

Ultrasensitive Electrochemical Biosensor Developed by Probe Lengthening for Detection of Genomic DNA in Human Serum

Jin-Yuan Chen,^{†,||} Zhou-Jie Liu,^{‡,§,||} Xue-Wen Wang,^{‡,||} Chen-Liu Ye,[‡] Yan-Jie Zheng,[‡] Hua-Ping Peng,[‡] Guang-Xian Zhong,[†] Ai-Lin Liu,^{*,‡} Wei Chen,^{*,‡} and Xin-Hua Lin^{*,‡}

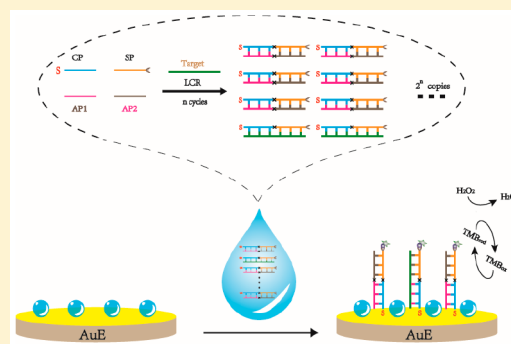
[†]The Centralab, The First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, China

[‡]Department of Pharmaceutical Analysis, Higher Educational Key Laboratory for Nano Biomedical Technology of Fujian Province, Faculty of Pharmacy, Fujian Medical University, Fuzhou 350122, China

[§]Department of Pharmacy, The First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, China

S Supporting Information

ABSTRACT: As an alternative to most of the reported nucleic acid amplification-based electrochemical DNA biosensors used for detection of trace levels of genomic DNA, we herein present a novel detection concept. The proposed system involves the conversion of two short double-stranded DNAs (dsDNAs), labeled with a thiol-tag or biotin-tag, into a single integrated dsDNA containing thiol and biotin at both terminals in the presence of target DNA through ligase chain reaction (LCR) and followed by the immobilization of these integrated dsDNAs on a bovine serum albumin (BSA)-modified gold electrode surface. Owing to rapid depletion of the two short dsDNAs via LCR, the integrated dsDNAs were generated in an exponential manner so that this sensing approach offered a limit of detection of 25 yoctomoles (15 copies in 50 μ L sample volumes), a high discrimination of single-base mismatch and a wide linear concentration range (across 6 orders of magnitude) for target DNA. Significantly, the proposed sensor, which has simplicity in operation and ease of miniaturization, detected the target of interest in total nucleic acid extracts derived from clinical serum samples with excellent results, thereby demonstrating its considerable diagnostic potential in fields ranging from virus detection to the diagnosis of genetic diseases.



The detection of trace amounts of DNA is of considerable significance in fields ranging from virus assays to the diagnosis of genetic diseases, and accordingly, numerous types of DNA detection systems have been developed, including those based on surface plasmon resonance (SPR),¹ quartz crystal microbalance (QCM),² fluorescence,^{3,4} colorimetry,⁵ and electrochemistry.^{6–10} Among these, electrochemical DNA (E-DNA) biosensors have the notable advantages of high sensitivity, simplicity, compatibility, miniaturization, and low cost.

In order to determine extremely low levels of genomic DNA in real samples, nucleic acid amplification methods have been employed in E-DNA biosensors, among which, polymerase chain reaction (PCR)-based techniques have been widely used.^{11–16} However, the various methods used for preparation of single-stranded target DNA amplicons in these PCR-based E-DNA biosensors, such as high-temperature denaturation, asymmetric PCR, and λ -exo digestion, have certain shortcomings. High-temperature denaturation has the inherent weakness of promoting predominant renaturation in solutions rather than hybridization on the sensor surface.^{11,12} Using an asymmetric PCR, it is difficult to optimize the ratio of primer amounts, and this technique necessitates the subsequent purification of single-stranded DNA (ssDNA) due to the

