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A universal biosensor for multiplex DNA detection based on hairpin probe assisted cascade signal amplification†

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A hairpin DNA probe mediated cascade signal amplification method was developed for visual and rapid DNA analysis with a detection limit of 100 aM. The implementation of tag/anti-tag DNA and gold nanoparticle reporters permits a universal platform for multiplex genotyping without instrumentation.

Simple and cost effective analysis of specific nucleic acids is highly sought in a number of fields such as clinical diagnostics, environmental monitoring, and food safety.[1] Target amplification is a commonly used strategy, including polymerase chain reaction (PCR) and some isothermal amplification techniques.[2] Compared with traditional blotting techniques,[3] the target amplification strategy offers high sensitivity and specificity, yet with its limitations in point-of-care applications. PCR requires thermocycling equipment that makes it unsuitable for providing cost effective access to end-users. The isothermal amplification methods involve sophisticated primer design, multiple enzymatic processes or initial heat denaturation, which bring potential problems in robustness.[2b] The design of point-of-care approaches for sensitive DNA analysis is in continuous demand.[1b]

Several signal amplification protocols have recently emerged as promising alternative to traditional target amplification because they are simple and thermocycler-free.[4] In addition, the implementation of hairpin DNA probes or padlock probes provides high specificity. These novel methods involve nicking endonucleases (NEase),[4a] exonucleases[4b] or polymerases[4c] that can create target-triggered biological circuits and yield enhanced fluorescent/electrochemical signals under isothermal conditions. Recently, a new signal amplification strategy based on NEase-assisted strand-displacement polymerization (NASDP) has been developed.[5a] NASDP is based on a hairpin probe-dependent biological circuit where the additional polymerization induced by NEase offers high sensitivity and short assay time. Using electrochemiluminescence, a detection limit of as low as 5 aM was reached.[5b] Although instrument-dependent readout, and lack of portability seriously hinder its applications in point-of-care formats, such a sensitive and simple strategy would be promising in the development of convenient household devices that can be practically suited for point-of-care applications.

Due to visual readout, short assay time, cost-effectiveness, and portable format, lateral-flow biosensors (LFBs) have been increasingly used for DNA analysis.[6] Compared with fluorescent/electrochemical platforms, LFBs minimize the requirements for highly qualified personnel and eliminate the complex procedures that involve expensive instrumentations.[7] To explore NASDP in point-of-care applications, it would be of great interest to combine LFB with NASDP.

Herein, for the first time, we developed a universal biosensor for visual and sensitive detection of DNA based on NASDP and LFB. In this study, a new hairpin probe with a biotinylated 5′-end and a loop containing a NEase (Nt.BbvCI) recognition site was developed for integrating NASDP with LFB, in addition, the implementation of multiple tag/anti-tag DNA pairs and gold nanoparticle reporters permits a universal lateral-flow platform for multiplex genotyping. The universal biosensor was first demonstrated by detecting a synthetic single-stranded target DNA, and then extended to analyze the genotype of the cytochrome P450 2D6 (CYP2D6) gene coding for one of the most important enzymes involved in drug metabolism.[8]

NASDP constitutes an integrated signal amplification reaction composed of two molecular circuits that are designed to detect and amplify target DNA (Scheme 1A). The first circuit (circuit 1) is designed to detect target DNA by a hybridization-induced conformational switch in a designed hairpin probe (HP1). In circuit 1, following hybridizing with target DNA (DNA1 in Table S1, ESI†), HP1 is open to a DNA tag-labelled primer (primer1 in Table S1, ESI†) that subsequently initiates DNA polymerization on the probe (step I). During DNA polymerization, the polymerase extends the primer and displaces the target (step II). As a result, a biotin- and DNA tag-labelled duplex DNA is formed, whereas the target is free to bind to another probe and initiates a new cycle of hybridization, polymerization and displacement (step III). The second circuit in NASDP is designed to initiate a signal amplification process (circuit 2). The 3′-region of HP1 contains a NEase recognition sequence (the red region in the probe) designed to initiate additional

