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Assistant DNA recycling with nicking endonuclease and molecular beacon for signal amplification using a target-complementary arched structure†

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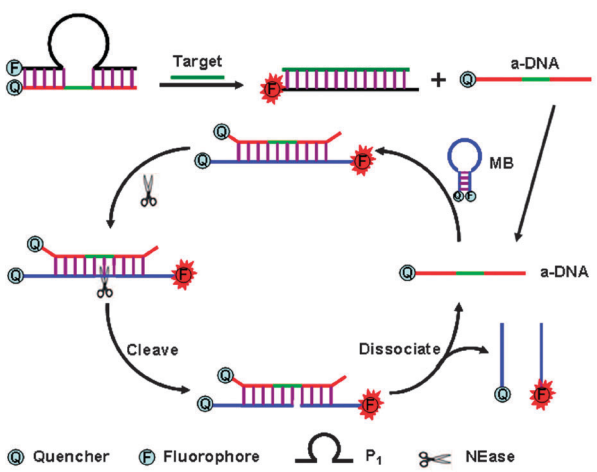
A simple and universal method for ultrasensitive "signal on" detection of DNA was developed with a target-complementary arched structure to release assistant DNA, which was recycled with nicking endonuclease to amplify the detectable fluorescent signal of molecular beacons.

Recently, signal amplification has become an important concept in the highly sensitive detection of specific DNA sequences for clinical diagnostics, gene therapy and environmental monitoring.[1] In general, signal amplification can be achieved by nanobiotechnology[2] and molecular biological protocols such as strand-replacement polymerization,[3] ligase chain reaction[4] and rolling circle amplification.[5] The latter have realized the detection of target DNA at femto- or attomolar levels with the aid of nuclease, *e.g.*, polymerase,[6] exonuclease,[7] and endonuclease.[8,9] Among these nucleases, nicking endonucleases (NEases) possess excellent cyclic efficiency.[10] A NEase assisted strand scission cycle has been designed for ultrasensitive detection of DNA and cancer cells,[11] and a NEase assisted nanoparticle amplification strategy has been proposed for homogeneous colorimetric detection of DNA by recognition of the long single-stranded oligonucleotides.[12] Due to the need for specific sequences for NEases, these methods can only be applied in the detection of sequence-specific target DNA. To overcome this shortcoming, several strategies based on the Y-shaped junction structure consisting of three complementary oligonucleotide branches have been introduced to release the target for recycling.[13–15] These strategies must optimize the ratios of three oligonucleotide sequences and the length of the assistant probe for obtaining a high signal to noise ratio. This work presented a novel concept of assistant DNA (a-DNA) recycling by using a target-complementary arched structure to release the a-DNA.

The arched DNA structure originates from the metal ion-based DNAzymes, which consist of the substrate and enzyme strands,[16] and has been employed to develop highly sensitive and selective biosensors for metal ions.[17] Here, the arched structure was formed by hybridization of fluorescent dye-labelled target-complementary probe 1 (P_1) with quencher-labelled a-DNA. In the absence of target DNA, the entire molecular recognition module was stable, and the formed arched structure did not emit any fluorescent signal. In the presence of target DNA, the target hybridized with the P_1 in the arched structure to release the quencher-labelled a-DNA, resulting in the restoration of fluorescence. Subsequently, the released a-DNA hybridized with the reporter molecular beacon (MB) to trigger the a-DNA recycling and restore the fluorescent signal from MB with the aid of NEase, leading to the amplification of fluorescent signal from MB (Scheme 1). The a-DNA recycling depended on the formation of a double-stranded recognition site for NEase, which cleaved the opened MB strand and released the a-DNA to initiate the next cycle of cleavage. The total amount of free a-DNA was decided by the amount of target DNA when the arched structure was in excess. Therefore, the enhanced fluorescence signal was related to the amount of target DNA, producing an ultrasensitive method for "signal on" detection of target DNA. The sequences of both the



Scheme 1 Schematic representation of assistant DNA recycling strategy with the arched structure and NEase for signal amplification.

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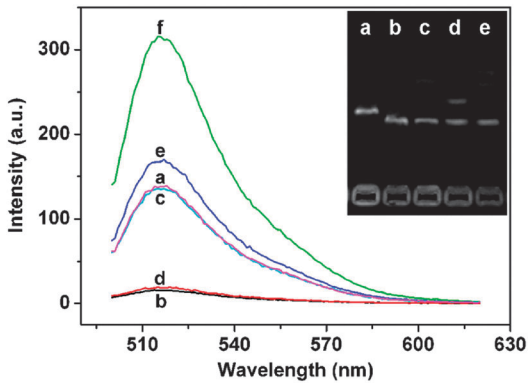

Fig. 1 (A) Fluorescence spectra of 1.0 μmol L^{-1} P_1 (a), *a* + 1.0 μmol L^{-1} BHQ-labelled a-DNA (b), *b* + 1.0 μmol L^{-1} target DNA (c), *b* + 5 U NEase (d), *c* + 10.0 μmol L^{-1} MB (e), and *d* + 10.0 μmol L^{-1} MB (f). (B) PAGE analysis of 1 μmol L^{-1} P_1 (a), *a* + 1 μmol L^{-1} BHQ-labelled a-DNA (b), *b* + 1 μmol L^{-1} target (c), *c* + 1 μmol L^{-1} MB (d), and *d* + 5U NEase (e).

