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A new mode for highly sensitive and specific detection of DNA based on exonuclease III-assisted target recycling amplification and mismatched catalytic hairpin assembly†

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A novel electrochemical sensing strategy was developed for highly sensitive and specific detection of DNA by combining the exonuclease III-assisted target recycling amplification with mismatched catalytic hairpin assembly. The proposed method showed high sensitivity and selectivity with a dynamic response range from 100 fM to 5 nM.

The highly sensitive and specific detection of low abundance of DNA has become imperative in clinical diagnosis, gene therapy, mutation analysis and pathogen detection.^{1–4} Over the past decades, different techniques and methods have been developed for DNA detection, such as DNA microarrays, colorimetric biosensors, chemiluminescent biosensors, electrochemical biosensors, surface plasmon resonance biosensors, and so on.^{5–9} Among these techniques, electrochemical biosensors have received increasing attention owing to their easy operation, rapid response, low cost, small dimensions and inexpensive instrumentation.^{10,11}

Recently, the signal amplification strategies for ultrasensitive DNA detection have attracted more and more attention, because they can overcome the inherent limitation of target-to-signal ratio of 1:1 in the traditional hybridization assay and acquire higher sensitivity.¹² So far, a number of amplification methods have been developed, such as the hybridization chain reaction,¹³ the strand-displacement amplification,^{14,15} rolling circle amplification (RCA)^{16,17} and the catalysis by nucleases.^{18–20} Among them, the use of various nucleases, such as exonuclease and endonuclease, has particularly received interest. Unlike nicking endonucleases,

a sequence-dependent enzyme, exonuclease III (Exo III), not requiring any specific recognition site, can catalyze the blunt or recessed 3'-terminus of double-stranded DNA with stepwise removal of mononucleotides and shows lower activity on single-stranded DNA or duplex DNAs with a protruding 3'-terminus.^{21,22} Thus, Exo III can provide a more universal platform for sensitive detection of DNA, RNA and proteins.^{23–26} On the other hand, catalytic hairpin assembly (CHA), a nonenzymatic amplification technique, has also been proven to be conducive in amplifying and transducing signals for nucleic acid analytes over the past few years.^{27,28} Hundred-fold catalytic amplification can be achieved by a CHA reaction. Meanwhile, CHA can readily convert analyte binding to fluorescent, electrochemical, colorimetric signals, and so on.^{29–32} Although CHA has a lot of advantages, the great background signal is the most troublesome problem due to the nonspecific CHA products in the absence of target, which may counteract the specificity of CHA-based signal amplification methods.^{33,34}

With the purpose of reducing nonspecific CHA products in the absence of target, this work introduces mismatched base pairs into the breathing sites of the hairpin substrates. Based on exonuclease III-assisted target recycling amplification (ERA) and mismatched CHA, a novel strategy has been developed for highly sensitive and specific detection of DNA for the first time. This biosensing strategy exhibits excellent analytical performance and may provide a simple and powerful platform for DNA screening in bioanalysis, clinical diagnosis, pathogen detection, and environmental monitoring.

As illustrated in Scheme 1, we designed three kinds of stem-loop DNA molecular beacons (denoted as H0, H1 and H2). H0 is ingeniously designed with the protruding 3'-end and cannot be digested by Exo III which specifically cleaves duplex DNA from a blunt or recessed 3'-terminus. H1 and H2 are designed referring to our recently published work (see ESI† for details).³⁴ The H0 consists of two regions, one region is complementary to target DNA at the 3'-terminus, the other region is complementary to part H1. In the presence of target DNA, H0 is opened by hybridizing with target DNA to form a DNA duplex with a blunt 3'-terminus. Exo III can catalyze the stepwise removal of mononucleotides from this terminus, releasing the target DNA and output DNA. The released target DNA is

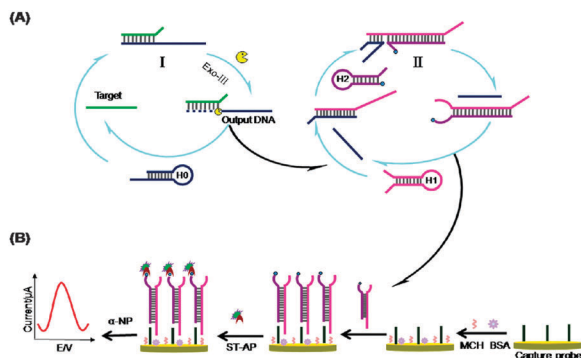
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Scheme 1 Schematic representation of the designed strategy for target DNA detection.

