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Electrochemical biosensing of DENV nucleic acid amplified with triplet nanostructure-mediated dendritic hybridization chain reaction

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ABSTRACT

A universal and highly sensitive electrochemical biosensing strategy was developed by using a triplet nanostructure-mediated dendritic hybridization chain reaction (HCR) for the analysis of Dengue virus (DENV) nucleic acid. A locked dsDNA was firstly recognized by the target DENV nucleic acid fragment to release the initiator, which was then captured by a capture DNA modified gold electrode to initiate the HCR in the presence of biotin labeled substrates A and B, and two helpers. After the recognition reactions of the captured initiator with substrate A and then helper 1, one DNA structure was formed to react with substrate B, which produced a triplet nanostructure to trigger the dendritic HCR by the toehold of the hybridization structure. After the dendritic product was bound with avidin labeled horseradish peroxidase (avidin-HRP), an amperometric signal could be obtained to achieve ultrasensitive electrochemical detection of DENV. The non-enzymatic isothermal amplification was driven only by entropy. The stable three-dimensional dendritic nanostructure with rigid double helix could assure the excellent performance of the designed electrochemical biosensing strategy. The proposed method showed a detection range of 1.6–1000 pM with a detection limit of 188 fM and ability to distinguish single-base mutation. By changing the recognition sequence of the initiator, the detection of different DENV nucleic acid fragments could be achieved with the same performance. Thus, this method had good extendability for other nucleic acids, providing a promising candidate for nucleic acid detection in early clinical diagnosis.

1. Introduction

Dengue virus (DENV), a member of the *Flavivirus* genus, is mainly transmitted through the bite of *Aedes* mosquitoes [1], which can lead to lethal complications, including hemorrhagic fever, shock syndrome, respiratory distress, and severe organ failure [2,3]. As early Dengue fever lacks obvious clinical symptoms, most initial infections (70–80 %) are asymptomatic [4], which leads to the urgent need of sensitive detection methods for early laboratory diagnosis. Currently, the clinical analysis methods for DENV diagnosis include reverse transcription polymerase chain reaction (RT-PCR) [5,6], NS1 antigen detection [7], and serum IgM antibody testing [8]. Although these methods permit quantitative analysis of DENV nucleic acid, antigen or antibody, the sensitivity for diagnosis of early infection stage is unsatisfactory [9], and the RT-PCR method is still limited by precision equipment, complex thermocycler step and high false positive rate [10].

Recently, DNA-based biosensors have attracted more and more attention. Various nucleic acid signal amplification technologies have also been explored to improve the detection sensitivity, such as loop-mediated isothermal amplification (LAMP) [11–13], catalytic hairpin self-assembly (CHA) [14–16], strand displacement amplification (SDA) [17,18], rolling circle amplification (RCA) [19–21] and hybridization chain reaction (HCR) [22–24]. In particular, entropy-driven catalysis by HCR has aroused wide interest of researchers due to the autonomous isothermal replication process under mild experimental conditions without any polymerases or other enzymes [25]. HCR eradicates complex thermal cycling steps to achieve highly sensitive nucleic acid amplification [26]. In a typical linear HCR, the initiator sequence triggers a cascade of hybridization events, leading to the formation of ordered DNA double helices. Signal probes can be easily bound on these ordered duplexes for performing efficient signal amplification [27]. However, traditional non-enzymatic HCR with linear self-assembly

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involves the analyte-triggered cross-opening of two DNA hairpins into long dsDNA copolymers, causing the inevitable shortcomings of weak biostability and relatively slow kinetics. Additionally, the amount of binding site is restricted on linear DNA toehold terminal. To address these defects, a three-dimensional DNA structure formed by hairpin-free dendritic HCR has become a promising candidate [28]. Compared with traditional HCR, dendritic HCR provides exponential growth of branched DNA nanostructures. Moreover, it can be faster performed and avoids the leakage of the "breathing" phenomenon between hairpins [29], as reported previously for the fast detection of DNA with low detection limit down to 850 fM [30].