generation of both ssDNA and double-stranded DNA (dsDNA).^{13,14} Despite its better performance compared with the previous two methods, λ -exo digestion requires a lengthy procedure.¹⁵ Given these various drawbacks, there have recently been a number of investigations that have sought to devise new nucleic acid amplification methods as viable alternatives to PCR for genomic DNA detection using E-DNA biosensors. For example, Hsieh et al. developed a microfluidic electrochemical quantitative loop-mediated isothermal amplification (MEQ-LAMP) system (an integrated microfluidic platform) for the detection of pathogenic DNA.¹⁷ However, due to the complexity of LAMP primer design, this technique necessitates the use of four to six different primers recognizing distinct regions of the target sequence, and it accumulates a large number of different amplification products, which complicates the design of sequence-specific detection probes. Barreda-García et al. combined helicase-dependent isothermal amplification with electrochemical detection to facilitate detection of the genomic DNA of *Mycobacterium*

Received: December 11, 2018

Accepted: March 6, 2019

Published: March 6, 2019



tuberculosis; this protocol, however, has to deal with the procedure of asymmetric amplification, the requirement of biotin–streptavidin separation, and the employment of a magnet.¹⁸ In addition, Li et al. reported an electrochemical biosensor method for Wnt7B gene detection based on an exponential amplification reaction (EXPAR) that combines polymerase strand extension and single-strand nicking.¹⁹ However, the specific restriction enzyme cutting site in dsDNA (Nt. BstNBI, 5'-GAGTCNNNN↓N-3') limits the wider application of this method for other DNA biomarkers. Furthermore, Yuan et al. demonstrated an electrochemical biosensing strategy for ultrasensitive detection of DNA in real samples based on defective T junction-induced transcription amplification (DTITA).²⁰ However, as this strategy requires a heating procedure to ensure denaturing of genomic DNA, the reannealing of the two separate strands during subsequent probe-target hybridization could substantially reduce the binding efficiency between probes and the target strand. Moreover, given that the Klenow fragment and T7 RNA polymerase are used in the reaction mixture, the buffer must be precisely adjusted for both enzymes. Clearly, the aforementioned protocols can detect trace levels of genomic DNA in real samples by producing large numbers of DNA copies via nucleic acid amplification; however, this is typically accompanied by nonspecific amplification.

In an E-DNA biosensor, the ssDNA probe is usually immobilized on an electrode surface which hybridizes with target DNA. To improve the recognition abilities of such heterogeneous surface probes, considerable attention has been focused on controlling the surface chemistry, conformation, and packing density of the probe molecules, as well as on the size and geometry of the surface, such as DNA tetrahedral nanostructures,^{21–23} bovine serum albumin (BSA)-based DNA probe carrier platforms,²⁴ and dual-thiol-modified hairpin DNA (dSH-HSP).²⁵ Nevertheless, difficulties remain in obtaining a hybridization efficiency approximating that in an homogeneous aqueous system.

With the aim of developing an efficient and practical electrochemical biosensor for highly sensitive and selective detection of genomic DNA in real samples without hybridization on a heterogeneous surface, we devised an ultrasensitive dsDNA-assembled electrochemical biosensor using a BSA-based DNA probe carrier platform.²⁶ In contrast to the previously developed nucleic acid amplification-based E-DNA biosensors, the system reported herein is based on a probe lengthening strategy where two short dsDNAs, labeled with a thiol-tag or biotin-tag, are converted into a single integrated dsDNA containing thiol and biotin at both terminals in the presence of target DNA through ligase chain reaction (LCR), followed by the immobilization of these integrated dsDNAs on the electrode. In order to guarantee the precise and specific assembly of dsDNA, a BSA-based DNA probe carrier platform developed by our group,²⁴ which has high resistance to the nonspecific adsorption of nucleic acids and proteins, was employed in this assay. During the LCR, the integrated dsDNAs are generated exponentially within a short time period. As a consequence, a sensitivity of 25 yoctomoles (yoctomole = 10^{-24} mole; 15 copies in 50 μL volume of sample), together with a high discrimination of single-base mismatch, and a wide linear range (across 6 orders of magnitude) were achieved for target DNA. Furthermore, the detection of DNA in real samples was realized with reliable results. Although this method requires the use of a

