction of DNA machine

First, the DNA stock solutions of probe 1 (P1, 10 μM), probe 2 (P2, 10 μM) and MB (10 μM) were heated at 90 °C for 10 min, and gradually cooling down to room temperature. To trigger the DNA machine, 40 μL target DNA at varying concentrations was mixed with 2 μL P1 (1 μM), 2 μL P2 (1 μM), 5 μL enzymatic reaction buffer (10 ×) and 1 μL TE buffer, and then incubated at 37 °C for 30 min. Subsequently, 5 U Exo-λ was added in the mixture, and incubated at 37 °C for 2.5 min. The enzymatic reaction was ended by adding 2 μL EDTA (0.5 M) to the reaction solution. At last, 2 μL MB (1 μM) was added into the mixture, and then incubated for 20 min at 37 °C prior to subsequent fluorescent detection.

3. Results and discussion

3.1. Design of strategy

In order to realize the sensitive detection of target DNA, this DNA machine involved one target recycling with the aid of Exo-λ for signal amplification and subsequent MB-based fluorescent enhancement as shown in Fig. 1. First, the P1 and the P2 hybridized each other to quench the fluorescence of fluorophore labeled on P1 by black hole quencher (BHQ) labeled on P2 [38]. In the absence of target, the Exo-λ did not exhibit any activity on the P1–P2 duplex due to the convex 5′-phosphorylated terminus of P1. Thus the DNA machine was static and nonfluorescent. When the target DNA was introduced to the machine, it hybridized with P1–P2 duplex to form a blunt dsDNA with a 5′-phosphorylated end, leading to selective digestion of the P1 by Exo-λ, which released target DNA and P2 to produce the fluorescence restoring of fluorophore labeled to P1. The released target DNA could hybridize with another P1–P2 duplex to trigger the target recycling, while the released P2 hybridized with MB to restore its fluorescence. The target recycling significantly amplified the fluorescence signal, and resulted in a wide linear range and a femtomolar detection limit. The DNA machine coupling with Exo-λ amplification provides a new possibility for sensitive detection of DNA in clinical application.

3.2. Feasibility of DNA machine

The feasibility of DNA machine was proved by stepwise fluorescence measurement as shown in Fig. 2. After excited at the maximal absorption wavelength (495 nm) of fluorescein (FAM), P1 showed strong fluorescence emission at 516 nm (curve *a*). When P2 was introduced, the fluorescence of P1 was greatly quenched (curve *b*) since the quencher labeled to P2 was nearby the FAM labeled to P1 after hybridization. Upon addition of the target, no obvious change of the fluorescence was observed

Table 1
Oligonucleotide sequences used in this work.

Oligonucleotide	Sequence
Probe DNA 1 (P1)	5′-PHOS-**TCAGTTGACCCTC**TTACATTGACGTATCTT-FAM-3′
Probe DNA 2 (P2)	5′-BHQ-AAG**ATACGTCAATGT**AA-3′
Molecular beacon (MB)	5′-FAM-ACCCTC**ACATTGACGTAT**GAGGGT-BHQ-3′
Target DNA	5′-**GAGGGTCAACTGA**-3′
Single-base mismatched DNA 1 (smDNA-1)	5′-**CAGGGTCAACTGA**-3′
Two-base mismatched DNA 1 (tmDNA-1)	5′-**CAGGCTCAACTGA**-3′
Single-base mismatched DNA 2 (smDNA-2)	5′-**GAGGCTCAACTGA**-3′
Two-base mismatched DNA 2 (tmDNA-2)	5′-**GAGGC*A*CAACTGA**-3′

PHOS, FAM and BHQ are phosphate group, fluorescein, and black hole quencher, respectively. Mismatched bases are highlighted in italic type.

(curve *c*). Subsequent addition of Exo-λ led to selective digestion of the 5′-phosphorylated strand of P1 in the dsDNA, which released the FAM to restore its fluorescence by 78.6% (curve *d*). The fluorescence restoring indicated the excellent enzymatic activity of the Exo-λ. After the introduction of MB, the fluorescence intensity of the DNA machine increased over 40.3% (curve *e*). At a target DNA concentration of 4.0 nmol L^{-1}, the fluorescent intensity in the presence of Exo-λ was 5.8 times higher than that in the absence of Exo-λ (curve *f*). Moreover, the DNA machine was hardly triggered in the absence of target DNA (curve *g*). Therefore, the high sensitivity was attributed to the target recycling with the aid of Exo-λ and subsequent MB-based fluorescent enhancement.