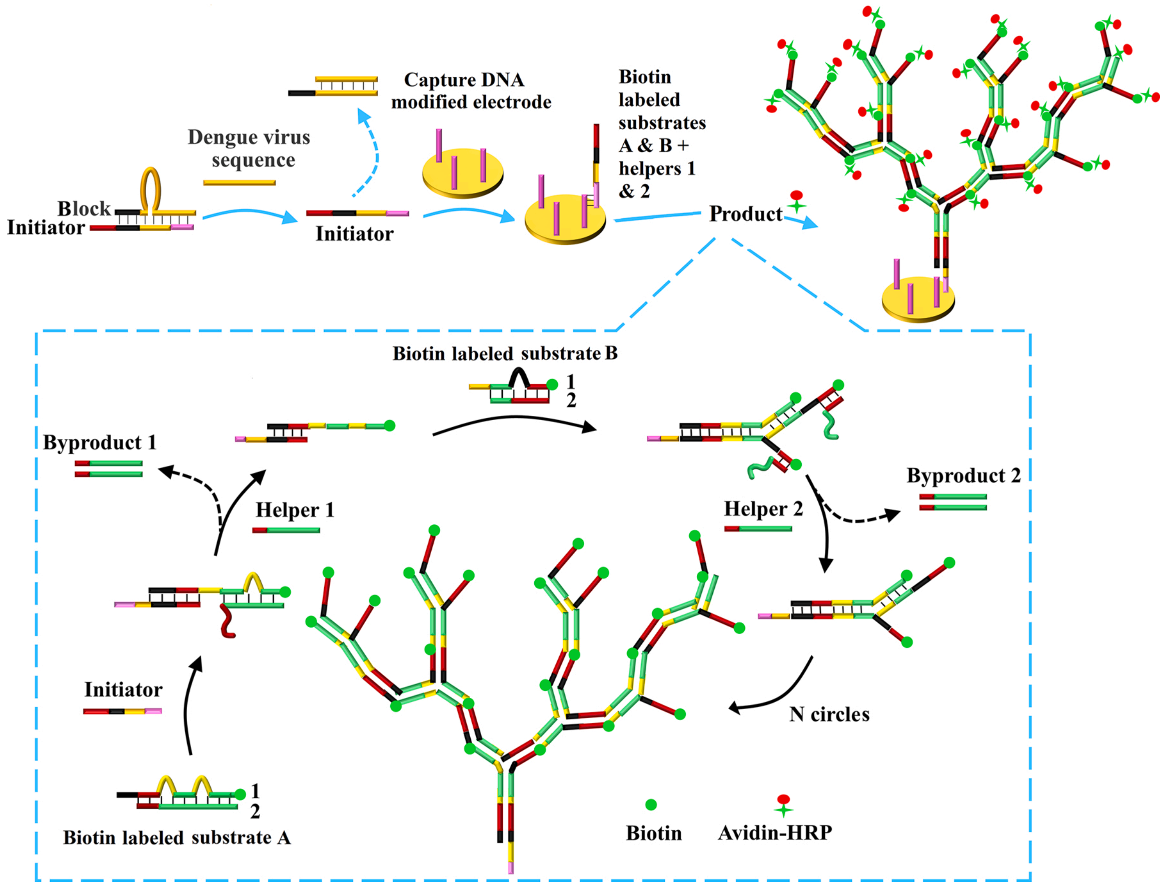
In order to meet the need of sensitive methods for detection of DENV nucleic acid, this work used triplet nanostructure-mediated dendritic HCR [28] to develop a universal and highly sensitive electrochemical biosensing strategy. As shown in Scheme 1, the locked dsDNA was firstly recognized by a target DENV DNA sequence to release the initiator. The released initiator was then captured by a capture DNA modified gold electrode, as the electrochemical biosensor, to initiate the surface assembly of triplet nanostructure. After helper 2 was introduced to expose the triplet nanostructure, the dendritic HCR could be triggered to form a branched DNA nanostructure with abundant biotin species, which provided the sites for binding avidin labeled horseradish peroxidase (avidin-HRP) and producing detectable amperometric signal in the presence of 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2. Besides its high sensitivity and good specificity, this strategy could be applied to the detection of different DNA sequences so long as the initiator contains the corresponding recognition sequence. This universal detection strategy provides promising applications in early laboratory diagnosis.

2. Experimental

2.1. Materials and reagents

Bovine serum albumin (BSA), avidin-HRP, premixed 10 × TBE powder, 30 % methylene acrylamide and all oligonucleotides listed in Table 1 and Table S1 were bought from Sangon Biotech Co. Ltd. (Shanghai, China). The sequences of substrates and helpers for dendritic HCR were the same as those reported previously [28]. Tris (2-carboxyethyl)-phosphine hydrochloride (TCEP), tris (hydroxymethyl)-aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), casein from bovine milk, mercaptohexanol (MCH) and N, N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). 20-bp DNA ladder, DNA loading buffer (6×) and 10,000 × SYBR Gold dye were purchased from Thermo Fisher Scientific. Co., Ltd. (Shanghai, China). The TMB kit including solutions A, B and C was obtained from Hefei BoMei Biotechnology Co. Ltd (Hefei, China). The DNA immobilization buffer was prepared with 10 mM Tris-HCl, 1 mM EDTA, and 0.3 M NaCl (pH 8.0). The binding buffer for association with avidin-HRP was 10 mM phosphate buffered saline (PBS, pH 7.2) containing 0.5 % casein, and the hybridization buffer was 0.5 M PBS (pH 7.4) containing 2.5 % BSA. Ultrapure water was obtained from a Millipore water purification system (18 MΩ, Milli-Q, Millipore) and used in the whole assay. The serum samples were obtained from Jiangsu Cancer Hospital.