MB and the a-DNA, which participated in the designed a-DNA recycling, were irrelevant to the target DNA, thus this method avoided the requirement of a NEase-specific recognition sequence in target DNA.

To confirm the detection mechanism of the a-DNA recycling strategy, the fluorescence intensity of the sensing system was first tested (Fig. 1A). P_1 showed an obvious fluorescent peak of carboxyfluorescein (FAM) at 517 nm (curve a). After adding the BHQ-labelled a-DNA into P1 solution, the fluorescence of FAM was quenched by the BHQ (curve b), indicating the nearness of the FAM group in P_1 to BHQ in the a-DNA due to the hybridization of P_1 with the a-DNA to form the arched structure. Upon the addition of target DNA into the mixture, the fluorescence intensity was restored (curve c). Interestingly, when the amounts of P1, BHQ-labelled a-DNA and target DNA were identical, the fluorescence could be restored to the same value as that of P_1 alone. This result suggested the a-DNA was released from the arched structure. Subsequently, after adding the MB probe, a slightly enhanced fluorescence peak was observed (curve e) since the released a-DNA could recognize the MB to open its cycle. Significantly, when 5 U NEase was added into this system, the fluorescence increased sharply (curve f). In contrast, when NEase was added into the solution of arched structure, no fluorescence response was observed (curve d). These phenomena demonstrated that the presence of target DNA led to the NEase aided a-DNA recycling after the double-stranded recognition site for NEase was formed, which could be efficiently used for amplifying the detection signal of target DNA.

Polyacrylamide gel electrophoresis (PAGE) analysis was used to investigate the viability of the sensing strategy (inset in Fig. 1). The mixture of P_1 and BHQ-labelled a-DNA showed only one band at the position different from the P_1 (lanes a and b), indicating the formation of the homogeneous arched structure due to the hybridization of P_1 with the a-DNA. After the target was added, a new weak band appeared (lane c), which corresponded to the released single stranded a-DNA. Upon addition of MB to the mixture for lane c, the free a-DNA could react with the MB to form a double-stranded complex with a faster migration rate than the arched structure (lane d). In the

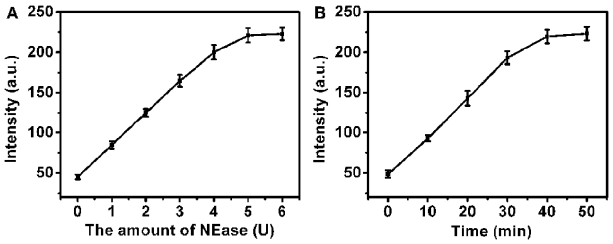

Fig. 2 Dependence of fluorescence intensity for 1 nmol L^{-1} target DNA on (A) the amount of NEase, (B) reaction time of a-DNA cycling. When one parameter changes the others are under their optimal conditions.

presence of NEase, three bands could be observed (lane e): two were similar to those in lane d, and another showed the fastest migration rate, which should be attributed to the small DNA pieces produced in the cleavage of MB by the NEase. The PAGE data demonstrated the feasibility of the designed strategy.

The amount of NEase and reaction time were optimized at 1.0 nmol L^{-1} target DNA and 10.0 μmol L^{-1} MB. At the reaction time of 1 h, the fluorescence intensity increased significantly with the increasing NEase concentration and reached a constant value at 5 U (Fig. 2A). Therefore, 5 U was chosen as the optimum concentration of NEase, at which the fluorescent intensity increased with the increasing reaction time up to 40 min (Fig. 2B). Therefore, 40 min was used for NEase-induced a-DNA recycling.