3.3. Optimization of detection conditions

To obtain the optimal detection performance of this DNA machine, the kinetic behaviors of the hybridization between P1 and P2 (Fig. 3A), and P2 and MB (Fig. 3B) were investigated by the time-dependent fluorescence emission spectra. After mixing P1 with P2 at an equal molar ratio, the maximum quenching efficiency occurred at 30 min, while maximum fluorescence restoring of MB could be observed at 20 min after mixing MB and P2. The effect of reaction time for enzymatic amplification on the fluorescence intensity was then examined at the optimal hybridization times. Considering the influence of nonspecific blank amplification [39], the ratio (F/F_0) of fluorescence intensities in the presence and absence of target DNA was used to optimize the reaction time (Fig. 3C). At 5 U of Exo-λ, the fluorescence intensity increased with the increasing reaction time, but the F/F_0 reached the maximum at 2.5 min. This result was contributed to the higher activity of Exo-λ to blunt dsDNA than 3′-recessed end of dsDNA. Therefore, 2.5 min was selected as the reaction time of Exo-λ.

3.4. DNA detection of DNA machine

At the three optimal hybridization and reaction times, the fluorescence intensity produced by DNA machine increased with the increasing target concentration (Fig. 4), indicating the target DNA acted as the fuel of the DNA machine which produced large signal for readout. The plot of the fluorescence intensity *vs.* the logarithmic value of target DNA concentration showed a linear relationship with through a 4-decade range from 0.4 pM to 4 nM (inset in Fig. 4). The detection limit of this method was 68 fM at a *S*/*N* ratio of 3, which was much lower than 2 nM for Exo-λ based isothermal electrochemical DNA biosensor [40], and 0.05 nM for Exo-λ based electrochemical impedance spectroscopic DNA biosensor [37]. The high sensitivity of this DNA machine was attributed to the target recycling based signal amplification and subsequent MB-based fluorescent enhancement.

3.5. Specificity of DNA machine

To evaluate the specificity of the DNA machine, the machine was used to detect perfect complementary target DNA and other bases mismatched DNAs at the same concentration (4 nM) (Fig. 5). For the 5′-terminal base mismatch, the fluorescence intensity of

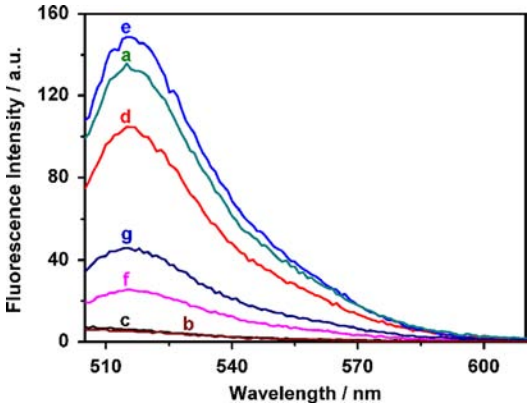

Fig. 2. Fluorescence emission spectra of P1 (a), P1+P2 (b), P1+P2+target (c), P1+P2 +target+Exo-λ (d), P1+P2+target+Exo-λ+MB (e), P1+P2+target+MB (f) and P1+P2 +Exo-λ+MB (g). The concentrations of P1, P2, MB, target and Exo-λ are 40, 40, 40, 4 nM and 5 U, respectively.

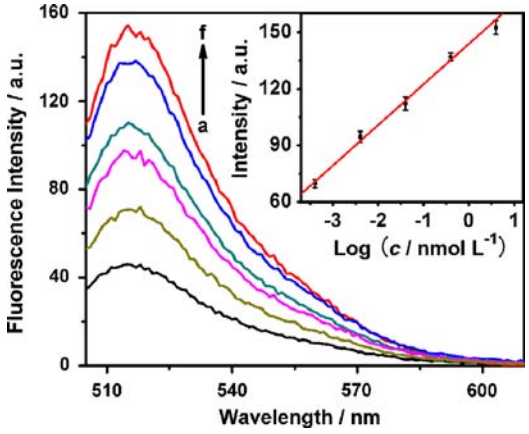

Fig. 4. Fluorescence emission spectra of the output of DNA machine for target DNA at 0, 4×10^{-13}, 4×10^{-12}, 4×10^{-11}, 4×10^{-10} and 4×10^{-9} mol L^{-1} (from a to f). Inset: plot fluorescence intensity *vs.* logarithm of target concentration.