Biotin labeled substrates A and B and locked dsDNA were prepared by mixing substrate A1 (1 μM) with substrate A2 (1.5 μM), substrate B1 (1 μM) with substrate B2 (1.5 μM), and block (1 μM) with the initiator (1 μM) at 85 °C for 5 min and then cooling to room temperature for 20 min, respectively. Excess substrates A2 and B2 in substrates A and B could be consumed by incubating them with helpers 1 and 2 (2 μM) for 30 min,



Scheme 1. Schematic illustration of triplet nanostructure-mediated dendritic HCR for electrochemical detection of DENV nucleic acid.

Table 1
Sequences of targets, blocks, initiators and capture DNAs used in this study.

Name	Sequences (5′-3′)
Target	AGGCTACCATACACAAACAGCAGGACCATGGCATCTAGGTAAGCTT
Block	**TTCGTCA**_AAG_***CTTACCTAGAT***_GCCATGGTCCTGCTGT_***TTGTGTATG***_GTAGCCT_**CAGAGC**
Initiator	**GCTCTGCATACACAAATCTAGGTAAGTGACGAA**CTAGTTGATGAAGCTG
Capture DNA 1	TTGTGTATGCAGAGCTTTTTT-$(CH_2)_6$-SH
Target 2	GAGATGGACTTTGATTTCTGCGAAGGAACCACAGTGGTGG
Block 2	**CGTCA**_CCAC_***CACTGTG***_GTTCCTTCGCAGAAAT_***CAAAGTCCATCTC*****CAGAG**
Initiator 2	**CTCTGGAGATGGACTTTGCACAGTGTGACG**AACTAGTTGATGAAGCTG
Capture DNA 2	CCATCTCCAGAGCTTTTTT-$(CH_2)_6$-SH
Target 3	TGACTGAGGACTGTGGAAATAGAGGACCCTCTTTAAGAAC
Block 3	**TTCGTCA**_**GTTCTTAAAG**_ _AGGGTCCTCTATTT_ _**CCACAGTCCTCAGTCA**_**CAGAGC**
Initiator 3	**GCTCTGTGACTGAGGACTGTGGCTTTAAGAACTGACGAA**CTAGTTGATGAAGCTG
Capture DNA 3	TCCTCAGTCACAGAGCTTTTTT-$(CH_2)_6$-SH

respectively.

2.2. Apparatus

All measurements were accomplished in a standard cell with a platinum wire counter electrode, Ag/AgCl reference electrode and a gold electrode with 5 mm diameter as working electrode. Chronoamperometric measurement was carried out with CHI 630D electrochemical workstation (CH Instruments Inc., U.S.A). Electrochemical impedance spectra (EIS) were measured with DH 7000 (Donghua Analysis Instruments Co., Ltd., Jiangsu, China). Electrophoresis was performed on an electrophoresis analyzer (Bio-Rad, USA) and imaged with Bio-Rad ChemDoc XRS (Bio-Rad, U.S.A.).

2.3. Fabrication of electrochemical biosensor

Prior to modification, the gold electrode was polished with 0.3 μm and 0.05 μm alumina slurry successively to obtain the mirror surface, and sonicated with water, ethanol, and water for 3 min respectively to dry with nitrogen. Afterward, this electrode was immersed in piranha solution for 30 s, rinsed with water, and dried with nitrogen.

The capture DNA was firstly treated with 10 mM TCEP for 1 h in the immobilization buffer to reduce disulfide bonds. It was then dropped on the treated electrode at room temperature overnight. After rinsing with 10 mM PBS (pH 7.2) and drying with nitrogen, the modified electrode was immersed in 1 mM MCH for 30 min to block the unmodified sites. The capture DNA modified electrode was finally washed with 10 mM PBS (pH 7.2) and dried with nitrogen for further use.

2.4. Detection of target DNA

The target DENV nucleic acid fragment was firstly incubated with locked dsDNA at 37 °C for 1 h. 8 μL of the resulting mixture was then dropped on the modified electrode to incubate at 37 °C for 1 h. Afterward, 8 μL of the mixture of biotin labeled substrates A (0.1 μM) and B (0.2 μM) and helpers 1 (0.2 μM) and 2 (0.4 μM) in hybridization buffer was added to the electrode surface for the triplet nanostructure-mediated dendritic HCR by incubating the electrode at 37 °C for 30 min. After washing with 10 mM PBS (pH 7.2), the electrode was incubated with 8 μL of avidin-HRP (2 μg mL^{-1}) in binding buffer for 15 min in the dark. The obtained electrode was finally rinsed and dried to perform chronoamperometric measurement in the mixture of the TMB kit at 100 mV, and the current was sampled at 100 s.