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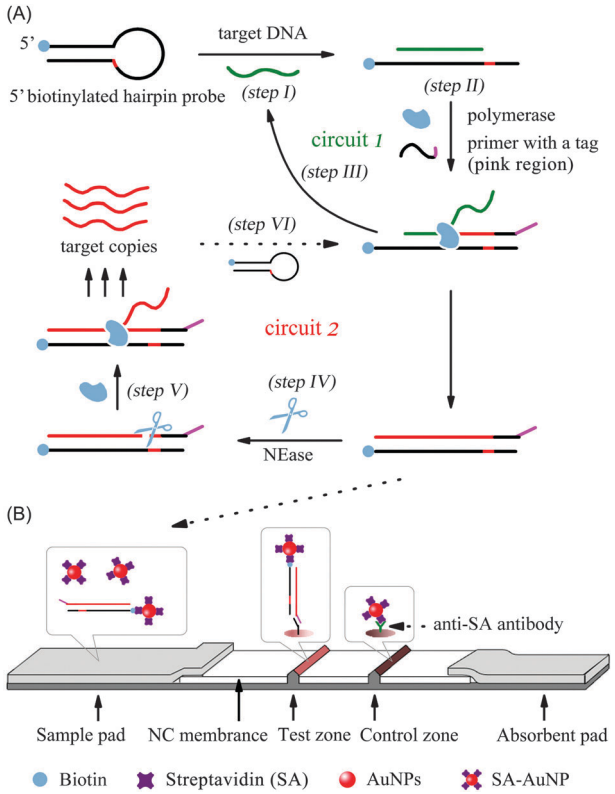

Scheme 1 (A) Schematic outline of the NEase-assisted strand-displacement polymerization (NASDP). (B) Schematic illustration of the lateral flow biosensor for visual analysis of the NASDP products.

polymerization reactions. In the presence of a target, a biotin- and DNA tag-labelled duplex DNA is produced and only then the recognition sequence is a suitable substrate for NEase 'nicking' (step II). After nicking of the duplex DNA (step IV), the 3′-end of the nicked DNA primes a subsequent cycle of DNA displacement and re-synthesis of a new cDNA in the presence of polymerase (step V). With each cycle of the circuit 2, new copies of target DNA are synthesized, which can open additional hairpin probes (step VI). Once circuit 2 is initiated, the nicking, polymerization and displacement reaction cycle continues to produce multiple copies of target DNA. This enables multiple probes to be open in a series of cyclic cascade reactions. Activation of the two circuits can yield large amounts of the duplex DNA characterized with a DNA tag and a biotin group, which are subsequently applied on a LFB for visual detection.

The LFB consists of three components: sample pad, nitrocellulose membrane, and absorption pad (Scheme 1B). Streptavidin–biotin–anti-tag DNA 1 (CP1, complementary to the tag DNA of primer1) and anti-streptavidin antibodies are dispensed on the nitrocellulose membrane to form a test and a control zone, respectively. The resulting products in NASDP are first mixed with streptavidin-modified gold nanoparticles (SA–AuNPs), forming the complexes of dsDNA–biotin–SA–AuNP, and then the mixture is applied on the sample pad. The solution migrates along the strip by capillary action. The resulting complexes are captured on the test zone by the reaction between tag DNA and the CP1. Consequently, the accumulation of AuNPs on the test zone is visualized as a characteristic red band. The excess of the sample continues to migrate and is captured on the control zone by the reaction between SA–AuNPs and anti-SA antibody, thus forming a second red band. In the absence of target DNA, no red band is observed on the test zone. In this case, a red band on the control zone shows that the biosensor works properly. Quantitative analysis is realized by recording the optical density of the band with a portable strip reader (ESI†).

