Fluorescence detection for DNA using hybridization chain reaction with enzyme-amplification†

Shuyan Niu, Yu Jiang and Shusheng Zhang*

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A signal amplification based on a combination of hybridization chain reaction (HCR) and enzyme-enhancement was applied in the study of a novel biosensor. The target sequence was added as catalyst which was simply transmitted but did not disappear as the DNA double strand grew on the surface of magnetic beads (MBs).

In gene chips, recognition is performed by single-stranded DNAs that are hybridized with complementary nucleic acid fragments, and a transduction process is usually performed by optical or electrochemical means.[1] Over the past years, enzyme-amplified nucleic acid assays were reported frequently to improve the sensitivity and the detection limit. For example, Xie and co-workers[2] have investigated the advantages of sandwich-type assays which did not require the chemical modification of the analyzed nucleic acids and for which the signal amplification was based on the combination of HRP and redox polymers. Del Giallo and co-workers studied the steric factors controlling the surface hybridization of PCR amplified sequences using an enzyme for the signal detection.[3]

Besides the modification of the hardware of the instruments like the modified electrode in electrochemical analysis or application of the enzyme which could catalyze redox reactions, polymerase chain reaction (PCR) is almost the most widely used in DNA detection. However, the polymerase chain reaction usually needs demanding conditions to keep the activity of polymerase, and on the other hand the PCR cannot produce modified strand directly.[4] To find a simple and sensitive method for specific sequences detection holds great promise in the development of biosensors.

As a matter of fact, single-stranded DNA is a versatile construction material[5] that can be programmed[6] to self-assemble into complex structures[7] driven by the free energy of base pair formation. Many academic researches are focusing on building synthetic molecular machinery from DNA. Such DNA nanomachines, made by self-assembly, usually include chain reactions or reaction cycles based on sequence-specific interactions. The typical research of this technology often involves a short single-stranded sticky end serving as a toehold for strand exchange. A complementary strand which first binds to the toehold can displace the shorter strand of the original duplex and the strand exchange proceeds by means of a random walk of the branch point separating regions hybridized to the competing strands. Yurke[8] and his co-workers studied the rate constant for such a reaction and the influence of the length of the toehold, and constructed a pair of DNA tweezers with two rigid double-stranded arms connected at one end by a flexible single-stranded hinge. Picuri and Frezza[9] made use of such strand exchange to develop a new nucleic acid diagnosis.

Since the DNA hairpin structure is a good reservoir of hybridization energy, Turberfield and co-workers introduced the idea that DNA loops could be used as a fuel.[10] In order to simplify FRET measurement of reaction rates, they used two-strand loop complexes. The hairpin loops were formed by hybridization of complementary domains at either end of a single strand,[11] however, the spontaneous hybridization rate of the two hairpins is slow compared with the cycle time of the motor, and so a means of catalyzing its hybridization is needed.[12]

Pierce and Dirks[13] first put forward the concept of hybridization chain reaction (HCR) and gave a detailed discussion of the free-running process, and we applied the modified fuels without any polymerase involved for the signal amplification in the DNA detection.

Two complementary hairpins (Bio-H1 and Bio-H2 in Scheme 1) with 18-base stems and 12-base loops both had 6-base sticky ends, which were stable hairpins respectively and would not open or hybridize with each other at room temperature. In detail, the stems of the hairpins of two kinds were totally complementary while one's sticky end and the other's loop had 6 bases complementary (see details in the supplementary information†). As is shown in Scheme 1, each hairpin was caught in a kinetic trap, preventing the system from rapidly equilibrating. Introduction of an initiator strand (T1) triggers a chain reaction of alternating kinetic escapes by the two hairpin species corresponding to "polymerization" into a nicked double helix. Amplification of the initiator recognition event continues until the supply of Bio-H1 or Bio-H2 is exhausted. Since the two fuels have been modified with biotin, we could use streptavidin-coated magnetic beads to immobilize the initiators and applied labeled horseradish peroxidase (avidin-HRP) to combine the enzyme-amplification and HCR together to achieve a better performance in the fluorescence detection.

It's important to note that in the hairpins, there were 6 successive "T" bases between sequence b–c or sequence b–a* in order to leave some space for the modifying groups during the hybridization.