3. Results and discussion

3.1. Principle of electrochemical biosensing

As shown in Scheme 1, the locked dsDNA of block and initiator was firstly recognized by a target DENV nucleic acid fragment by the exposed toehold of a block to release the initiator. The released initiator was then captured by the capture DNA modified gold electrode to expose a sequence for hybridization with the single strand part of biotin labeled substrate A, which provided a toehold to recognize helper 1 and form byproduct 1 and a DNA nanostructure. The surface captured nanostructure contained three parts, one for initiator capture, and two for binding with two biotin labeled substrate B to produce a triplet nanostructure on the electrode surface. After helper 2 was introduced to expose the triplet nanostructure, the dendritic HCR could be triggered to form a branched DNA nanostructure with abundant biotin species, which provided sites for binding of avidin-HRP to produce detectable amperometric signal in the presence of TMB and H_2O_2. Thus, each target DENV nucleic acid fragment could produce a dendritic HCR product with abundant HRP to catalyze the oxidation of TMB and H_2O_2, leading to greatly amplified amperometric signal for highly sensitive target analysis. It is worth noting that substrates A and B, and helpers 1 and 2 could coexist constantly in the absence of the target.

3.2. Feasibility of dendritic HCR and biosensing strategy

To validate the feasibility of dendritic HCR, we performed 8% polyacrylamide gel electrophoresis (PAGE) analysis in 1 × TBE running buffer at 100 V for 50 min. The gel was stained with the SYBR Gold dye and placed in the dark for 20 min. It was then photographed under UV irradiation. The results showed the respective bands of substrates A and B, and byproducts 1 and 2 (Fig. 1, lanes 1–4). The mixture of substrates A and B, and helpers 1 and 2 did not show any new band (Fig. 1, lane 5). However, after the initiator was added in the mixture, a large number of bands with low mobility could be observed, and the bands for byproducts 1 and 2 were also observed (Fig. 1, lane 6), indicating the formation

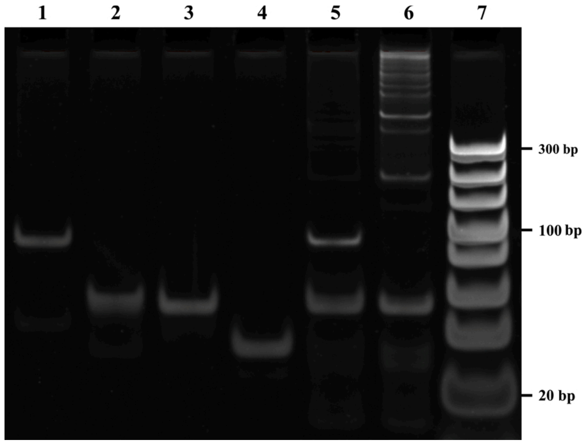


Fig. 1. PAGE analysis of dendritic HCR feasibility: (lane 1) substrate A, (lane 2) substrate B, (lane 3) byproduct 1, (lane 4) byproduct 2, (lane 5) substrates A & B + helpers 1 & 2, (lane 6) substrates A & B + helpers 1 & 2 + initiator, (lane 7) DNA ladder marker.

of dendritic DNA nanostructure with low mobility, which consumed a lot of substrates A and B, and produced byproducts 1 and 2.

EIS was used to characterize the stepwise modification of the gold electrode and the surface dendritic HCR (Fig. 2A). The bare gold electrode showed good conductivity (curve a). After the capture DNA was assembled on the electrode, it showed an electron transfer resistance of 590 Ω due to the repulsion of the negatively charged nucleic acid to $[Fe(CN)_6]^{3-/4-}$ (curve b). After MCH was assembled to the electrode to block the unbound sites, the electron transfer resistance increased to 2390 Ω (curve c). The formation of the dendritic DNA nanostructure on electrodes led to a resistance of 4095 Ω (curve d), which was ascribed to both the repulsion of the negatively charged phosphate skeleton of DNA to $[Fe(CN)_6]^{3-/4-}$ and the large steric hindrance effect of the DNA dendrimer. The binding of avidin-HRP to the nanostructures greatly increased the resistance to 7820 Ω due to the steric hindrance of avidin-HRP (curve e).

