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## Triplex DNA: A new platform for polymerase chain reaction – based biosensor

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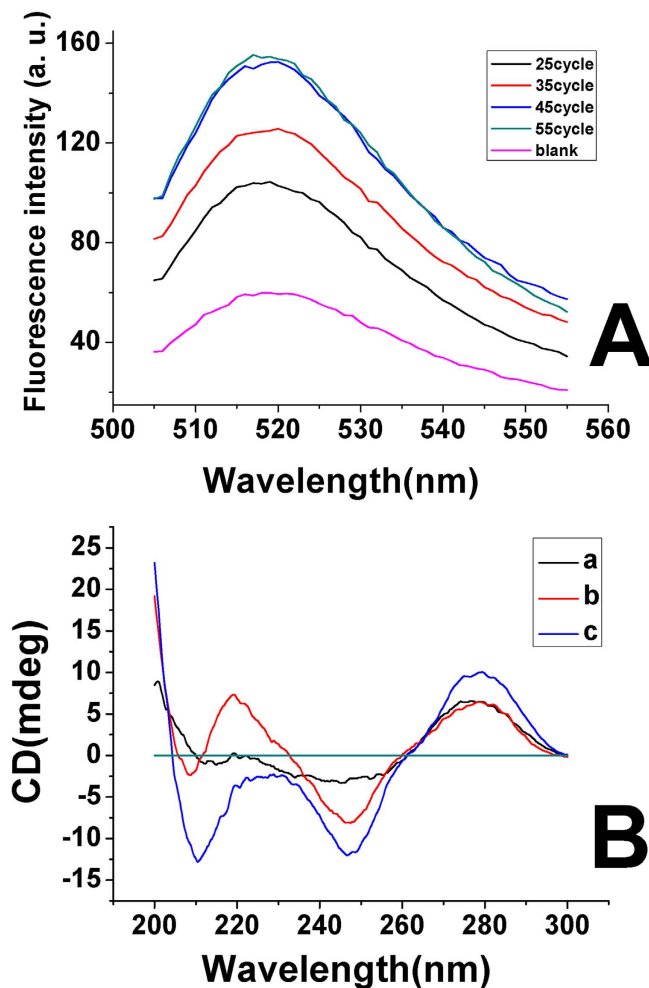
Non-specific PCR amplification and DNA contamination usually accompany with PCR process, to overcome these problems, here we establish a sensor for thrombin by sequence-specific recognition of the PCR product with molecular beacon through triplex formation. Probe A and probe B were designed for the sensor, upon addition of thrombin, two probes hybridized to each other and the probe B was extended in the presence of Klenow Fragment polymerase and dNTPs. The PCR amplification occurred with further addition of Taq DNA Polymerase and two primers, the PCR product was recognized by molecular beacon through triplex formation. The fluorescence intensity increased with the logarithm of the concentration of thrombin over the range from  $1.0 \times 10^{-12}$  M to  $1.0 \times 10^{-7}$  M, with a detection limit of 261 fM. Moreover, the effect of DNA contamination and non-specific amplification could be ignored completely in the proposed strategy.

The polymerase chain reaction (PCR) technology is an essential tool for gene research, because the exponential amplification efficiency enables the detection of small numbers of nucleic acid<sup>1</sup>. To extend the scope of PCR to high sensitive detection of protein, Sano *et al.* established the immuno-PCR (IPCR) method by combines antibody recognition and PCR amplification<sup>2-6</sup>, which could lead to about 100 - 10000 fold increase of sensitivity over the conventional ELISA for protein<sup>7-9</sup>. Owing to the exponential amplification and high sensitivity of PCR, it has been widely applied in detection of gene mutation<sup>10-13</sup>, pharmacogenetics<sup>14,15</sup>, gene expression analysis<sup>16-18</sup>, recognition of methylation specific loci<sup>19-21</sup>, protein<sup>22-26</sup>, detection of metal ion<sup>27</sup> and constructing of DNA nano-structures<sup>28</sup>. However, non-specific amplification is inevitable during the process of PCR amplification. Therefore, the sensitivity and selectivity of PCR technique is controlled by detection method for PCR product. There are mainly five kinds of methods. Firstly, gel electrophoresis, it is generally cumbersome and time-consuming, which has been successfully used in the IPCR<sup>29-31</sup>. Secondly, Detection electrochemical signal of guanine nucleobase of PCR product after separation<sup>32</sup>. Thirdly, detection of PCR products with the TaqMan probes<sup>33</sup>. Fourthly, PNA or LNA modified molecular beacon through displacement hybridization<sup>34</sup>. Fifthly, intercalative molecules, such as Ethidium bromide and SYBR Green I etc., their fluorescence increase upon addition of double-stranded DNA, but they cannot distinguish PCR product from non-specific amplification product such as primer-dimers<sup>35</sup>. It is still a challenge to sequence-specifically recognize the duplex structure of PCR product.