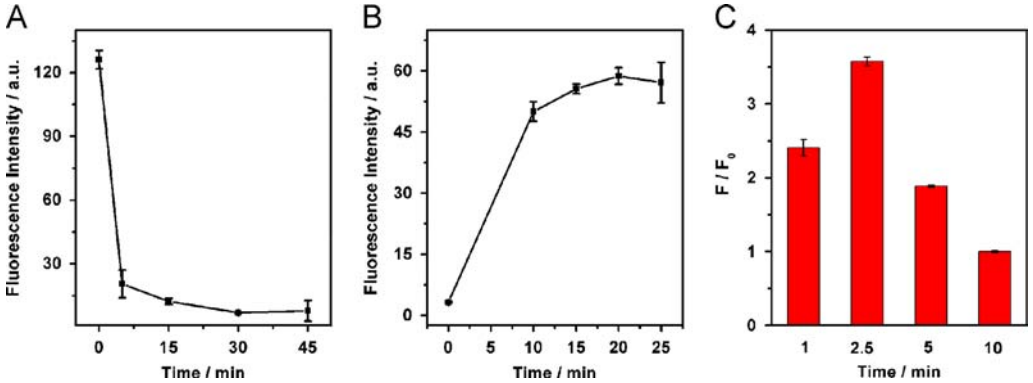

Fig. 3. Plots of fluorescence intensity *vs.* incubation time for the hybridization of (A) P1 (40 nM) and P2 (40 nM), and (B) MB (40 nM) and P2 (40 nM) at reaction temperature of 37 °C. (C) Dependence of F/F_0 on reaction time of Exo-λ in the presence and absence of 4 nM target DNA at 1, 2.5, 5 and 10 min. The other parameters are under their optimal conditions.

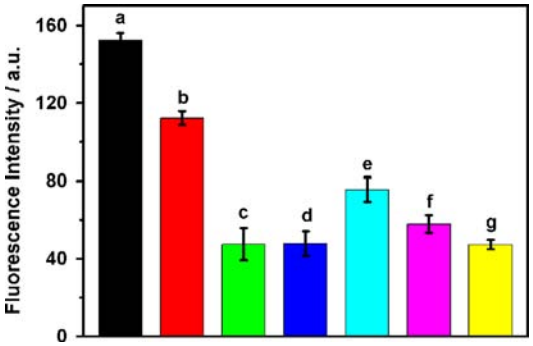

Fig. 5. Specificity of this DNA machine analyzing target DNA at 4 nM (a) and 0.04 nM (b), smDNA-1 (4 nM) (c), tmDNA-1 (4 nM) (d), smDNA-2 (4 nM) (e), tmDNA-2 (4 nM) (f) and blank (g).

complementary target DNA was about 3.5 times higher than the responses of both smDNA-1 and tmDNA-1 which were closed to that of blank. For the base mismatch inside DNA sequence, the fluorescence intensity of smDNA-2 was about 1.6 times higher than that of the smDNA-1. These results may be attributed to that the 5′-terminal mismatch reduced the Exo-λ reaction efficiency. Meanwhile, compared with that of smDNA-2, the fluorescence intensity of tmDNA-2 was low to 76.9% due to the relative weak stringency of the hybridization reaction between tmDNA-2 and P1. In addition, even the concentration of complementary target DNA was 1/100 of the mismatched DNAs, the DNA machine also showed much higher fluorescence response. The selectivity of the method resulted from the specific recognition site of Exo-λ to dsDNA and the specific hybridization of target DNA with P1–P2 duplex.

4. Conclusions

A novel DNA machine was successfully constructed with three assistant DNAs and Exo-λ for sensitive homogeneous fluorescent detection of DNA. This DNA machine involved one target recycling for signal amplification and subsequent MB-based fluorescent enhancement. The specific recognition site of Exo-λ to blunt or recessed 5′-phosphorylated strand in dsDNA led to high selectivity of the proposed method. The release of target DNA in the digestion process of dsDNA formed by the hybridization of target DNA with P1–P2 duplex triggered the target recycling. This DNA machine achieved a linear range of 4 orders of magnitude and a femtomolar detection limit. Besides, one advantage of this method over polymerase chain reaction is its feasibility without temperature cycling and thus no need for expensive instruments. The DNA machine using target DNA as the fuel with molecular biological amplification technique, provides a sensitive, fast, and specific homogeneous protocol for detection of target DNA, and has the potential application in single-nucleotide polymorphism probing without optimizing the hybridization stringency.

Acknowledgments

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