In this work, the SA–AuNP conjugates were prepared with a mixed solution of SA and BSA because a higher LFB response was observed compared with the traditional procedures where AuNPs are first coated with SA and then with a blocking agent such as bovine serum albumin (BSA).[9] Thus, SA/BSA ratio was investigated at a fixed SA concentration of 5 g L^{-1} that gave maximum peak area as shown in Fig. S1 of ESI.† As shown in Fig. 1A, the peak area increased with reducing molar ratios from 100 to 1; further decreasing BSA led to a drop in the peak area. Hence, SA–AuNP conjugates were prepared using equal amounts of SA and BSA. The stability of the resulting conjugates was tested using various concentrations of Na^+ ions, and the conjugates remained stable even after the addition of 1 M [Na^+] (Fig. S2, ESI†). The volume of the conjugates used was optimized because it affects the sensitivity of the LFB. According to Fig. S3 in ESI,† 5 μL of conjugates offered maximum peak area, thus, was used in subsequent experiments.

Under optimum LFB assay conditions, the NASDP was optimized by detecting 1 nM of target DNA on LFB. To ensure that the hairpin probe remains thermally stable during the assay, the optimum [Na^+] was investigated by comparing S/N ratios. As shown in Fig. 1B, the S/N ratios rose with the increase in [Na^+] but decreased after [Na^+] exceeding 200 mM. This was attributed to the low stability of the probe stem at low salt concentrations and the primer-induced nonspecific signal amplification. Temperature is an important factor that affects the annealing of primers and stem stability of the probes, thus, it was investigated by comparing the S/N ratios at four different temperatures. It was observed that the ratio increased with temperature from 25 °C to 37 °C, and decreased after 37 °C (Fig. S4, ESI†). The decrease in the S/N ratio can be explained by high background that resulted from target-independent conformational switch (step I in Scheme 1A) at high temperature and subsequent nonspecific amplification. Hence, 200 mM [Na^+] and 37 °C were used in the following experiments. The sensitivity was strongly affected by the incubation time of NASDP. Within the first 60 min the LFB response elevated gradually as the incubation time increased, and a further increase in the time led to decreased S/N (Fig. S5, ESI†). Thus, 60 min was the optimum incubation time.

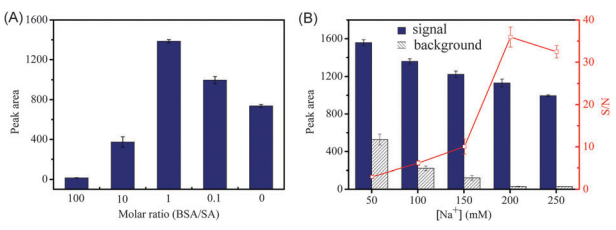

Fig. 1 (A) Effect of the molar ratio of BSA to SA on the responses of 1 μM target DNA on the LFB without NASDP. (B) Effect of [Na^+] on the responses of 1 nM target DNA on the LFB with NASDP. Values represent the intensity means ± s.d. of triplicate reactions.

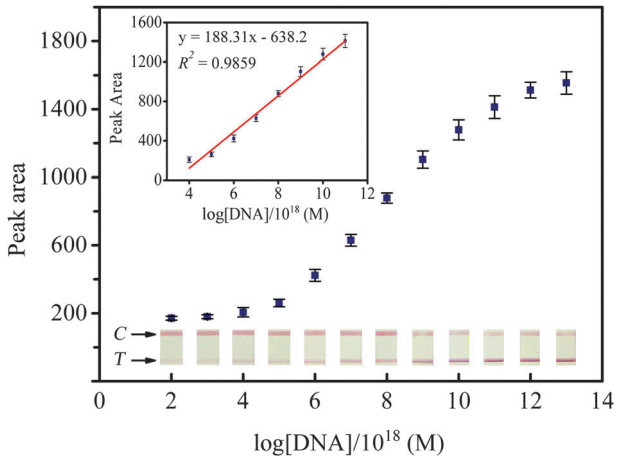

Fig. 2 Calibration curve of the assay with different concentrations of target DNA. The inset shows corresponding photo images of LFBs after applying the NASDP product. Values represent the intensity means ± s.d. of triplicate reactions.