Homopurine-Homopyrimidine duplex DNA can be sequence-specifically recognized by homopyrimidine Oligonucleotide (or homopurine Oligonucleotide) through triplex formation<sup>36,37</sup>. Study of triplex DNA mainly focus on following aspect: firstly, biological function of triplex DNA, such as their effects on the process of gene translation<sup>38</sup>, DNA transcription, replication and cleavage<sup>39,40</sup>; Secondly, effect of hole transport<sup>41-44</sup>; Thirdly, pH controlled switchable conformation change between duplex DNA and triplex DNA<sup>45-48</sup>; Fourthly, sequence-specific recognition of double-stranded DNA<sup>49-54</sup>; Fifthly, biosensing for other molecules<sup>55-60</sup>. However, there had little biosensor that based upon triplex formation and PCR amplification.

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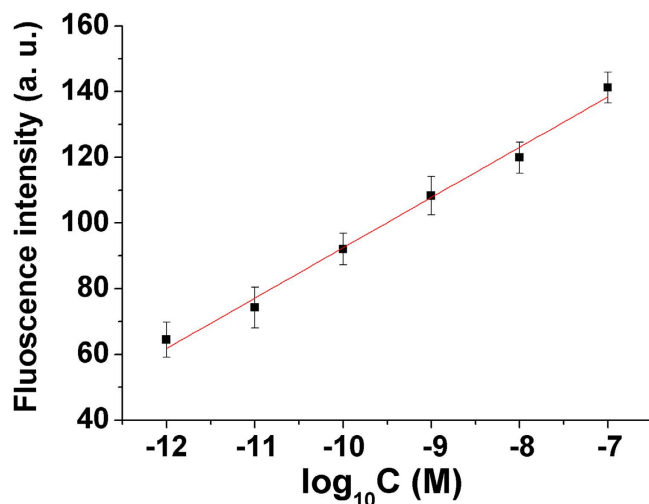


**Figure 2.** (A) Fluorescence spectroscopy of molecular beacon after hybridization with different cycle of PCR product. The PCR amplification was carried out under the condition of 30 s denaturation at 94 °C, 30 s of annealing temperature at 60 °C, and 10 s of extending temperature at 72 °C. MB: 200 nM, spermine: 0.4 mM, pH: 5.0. (B) Circular dichroism (CD) spectroscopy of Oligonucleotides under different conditions. a. MB:  $1.0 \times 10^{-5}$  M; b. R1•R2:  $1.0 \times 10^{-5}$  M; c. Mixture of  $1.0 \times 10^{-5}$  M MB and  $1.0 \times 10^{-5}$  M R1•R2. Spermine: 0.4 mM, pH: 5.0.

the fluorescence intensity), with a detection limit of 261 fM, which was obtained from the equation of  $DL = 3\sigma/slope$ . The comparison of the proposed method with others was listed in Table 1, it had merit of wide linear range.

Non - specific PCR amplification usually accompanied with the PCR amplification process. DNA contamination might easily occurred during PCR amplification process as well. Both DNA contamination and non - specific amplification PCR product were double - stranded DNA, which was similar to that of PCR product, so it was difficult to distinguish the signal for non - specific amplification and DNA contamination from that for PCR product, and then they usually affect the detecting result. To investigate the effect of non - specific PCR amplification and DNA contamination on the proposed strategy, the fluorescence intensity of the PCR product were measured with the proposed strategy and the common probe (SYRB - Green) for real - time PCR respectively. Here lambda - DNA was used to act as non - specific amplification product and DNA contamination due to its duplex structure. As shown in Fig. 4, the fluorescence intensity increased with concentration of lambda - DNA by using the probe of SYRB - Green, while the fluorescence intensity had little change when the concentration of lambda - DNA increased from 0 to 1.6 μg/mL by using the proposed strategy. These results indicated that the proposed strategy could overcome the effect of non - specific PCR amplification and DNA contamination completely, and the effect of non - specific amplification could be ignored in the proposed method. These results mainly due to the stringent sequence - specific recognition between PCR product and MB through triplex formation<sup>49–54</sup>.