free to participate in the next cycle of hybridization and cleavage process (Cycle I). The output DNA can hybridize with H1 and unfold the hairpin structure of H1. The opened H1 assembles with biotin-tagged H2 to displace the output DNA. The liberated output DNA again hybridizes with the hairpin sequence of H1 and initiates the mismatched CHA cycles, resulting in the generation of massive H1–H2 complexes (Cycle II). Afterwards, the capture probes immobilized on the gold electrode can specifically hybridize with the H1–H2 complexes. The streptavidin-alkaline phosphatase (ST-AP) is labeled on the capture probe–H1–H2 complexes by the specific recognition of ST and biotin, which produces enzymatic electrochemical signal readout. The dual signal amplification strategies might provide a novel sensing platform for sensitive and specific detection of DNA.

In order to evaluate the stepwise biosensor fabrication, different modified processes were characterized by electrochemical impedance spectroscopy (EIS) and square wave voltammetry (SWV) (see ESI†). Both results of EIS and SWV proved that the biosensor worked indeed as described in the principle scheme.

To verify whether the ERA and mismatched CHA can proceed as designed, a native polyacrylamide gel electrophoresis (PAGE) experiment was carried out (Fig. 1). The mixture of H0 and target showed two bands (Fig. 1A, lane b) at two positions different from the H0 (lane a), which resulted from the hybridization of H0 with target (H0–T complexes), leading to a new band. After the reaction mixtures were incubated for 105 min with participation of Exo III, some bands for residual sequences appeared, instead of the band for H0–T complexes (lane c), revealing that the ERA was carried out as expected. In the absence of target, the band of H1–H2 complexes was very weak (lane d). However, the strong band of H1–H2 complexes was observed in the presence of target (lane e), indicating that mismatched CHA could occur as expected and only a few of H1 and H2 initiated CHA without the participation of target. In this strategy, Exo III played an important role in signal amplification. The DPV response of 500 pM target in the presence of Exo III was larger than that in the absence of Exo III, because Exo III could assist recycling amplification (Fig. 1B). Hence, the biosensing platform was established for the highly specific and sensitive determination of target DNA.

Under the optimal experimental conditions (see ESI† for details), the differential pulse voltammetry (DPV) responses for

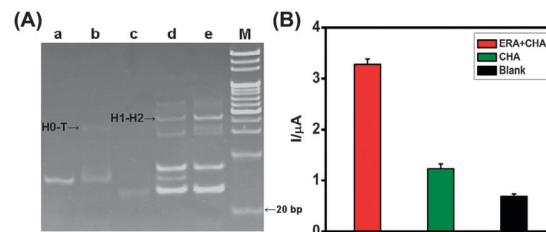


Fig. 1 (A) The native PAGE analysis: lane a: H0, lane b: H0 + target, lane c: H0 + target + Exo III, lane d: H0 + Exo III + H1 + H2, lane e: H0 + target + Exo III + H1 + H2 and lane M: 20 bp DNA Ladder Marker. The 12% nated PAGE analysis was carried out in $1 \times$ TBE/Mg²⁺ (pH 8.0). (B) Comparison of DPV peak currents in the absence (blank) and presence of 500 pM target with CHA (without ERA) and ERA-CHA.

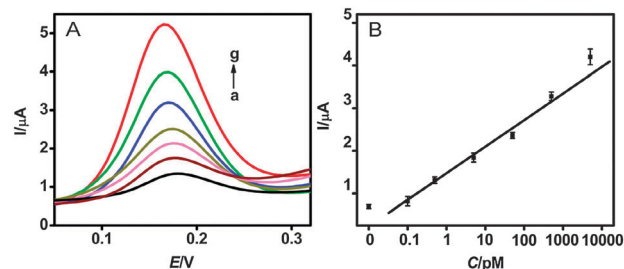


Fig. 2 (A) DPV curves responding to 0, 0.1, 0.5, 5, 50, 500, 5000 pM target DNA (from a to g). (B) Quantitative dynamic range of the designed strategy. The error bars represent the standard deviations calculated from three different spots.