To determine the sensitivity of the developed biosensor, a series of concentrations of target DNA were examined. Quantitative analysis was performed by recording the intensities of the test lines. As shown in Fig. 2, the peak area increased with the increase in the logarithm of DNA concentration, and it showed a good linear correlation with the logarithm of the DNA concentration from 10 fM to 100 nM. For qualitative analysis, a red band in the test zone was observed with as low as 0.1 fM of target DNA (inset photos, Fig. 2). In 1 mL of whole blood there are approximately 0.75–1.83 fM of genomic DNA,[10] thus, the sensitivity of the biosensor is applicable in the clinical practices.

The specificity was examined by detecting 1 nM of synthetic target DNA (DNA1), single-base mismatched target DNA (DNA2), and two base mismatched target DNA (DNA3). No red band was observed in the test zone in the presence of the mismatched targets (Fig. 3A). In addition, the system displayed good specificity when mismatched DNA is in 10× and 100× excess of matched DNA (Fig. S6, ESI†). These indicated that the biosensor has an excellent specificity which could distinguish target DNA from non-target DNA that differs in only a single nucleotide. The reproducibility of the assay was assessed by testing different samples. The coefficients of variation of the tests (peak areas) for 1 nM DNA1, 1 nM DNA2, 50 nM non-complementary DNA and 0 nM DNA were 5.8%, 7.4%, 6.5%, and 4.6%, respectively (n = 10). The overall relative standard deviation was less than 8.0%, which indicates good reproducibility. CYP2D6*4 is one of the most common polymorphic loci in CYP2D6 gene.[8] To evaluate the analytical performance of the biosensor in multiplex detection, two allele-specific hairpin probes (HP2 and HP3 in Table S1, ESI†) and the corresponding primers (primer2 and primer3 in Table S1, ESI†) were designed, targeting two alleles of CYP2D6*4, namely, the wild and the mutant alleles (A1 and A2 in Table S1, ESI†). The anti-tags (CP2 and CP3 in Table S1, ESI†) for HP2 and HP3 were immobilized on the LFB to form the test zones (T1 and T2 in Fig. 3B) of the CYP2D6*4 alleles. As shown in Fig. 3B, two test bands were observed on the LFB in the presence of 1 nM of both alleles; only one test band appeared when 1 nM of wild or mutant allele was present respectively. These results indicated that the proposed biosensor was able to differentiate between homozygous and heterozygous mutants at CYP2D6*4 locus in a single assay with allele-specific hairpin probes and tag–anti-tag pairs.

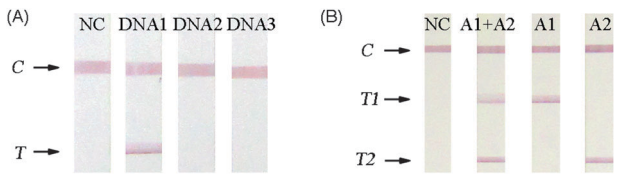

Fig. 3 (A) Photo images of the LFBs for specificity analysis using 1 nM of target DNA (DNA1), single-base mismatched target DNA (DNA2), and two base mismatched target DNA (DNA3). (B) Photo images of the LFBs for genotyping the wild and the mutant alleles (A1 and A2) of the CYP2D6*4 locus.

In previous studies of lateral-flow approaches,[11] the extent of multiplex was limited by the availability of post-amplification manipulations,[11a] cross-reactions among multiple primers,[11b] and the number of protein capture agents,[11c] in contrast, the multiplex capability of this approach would be easily extended with additional probes and tag–anti-tag pairs.

In conclusion, a universal lateral-flow biosensor for multiplex DNA detection was developed. The proposed approach eliminates the need for any instrument, and allows simple and visual analysis of nucleic acids with a detection limit of 100 aM within 75 min. The use of lateral flow biosensor, coupled with gold nanoparticles, tag/anti-tag DNA and NASDP, can lead to a novel range of detection systems capable of multiplex and cost effective genotyping without any specialized instrument and highly qualified personnel. This work will be the basis for future work aiming at the development of convenient household devices to sensitively detect the genetic variations related to genetic diseases and drug metabolism.

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