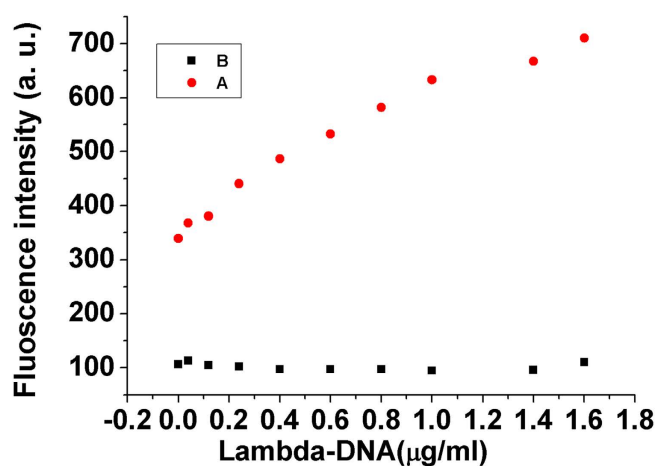
In order to test the selectivity of the proposed sensor, the effect of other possible interferences were investigated.  $1.0 \times 10^{-7}$  M of lysozyme, hemoglobin and apo - transferrin human were used to replace the



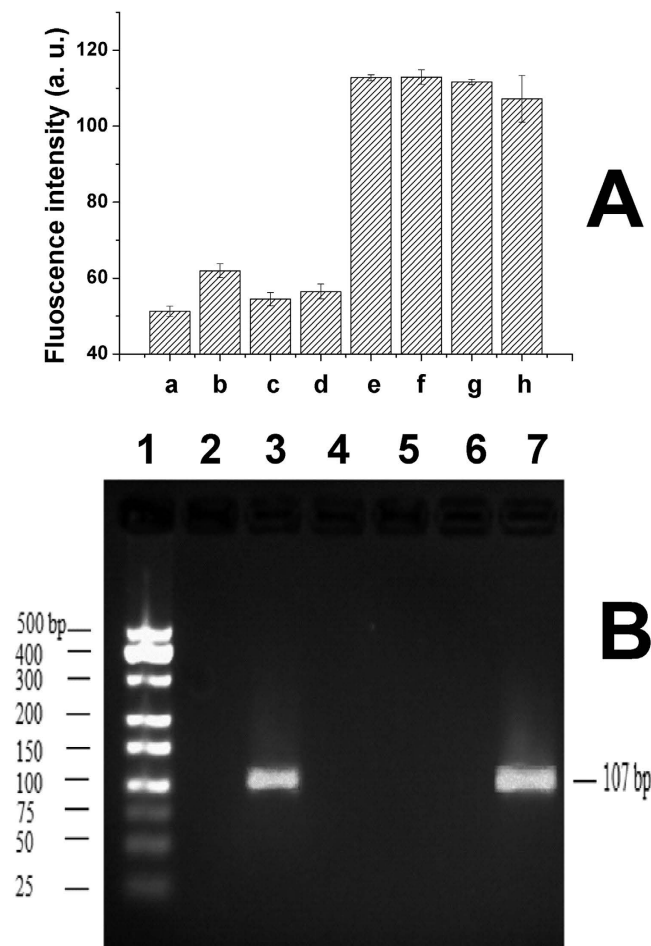
**Figure 3. Calibration curve for the biosensor.** 45 cycles of PCR amplification was carried out by using 30 s of denaturation at 94 °C, 30 s of annealing temperature at 60 °C, and 10 s of extending temperature at 72 °C. MB: 200 nM, spermine: 0.4 mM, pH:5.0. Each point was the mean of three measurements. The error bars are the standard deviation.

Method	Analytical range	LOD	Application to samples	Ref.
Electrochemistry	0.5 pM–20 nM	0.15 pM	no	63
Fluorescence	10 pM–10 nM	8.06 pM	yes	26
Colorimetry	0.1 pg/mL–50.0 pg/mL	0.083 pg/mL	yes	64
Surface enhanced Raman scattering	0.1 nM–10 nM	20 pM	yes	65
Surface plasmon resonance	25 fM–2 pM	25 fM	no	66
The proposed method	1.0 pM–100 nM	261 fM	yes	This work

**Table 1. Comparison of the proposed method with others.**



**Figure 4. Effect of lambda - DNA on the fluorescence of the PCR product of  $1.0 \times 10^{-8}$  M thrombin.** (A) Detecting PCR product by using SYRB - Green; SYRB - Green:  $1 \times$ , spermine: 0.4 mM, pH:5.0. (B) Detecting PCR product with the proposed method (detecting PCR product through triplex formation); MB: 200 nM, spermine:0.4 mM, pH:5.0.