In order to demonstrate the growth of the double helix, gel electrophoresis was applied to reveal the relation between the

Key Laboratory of Eco-chemical Engineering, Ministry of Education, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, Shandong, P. R. China. E-mail: shushzhang@126.com; Fax: (+86) 532-84022750; Tel: (+86) 532-84022750

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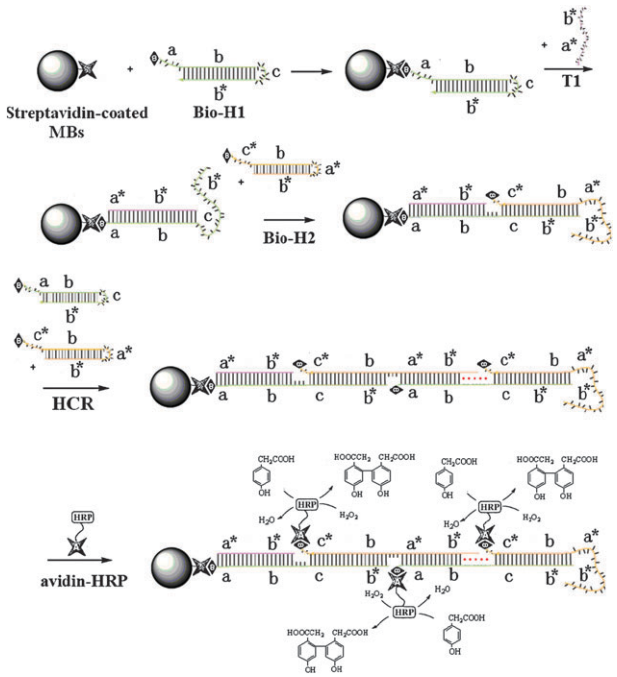

Scheme 1 When the T1 was captured by the MBs, the c–b* fragment was free, which would trigger the opening of Bio-H2. These two steps represent one cycle of the chain reaction where the target sequences a*–b* were regenerated. After the double helix growth was completed, avidin-HRP was added for the next enzyme-enhanced fluorescence detection.

average molecular weight and the amount of target. As is shown in Fig. 1, the less the initiator was, the larger the average molecular weight was. The average molecular weight of the resulting polymers, which is determined by the amount of the fuels distributed to one initiator, is inversely related to the initiator concentration when the amount of fuels is consistent.

Even if the growth of the double helix will not stop in terms of kinetics and thermodynamics until the fuels are exhausted, the length of the strand which grows along with the time passing will limit the freedom of the reactants and the system's instability also increases, which leads to deceleration of the reaction speed. Besides, during the analytical study, the long-playing operation will greatly affect the accuracy and

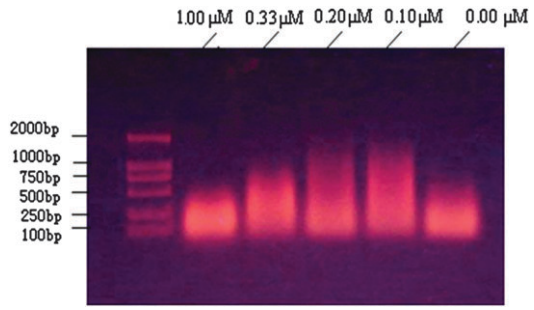

Fig. 1 The effect of initiator concentration on HCR amplification. Lanes 2–6: five different concentrations of initiator (1.00, 0.33, 0.20, 0.10, and 0.00 μM) in a mixture of 1 μM Bio-H1 and 1 μM Bio-H2. Lane 1: DNA markers with 100-bp to 2000-bp increments, respectively.)

reproducibility of the data. Therefore, we studied the optimized time of the growth in the system as shown in Fig. S4 (see supporting information†). We can clearly see the fluorescence intensity increased rapidly in the first 30 min and hardly rose after 2 h. Thus 1 h was determined as the HCR growing time.

Subfemtomolar concentration detection limits in DNA analysis have been achieved using an HCR system with enzyme-amplification. Fig. 2 shows representative fluorescence responses when the concentrations of target DNA hybridized were increasing under optimal experimental conditions. The HCR system raises the amount of HRP bound to the MBs and on the other side, the extra fuels left in the solution which exist in solution as long-stem hairpins can hardly have physisorption with the MBs. Thus the blank fluorescence response was negligible. The results show that the fluorescence intensity increased with the increase of target concentration in the range from 2 fM to 100 fM with a detection limit of 8.10×10^{-16} M as estimated by the 3σ rule. The calibration curve is shown in Fig. 2(B). The regression equation was $F = 19.79 + 8.819(C_{T1}/\text{fM})$ (F was the fluorescence intensity of bi-*p*,*p*′-4-hydroxyphenylacetic acid, background fluorescence was subtracted; C_{T1} was the concentration of the target) during the range from 2 fM to 100 fM and the correlation coefficient *r* was 0.9981.