3.3. Condition optimization for target detection

To obtain excellent performance of the designed biosensing strategy for the analysis of DENV nucleic acid, the concentration of capture DNA for biosensor preparation, avidin-HRP concentration and dendritic HCR time were optimized at the initiator concentration of 1 nM. The signal-to-background (S/B) value was used as the evaluation index. The background signal was obtained in the absence of the initiator. Initially, we examined the effect of capture DNA concentration on the current response. With increasing capture DNA concentration, the signal gradually increased and reached the maximum current at 1 μM of capture DNA, at which the highest S/B value was observed (Fig. 2B). At concentrations higher than 1 μM, the signal decreased due to the high density of capture DNA on the electrode surface, which hindered the capture of the initiator and subsequent dendritic HCR. Hence, 1 μM capture DNA was applied to the following experiments.

On the DNA nanostructure modified electrode, the effect of avidin-HRP concentration on the current response was also examined. With increasing avidin-HRP concentration, more HRP molecules were assembled on the electrode surface to catalyze the oxidation of TMB by H_2O_2, thus the current response increased (Fig. 2C), though the electron transfer resistance also increased due to the presence of more protein molecules (Fig. 2A, curve e). However, high avidin-HRP concentration brought high background, which led to a decreasing S/B value at avidin-HRP concentrations higher than 2 μg mL^{-1}. This could be attributed to the nonspecific adsorption of avidin-HRP at high concentration due to the incomplete backfilling or surface imperfection [31–33]. Thus, 2 μg mL^{-1} of avidin-HRP was used for target detection.

The amplified signal depended on the formation of dendritic DNA nanostructure, which obviously related to the incubation time. After the reaction mixture was dropped on the initiator/capture DNA modified electrode to incubate at 37 °C, the current signal quickly increased and reached the maximum value at 30 min (Fig. 2D), which could be defined as the optimum HCR incubation time. A long incubation time resulted in larger dendritic DNA nanostructure, which should be unfavorable to both the binding of avidin-HRP and electron transfer, thus the current response decreased.

3.4. Signal amplification by dendritic HCR

The signal amplification performance of dendritic HCR could be investigated at 1000 pM initiator by changing the composition of the reaction mixture. When the reaction mixture contained only substrate A and helper 1, the dendritic HCR did not occur, and only a DNA nanostructure could be formed for each initiator captured on the electrode surface, which showed relatively low current response (Fig. 3A). The subsequent incubation of the DNA nanostructure modified electrode with a reaction mixture containing only substrate B and helper 2 produced a triplet nanostructure modified electrode, which showed an