synthetic target oligonucleotides at different concentrations are shown in Fig. 2A. The DPV peak currents increased with increasing concentrations of target oligonucleotides. To investigate the analysis capability of this designed method, the dynamic range for detection of target DNA concentration was examined. The biosensor showed a good linear relationship between the plot of the response and the logarithm of target DNA concentration in the range from 100 fM to 5 nM (Fig. 2B). The resulting linear equation was $I(\mu\text{A}) = 1.47 + 0.62 \times \lg c$ (c is the concentration of target DNA (pM)) with a correlation coefficient of 0.9935. Additionally, the limit of detection (LOD) was 92 fM at a signal-to-noise ratio of 3, which was much lower than most of the previous reported methods based on ERA or CHA (Table S2 in ESI†).^{35–39} Compared with Zou's work about CHA and ERA,⁴⁰ this method had a higher signal-to-noise ratio, which was attributed to mismatched CHA and the position of ERA before CHA.

To investigate the specificity of the biosensor to different oligonucleotides, full-complementary oligonucleotides (a), single-base-mismatched oligonucleotides (b), two-base-mismatched oligonucleotides (c) and non-complementary oligonucleotides (d) were analyzed. Fig. 3 displays the typical DPV curves (A) and DPV peak currents (B) responding to 500 pM of four different oligonucleotides and the background. The DPV response of (a) was much larger than those of (b)–(e), which demonstrated that the designed biosensor could effectively discriminate different DNA sequences and displayed excellent selectivity. To evaluate the repeatability of the developed biosensor, the synthetic target DNA at 100 fM and 1 nM was examined 5 times. The coefficients of variation for

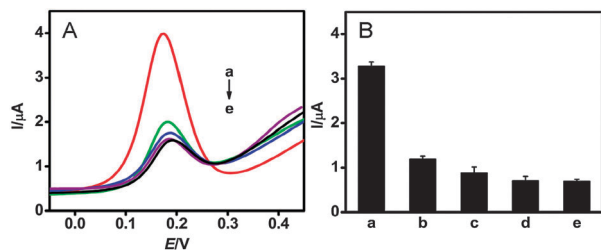


Fig. 3 (A) Typical DPV curves and (B) DPV peak currents responding to 500 pM of target oligonucleotides (a), single-base-mismatched oligonucleotides (b), two-base-mismatched oligonucleotides (c), non-complementary oligonucleotides (d) and blank (e).

Table 1 The recoveries determined using the proposed method via spiking synthetic DNA into human total DNA and serum samples^a

Sample no.	Spiking value (pM)	Assayed value (pM)	Reproducibility (%)	Recovery (%)
1	0.50	0.50	1.3	100.0
2	5.00	4.94	2.1	98.8
3	50.00	51.25	3.2	102.5
4	0.50	0.48	2.7	96.0
5	5.00	5.14	2.0	102.9
6	50.00	49.56	1.6	99.1

^a 1–3 for total DNA samples, 4–6 for serum samples.

both concentrations were less than 5%, which showed that this method had an acceptable stability and reproducibility.

To demonstrate the practical applicability of the established biosensor in real sample analysis, we performed the DNA assay using total DNA of human normal cells and serum samples. Total DNA of human normal cells was extracted by TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0. Human serum samples were obtained from the First Affiliated Hospital of Chongqing Medical University. Various amounts of synthetic DNA were spiked into total DNA of human normal cells and serum samples, respectively, and analyzed by the designed method. The results are listed in Table 1. The recoveries were both between 95% and 105% from 0.5 pM to 50 pM in 200 pg mL⁻¹ total DNA and 1 : 5 serum samples, which indicated that the analysis capability of biosensors in complex mixtures was almost unaffected. Thus, the developed strategy may become a potential approach for DNA assay in real biological samples.

In summary, a novel biosensor has been developed for DNA detection by coupling the exonuclease III-assisted target recycling amplification with mismatched catalytic hairpin assembly. The biosensor shows a broader dynamic range, high sensitivity, good specificity and reproducibility, not requiring thermal cycling and complicated amplification labels. Also, this assay has been successfully applied to detect DNA from total DNA of human normal cells and serum samples. The developed dual amplification strategies may be further extended for DNA detection in the area of clinical diagnosis and therapy, pathogen detection and environmental monitoring in the future.

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