To investigate the length of the product strand after HCR and compare the signal amplification by the HCR system with enzyme-amplification to a one-step enzyme-amplification DNA detection, comparison experiments were carried out by removing one kind of fuels in the HCR system to interrupt the chain reaction. After the probe and target were immobilized on the MBs, only Bio-H2 was added into the system. With full reaction, Bio-H2 was opened and a*–b* sequences were free. Since there was no a–b fragment existing in the solution, the hybridization reaction came to a halt. That means one target

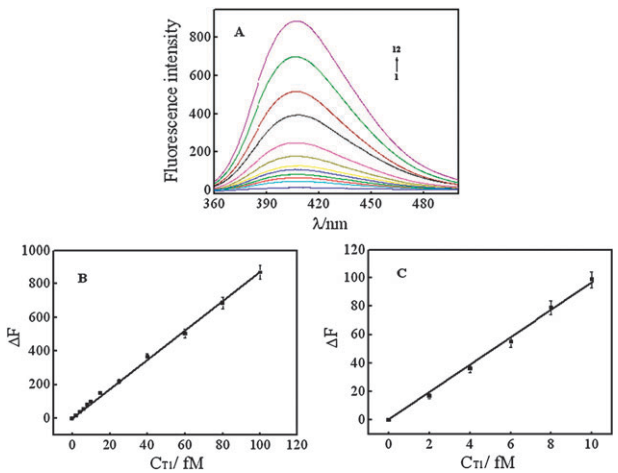

Fig. 2 (A) The fluorescence response of different concentrations of target DNA after the separation. (1) 0.00 M; (2) 2.00×10^{-15} M; (3) 4.00×10^{-15} M; (4) 6.00×10^{-15} M; (5) 8.00×10^{-15} M; (6) 1.00×10^{-14} M; (7) 1.50×10^{-14} M; (8) 2.50×10^{-14} M; (9) 4.00×10^{-14} M; (10) 6.00×10^{-14} M; (11) 8.00×10^{-14} M; (12) 1.00×10^{-13} M. Background fluorescence had been subtracted for each value. (B) Calibration curve of the DNA sensor, where the definition of signal is the same as that in (A). (C) is the amplification of the dots 1–6 in (B).

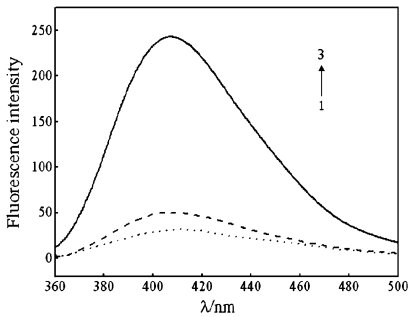

Fig. 3 The fluorescence responses after the separation; reaction with (1) 2.00×10^{-14} M three bases mismatch DNA; (2) 2.00×10^{-14} M single-mismatch DNA; (3) 2.00×10^{-14} M target DNA. Background fluorescence had been subtracted for each value.

corresponds to one labeled DNA strand only. Through the comparison experiments, the number of the labeled DNA combined on to the MBs, namely, the length of the double strand product on average could be worked out. The experimental results (see Fig. S5 in the supporting information†) showed that there were about 30 hairpins opened and combined into one long double helix, namely, 30-fold magnification compared with the one-step enzyme-amplification (we choose the lowest concentration of the linear range as the comparison standard). Besides, the comparison to other DNA detection methods, like single enzyme-amplification with fibers or Au nanoparticles-amplification, is also discussed in Table S1 in the supplementary information.† Apparently, biosensors with super sensitivity often need complex modification and the typical advantages of this HRP-amplification biosensor are the simple operation and competitive sensitivity.

The HCR system has also provided excellent capability in discriminating two target DNA sequences that differed by only one base upon DNA hybridization. The selectivity of the present biosensor was investigated by using the same concentration of complete complementary target DNA sequences, the one-base mismatched DNA sequences and the three-base DNA sequences as initiator, respectively. As is shown in Fig. 3, a well-defined fluorescence signal of bi-*p*,*p*′-4-hydroxyphenylacetic acid was obtained for the complementary sequences. The fluorescence intensity for one-base mismatched sequences was only about 20% of that for complementary DNA at the same concentration, while for the three-base mismatched sequences it was about 10%. The results showed that the mismatch ssDNA can hardly open the hairpin and trigger the chain reaction, because the replacement of the random walk of the branch point separating regions can hardly proceed if the displacement strands are not competitive enough, which means the HCR growth has a demanding requirement for the specific sequences of the initiator.

In conclusion, we designed a DNA nanomachine system for a novel biosensor and demonstrated the superiority of its utilization combined with enzyme-amplification in the fluorescence quantitative analysis of DNA detection. The growth of the double-strand DNA has been demonstrated by gel electrophoresis and fluorescence detection. The target DNA triggered the HCR system in which the different labeled hairpins were combined into one long double helix and consequently separated by magnetic beads. The quantitative analysis of target ssDNA was thus carried out by measuring the fluorescence intensity of bi-*p*,*p*′-4-hydroxyphenylacetic acid generated by HRP catalyzing HPA in the system and obtained 8.10×10^{-16} M as detection limit estimated by the 3σ rule and a linear range from 2 fM to 100 fM.

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