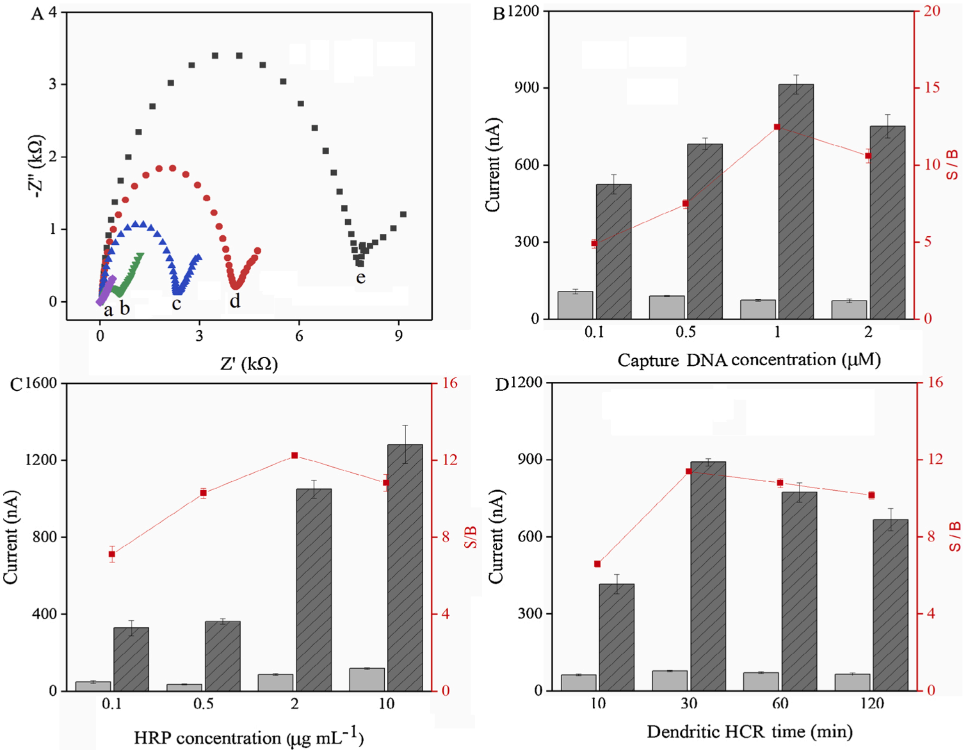


Fig. 2. (A) Nyquist plots for (a) bare gold electrode, (b) capture DNA, (c) capture DNA/MCH, (d) capture DNA/MCH/DNA dendrimer and (e) capture DNA/MCH/DNA dendrimer/HRP modified electrodes in 0.1 M KCl solution containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ from 0.1 Hz to 10,000 Hz with an amplitude of 0.01 V. Optimization of (B) capture DNA concentration, (C) avidin-HRP concentration and (D) dendritic HCR time. Error bars show the standard deviations of three measurements.

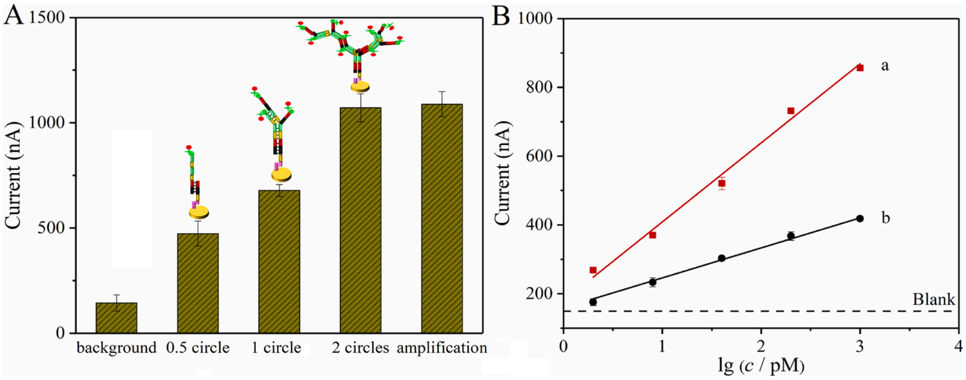


Fig. 3. (A) Chronoamperometric responses at 100 mV and 100 s for various reaction circles. Error bars show the standard deviations of measurements taken from three tests. (B) Plots of current signal at 100 s vs logarithm of initiator concentration, detected with (a) and without (b) dendritic amplification. Error bars represent the standard deviations of three tests.

increasing current signal due to the fact that three HRP molecules could be bound to each captured initiator. As expected, the formation of the dendritic DNA nanostructure by two circles further increased the current response, which reached platform value obtained from the reaction mixture containing all four components. It meant that the dendrimer reached a stable structure after two circles due to limited space on the electrode surface.

The formation of the dendritic DNA nanostructure obviously improved the sensitivity of target DNA detection. As shown in Fig. 3B, the calibration curve for initiator DNA with dendritic HCR amplification showed a linear regression equation of $I = 230 \log C + 179$ ($R^2 = 0.9909$), while the linear regression equation without dendritic HCR amplification was $I = 87.2 \log C + 158$ ($R^2 = 0.9812$).

3.5. *Detection of DENV DNA*

To evaluate the proposed strategy, different concentrations of DENV nucleic acid were analyzed under optimized conditions. The chronoamperometric signal increased with increasing target concentration from 1.6 pM to 1000 pM (Fig. 4A), and the plot of the current signal vs the logarithm of concentration showed a satisfying linear correlation in this concentration range (Fig. 4B). The linear regression equation was $I = 84.8 \log C + 207$ ($R^2 = 0.9950$). The detection limit was estimated at three times the standard deviation of the blank control signal to be 188 fM. Compared with other nucleic acid detection methods [34–38], the proposed biosensing strategy displayed a wide linear range and the lowest detection limit (Table S2). After the DNACp + MCH-AuEs were stored at 4 °C for 2 weeks, the chronoamperometric signals showed the same results as their initial values, indicating good storage stability.

3.6. *Extendability for other nucleic acids*

The extendability of the proposed strategy was investigated by detecting other fragments of DENV nucleic acid, target DNA 2 and DNA 3, which were sequential sequences in the Dengue genome. Except for the design of the block and initiator, other reaction chains were consistent with those described above. Fig. 5A shows the chronoamperometric responses to different concentrations of target DNA 2. The linear regression equation for the logarithm of target DNA 2 concentration is $I = 91.1 \log C + 189$ ($R^2 = 0.9974$) (Fig. 5B). Similarly, target

DNA 3 also showed an increasing chronoamperometric response (Fig. 5C) and a linear regression equation of $I = 93.9 \log C + 177$ ($R^2 = 0.9945$) (Fig. 5D). The similar responses and close slopes to target DNA demonstrated the good extendability of the proposed strategy.