**Figure 5. Selectivity of the sensor and effect of interference molecules.** (A) a: blank; b:  $1.0 \times 10^{-7}$  M of lysozyme; c:  $1.0 \times 10^{-7}$  M of hemoglobin; d:  $1.0 \times 10^{-7}$  M of apo-transferrin human; e:  $1.0 \times 10^{-8}$  M of thrombin; f: mixture of  $1.0 \times 10^{-8}$  M of thrombin and  $1.0 \times 10^{-7}$  M of lysozyme; g: mixture of  $1.0 \times 10^{-8}$  M of thrombin and  $1.0 \times 10^{-7}$  M of hemoglobin; h: mixture of  $1.0 \times 10^{-8}$  M of thrombin and  $1.0 \times 10^{-7}$  M of apo-transferrin human. Every point was the mean of three measurements, error bar was the standard deviation. (B) Electrophoresis photograph of PCR product. 1. 500 bp DNA ladder; 2. Blank; 3.  $1.0 \times 10^{-8}$  M of thrombin; 4.  $1.0 \times 10^{-7}$  M of lysozyme; 5.  $1.0 \times 10^{-7}$  M of hemoglobin; 6.  $1.0 \times 10^{-7}$  M of apo-transferrin human; 7. mixture of  $1.0 \times 10^{-8}$  M of thrombin and  $1.0 \times 10^{-7}$  M of lysozyme, hemoglobin, apo-transferrin human; EB:  $0.5 \mu\text{g/mL}$ ; Agarose: 3%.

$1.0 \times 10^{-8}$  M of thrombin for PCR amplification respectively, As demonstrated in Fig. 5A, each of their fluorescence intensity was almost at the same level of the blank. Moreover, the existence of 10-folded lysozyme, hemoglobin and apo-transferrin human had little effect on the fluorescence intensity of thrombin. To further certificate the selectivity of the sensor, electrophoresis photograph of PCR product was investigated, as demonstrated in Fig. 5B, there were no bands with addition of lysozyme, hemoglobin and apo-transferrin human, while there was the correct size band in the thrombin, which indicated that these proteins had no effect on detection of thrombin. These results indicated that the proposed biosensor had good selectivity.

To assess the analytical application of the sensor, the method was used to detect thrombin in human sera. Since no thrombin was found from the human sera, addition and recovery experiment was performed to estimate the application of the assay in complex sample. As demonstrated in Table 2,  $5.0 \times 10^{-11}$ – $5.0 \times 10^{-8}$  M of thrombin was added into each human sera, the recovery ranges from 87.6% to 112.6%, and the relative standard deviation values were in the ranges of 4.3%–9.8%, which indicated that the method had good analytical application in human sera.

In summary, we have developed an ultrasensitive and selective sensor for thrombin by combining the PCR amplification and triplex formation. The fluorescence intensity was proportional to the logarithm of the concentration of thrombin over the range of  $1.0 \times 10^{-12}$  M– $1.0 \times 10^{-7}$  M, with a detection limit of 261 fM. The most important contribution is that the proposed strategy can overcome the effect of non-specific amplification and DNA contamination completely, which was realized by detecting PCR

Serum samples	Added thrombin(M)	Founded thrombin(M)	Recovery(%)	Relative standard deviation (%)
1	$5.0 \times 10^{-11}$	$5.63 \times 10^{-11}$	112.6	9.8
2	$5.0 \times 10^{-10}$	$4.66 \times 10^{-10}$	93.2	7.5
3	$5.0 \times 10^{-9}$	$4.38 \times 10^{-9}$	87.6	8.9
4	$5.0 \times 10^{-8}$	$5.44 \times 10^{-8}$	108.8	4.3

**Table 2. Recoveries of thrombin from the spiked human serum samples.** The values shown here are the average values from three measurements.

product with MB through triplex formation. Moreover, the proposed strategy may open a new avenue for the application of triplex DNA, it may be extend to immune - PCR and other aptasensor in the future.