Under the optimal assay conditions, the fluorescent intensity increased with the increasing concentration of target DNA up to 10 nmol L^{-1} (Fig. 3), which implied that more a-DNA in the arched structure were replaced by the target to initiate the NEase-induced a-DNA recycling, which led to more cleaved MB strands to emit the fluorescence. The logarithm of $F–F_0$ was proportional to the logarithm value of target concentration in the range from 1.0×10^{-12} to 1.0×10^{-8} mol L^{-1} due to the exponential amplification of fluorescence signal by the a-DNA recycling (inset in Fig. 3). Here, F_0 and F are the fluorescence intensities detected in the absence and presence of target DNA, respectively. The detection limit was 0.21 pmol L^{-1} at 3σ. This detection limit was lower than that of the Y-shaped junction structure (50 pmol L^{-1})[14] and conventional NEase signal

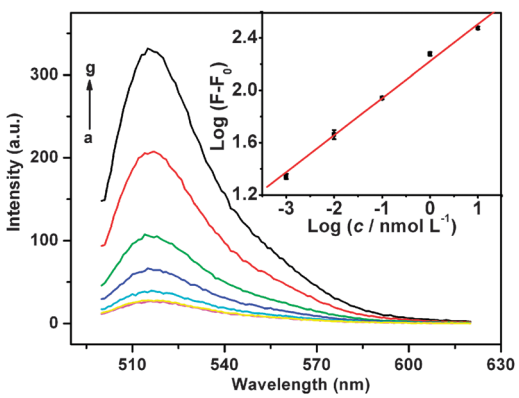

Fig. 3 Fluorescence spectra for blank (a) and target DNA at 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} and 10^{-8} mol L^{-1} (from b to g) with a-DNA recycling strategy for signal amplification. Inset: plot of logarithm of $F–F_0$ *vs.* logarithm of target concentration.

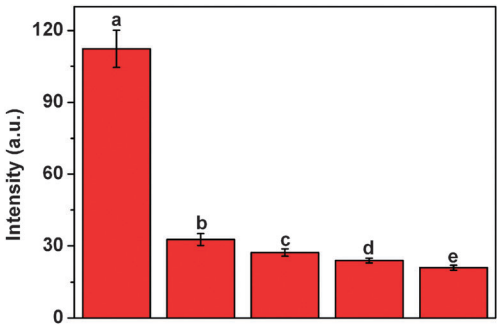

Fig. 4 Histograms of fluorescence intensity for 0.1 nmol L^{-1} complementary (a), single-base mismatched (b), two-base mismatched (c), three-base mismatched (d) DNA, and blank (e).

amplification (20 pmol L^{-1})[8] with fluorescence measurement. To confirm the source of the high sensitivity, a control experiment in the absence of NEase was carried out under similar conditions. The plot of the fluorescent intensity *vs.* the logarithmic value of target concentration showed a good linearity from 1.0×10^{-9} to 1.0×10^{-6} mol L^{-1} (Fig. S1, ESI†), and the detection limit at 3σ was 0.16 nmol L^{-1}, which was about 760 times higher than that obtained in the presence of NEase. At the same target DNA concentrations of 1.0 and 10 nmol L^{-1}, the fluorescent intensity in the presence of NEase was 4.97 and 4.32 times higher than those in the absence of NEase, respectively. Therefore, the high sensitivity was attributed to the a-DNA recycling with NEase and MB.

Different kinds of DNA including complementary DNA, single-base mismatched DNA, two-base mismatched DNA, and three-base mismatched DNA were chosen to investigate the selectivity of this approach. The peak intensity at 517 nm for perfectly matched, single-base mismatched, two-base mismatched, and three-base mismatched DNA was about 5.33, 1.55, 1.28, and 1.14 times higher than the background signal, respectively (Fig. 4). Although the fluorescence intensity increased with the increasing concentration of the mismatched DNA (Fig. S2, ESI†), both the slopes and the fluorescence signals for mismatched DNA were much lower than those for the perfectly matched target at the same concentration, and only 1 μmol L^{-1} single-base mismatched DNA showed a signal similar to that of the perfectly matched target at 1 pmol L^{-1}, indicating a good selectivity of the proposed method. Therefore, this DNA sensing system could be used to discriminate perfectly matched and mismatched DNA, which should be attributed to the relatively long loop of the P_1, which formed a thermodynamically stable arched structure. The high specificity of this method led to great potential for single nucleotide polymorphism analysis. To evaluate the potential application, recovery testing was carried out by spiking target solution into human serum. At the concentrations of 10^{-11} and 10^{-9} mol L^{-1}, the recoveries were 98.5 ± 0.3% and 104.1 ± 0.7% (n = 3), respectively, indicating acceptable precision and the possibility for real analytical application.

In summary, the target-complementary arched structure was successfully designed to trigger the a-DNA recycling with NEase and MB for signal amplification, which led to a highly sensitive method for fluorescent detection of target DNA. The entire detection time was less than 2 h. Based on the a-DNA recycling, the strategy showed a detection limit at the sub-picomolar level, which was about 3 orders of magnitude lower than that of the conventional hybridization without NEase-based amplification. Moreover, since the a-DNA recycling replaced the target DNA recycling, the proposed strategy provided a universal strategy in DNA detection without the requirement of a NEase-specific recognition sequence in the target DNA. The novel concept of an a-DNA recycling strategy can be expected to design an integrated, portable and low cost device for DNA detection based on the arched structure.

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