3.7. *Specificity and recovery test*

Mutation can cause great changes in nucleic acid structure. Consequently, it is necessary to develop a strategy for specific identification of base mismatch, which requires high specificity of the biosensor. As a result, the distinction between the unmatched and perfectly matched target DENV nucleic acid fragment was examined at 1000 pM. Single-base, double-base, three-base, five-base mismatched DNA and completely mismatched DNA showed obviously lower response than the perfectly matched target (Fig. 6), indicating that our method could

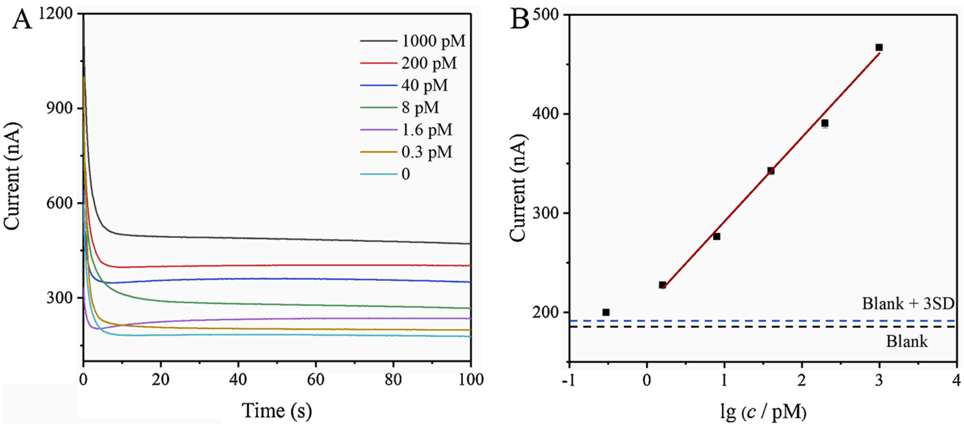


Fig. 4. (A) Chronoamperometric responses of the biosensor at 100 mV to 0, 0.3, 1.6, 8, 40, 200 and 1000 pM target DNA. (B) Plot of current signal at 100 s vs logarithm of target DNA concentration. Error bars show the standard deviations of three measurements.

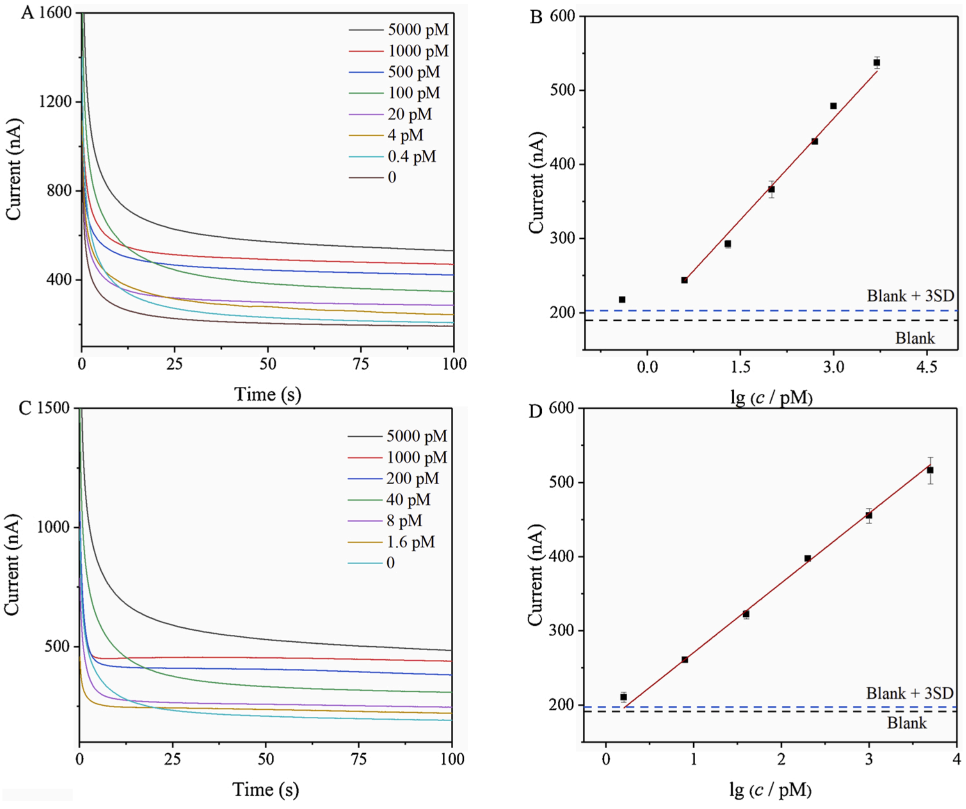


Fig. 5. (A) Chronoamperometric responses of the biosensor at 100 mV to 0, 0.4, 4, 20, 100, 500, 1000, and 5000 pM target DNA 2. (B) Plot of current signal at 100 s vs logarithm of target DNA 2 concentration. (C) Chronoamperometric responses of the biosensor at 100 mV to 0, 1.6, 8, 40, 200, 1000 and 5000 pM target DNA 3. (D) Plot of current signal at 100 s vs logarithm of target DNA 3 concentration. Error bars represent the standard deviations of three tests.