## Methods

**Materials and Reagents.** All Oligonucleotides were purchased from Sangon Bioengineering Technology and Services Co. Ltd. (Shanghai, China). Human thrombin, lysozyme (LYS), apo-transferin human (ATH), hemoglobin (HB), spermine were purchased from Sigma-Aldrich (USA). Klenow Fragment (exo-) polymerase was purchased from Thermo scientific Inc (USA). Taq DNA Polymerase and dNTPs were obtained from Sangon Bioengineering Technology and Services Co. Ltd. (Shanghai, China). SYBR Green I (20 $\times$ ) was purchased from Bio Teke Corporation (Beijing, China). Nanopure water (18.1 M $\Omega$ ) was obtained from a 350 Nanopure water system (Guangzhou Crystalline Resource Desalination of Sea Water and Treatment Co. Ltd.) and was used in all experiments. All chemicals were analytical grade unless stated.

Tris-HAc buffer (20 mM, pH 7.9, 50 mM KAc, 10 mM Mg (Ac)<sub>2</sub>, and 1.0 mM DTT) was used throughout the recognition of thrombin with aptamer. Phosphate buffered solution (PBS) (pH 5.0, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, and 0.1 M NaAc) was used for triplex formation between PCR product and MB.

**Apparatus.** The PCR amplification was performed on a ETC 811 PCR Instrument (Eastwin Life Sciences, Inc., China). The fluorescence signal was measured on a RF-5301PC spectrofluorimeter (Shimadzu, Japan). The circular dichroism (CD) spectroscopy was obtained from a J-810-150S spectropolarimeter (JASCO International Co. Ltd., Japan).

**Recognition of thrombin and preparation of PCR template.** Probe A and probe B were denatured at 90 °C for 5 min, then cooled to 0 °C rapidly. 2  $\mu$ L of thrombin solution was mixed with 2  $\mu$ L 4.0  $\times 10^{-10}$  M probe A and probe B in Tris-HAc buffer, kept at 37 °C for 30 min. After that, 0.5 U Klenow Fragment (exo-) polymerase and 0.9  $\mu$ L 2.5 mM dNTPs were added, and the total mixture volume reached 10  $\mu$ L, kept the mixture at 37 °C for 30 min, along with heating to 95 °C to inactivate the Klenow Fragment (exo-) polymerase. Then the inactivated mixture was acted as PCR template for following PCR amplification.

**PCR amplification.** The PCR amplification was carried out in a 200  $\mu$ L PCR tube that containing 5.0  $\mu$ L PCR template, 2.0  $\mu$ L 10  $\mu$ M of two primers, 5  $\mu$ L of 10  $\times$  PCR Buffer, 1  $\mu$ L 5 U/ $\mu$ L of Taq DNA Polymerase, 3  $\mu$ L 25 mM of MgCl<sub>2</sub>, 1  $\mu$ L 10 mM of dNTPs and 31  $\mu$ L of Nanopure H<sub>2</sub>O. The thermal program was comprised of an initial denaturation at 95 °C for 10 min, 45 cycles of PCR amplification was carried out by using 30 s of denaturation at 94 °C, 30 s of annealing temperature at 60 °C, and 10 s of extending temperature at 72 °C. All the reactions were run in triplicates, and the control experiment was carried out with same reaction mixture in the absence of thrombin.

**Measurement of fluorescence spectrum.** 10  $\mu$ L of 10  $\mu$ M MB were mixed with 490  $\mu$ L PBS buffer solution which contained different amounts of PCR product. After 1.5 hours of incubation (25 °C), the fluorescence signal was measured with spectrofluorimeter. Slit widths were both 5.0 nm, and the excitation and emission wavelengths were set at 495 and 518 nm, respectively.

**Measurement of CD spectroscopy.** The circular dichroism (CD) spectroscopy was measured at room temperature and performed over the wavelength range from 200 to 300 nm in 0.1 cm path length cuvettes. The result was obtained by averaging 3 scans at the scanning rate of 100 nm per minute with a response time of 1.0 s and the bandwidth of 1.71 nm.

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## Author Contributions

L.S.Ling and X.M.Miao designed the experiments. Y.B.Li performed experiments and data analysis. L.S.Ling and Y.B.Li wrote the main text. All authors reviewed the manuscript.

## Additional Information

**Competing financial interests:** The authors declare no competing financial interests.

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