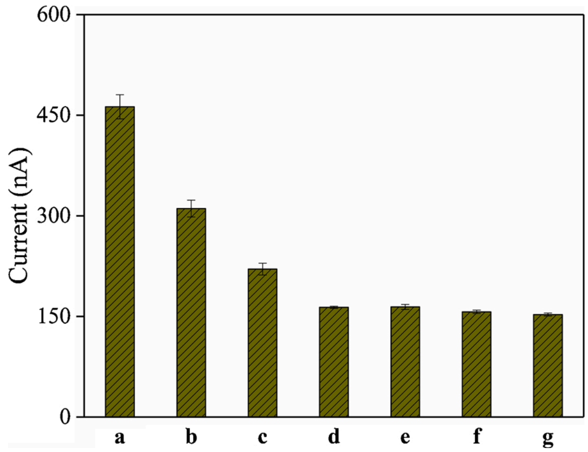


Fig. 6. Responses of the biosensor to (a) perfectly matched target, (b) single-base, (c) double-base, (d) three-base and (e) five-base mismatched DNA, (f) completely mismatched DNA and (g) blank. Error bars represent the standard deviations of three tests.

Table 2
Recovery for detection of DENV nucleic acid in diluted serum samples.

Samples	Added (pM)	Found (pM) [a]	Recovery (%)	RSD (%) [a]
1	2	1.9	105.3	6.44
2	10	10.5	95.7	5.30
3	50	50.9	98.2	7.04
4	200	199.5	100.2	5.80
5	500	521.7	95.9	1.29

[a] The mean of three measurements.

correctly identify the mismatched base.

The practical application of the biosensing strategy was verified by recovery tests. Five concentrations of target DENV DNA were detected in 10-fold diluted serum samples with the linear regression equation (Fig. 4B) and the current responses obtained with different DNACp + MCH-AuEs. As listed in Table 2, the recoveries ranged from 95.7 % to 105.3 % and the RSDs (relative standard deviations) ranged from 1.29 % to 7.04 %, indicating the good accuracy and precision for analysis of target DNA in clinical samples. Thus, the reproducibility of the biosensors prepared in the same way for a given concentration of synthetic DNA was acceptable, and the matrix had little effect on target DENV DNA detection with the proposed method.

4. Conclusion

This work demonstrates a highly sensitive electrochemical biosensing strategy for the analysis of DENV nucleic acid. The detection can conveniently be performed on a capture DNA modified electrode by using the target DNA to release the designed initiator from a locked dsDNA, and then incubating the initiator captured electrode with a reaction mixture, during which a triplet nanostructure-mediated dendritic HCR is triggered to amplify the detection signal. The non-enzymatic entropy-driven amplification reaction allows only DNA assembly to form a stable three-dimensional dendritic nanostructure with rigid double helix, which assures cost-efficient for high precision analysis of nucleic acid. This proposed strategy possesses relatively wide detection range and low detection limit, and can correctly identify mismatched sequence from the target sequence. The recovery tests demonstrate its good accuracy for the analysis of target DNA in clinical samples. Meanwhile, this strategy has good extendability for other nucleic acids so long as the initiator contains the corresponding recognition sequence.

Therefore, this work provides a potential tool for nucleic acid detection in early clinical diagnosis.

CRediT authorship contribution statement

Jinling Fu: Conceptualization, Data curation, Writing - original draft. **Jie Wu:** Methodology, Writing - review & editing. **Rui Zhang:** Formal analysis. **Qiang Wu:** Conceptualization, Funding, Writing. **Huangxian Ju:** Conceptualization, Methodology, Writing - review & editing.

Author contribution

We confirm that the manuscript has been read and approved by all named authors. We further confirm that the order of authors listed in the manuscript has been approved by all of us. The contribution of authors are described as follows:

Jinling Fu Conceptualization, Data curation, Writing - original draft.

Jie Wu Methodology, Writing - review & editing.

Rui Zhang Formal analysis.

Qiang Wu Conceptualization, Funding, Writing.

Huangxian Ju Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2021.130436.

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