thermocycler for amplification, it should be pointed out that recent work has focused on thermocycling platforms that are cheap, portable, and battery operated, some of which are now commercially available, including Palm PCR (Ahram Biosystems Inc.), Freedom4 (Ubiquitome), and miniPCR (Amplifyus).²⁷ Accordingly, there no longer exist restrictions regarding application for the proposed sensor in a low-resource settings.

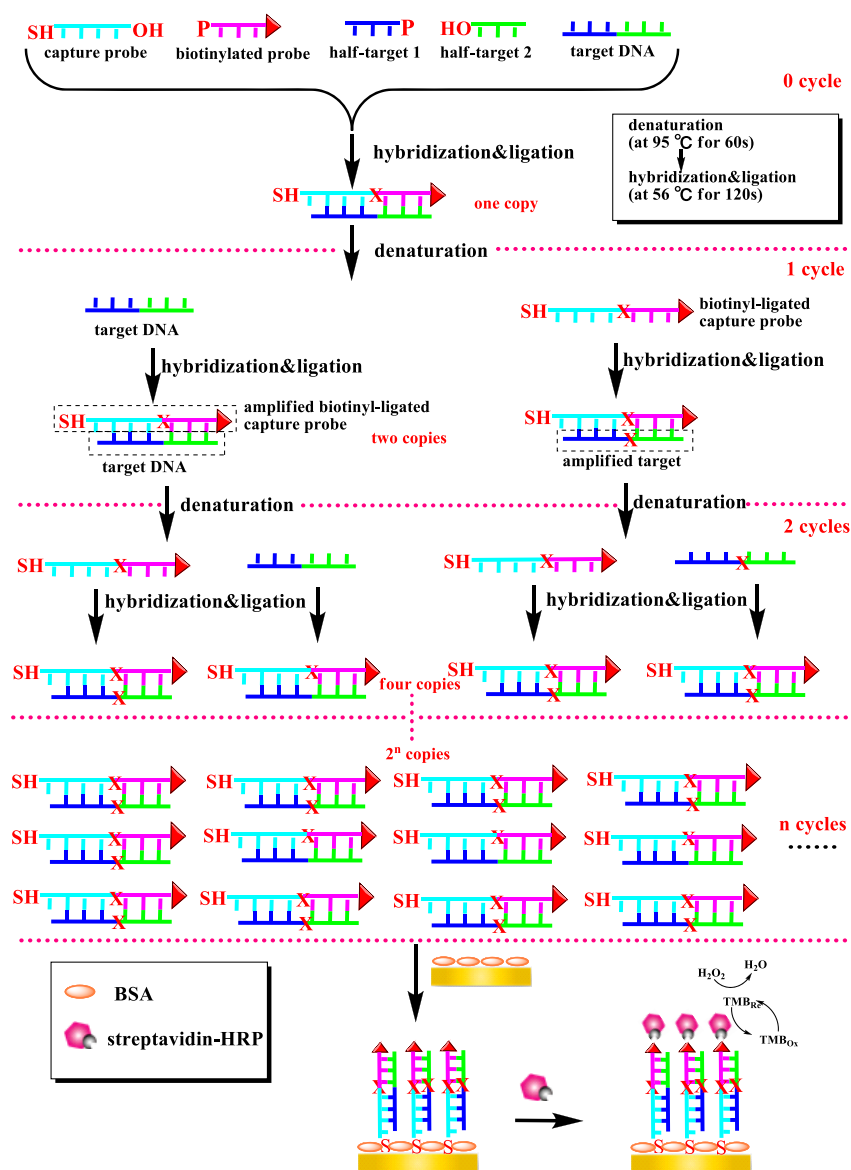
■ EXPERIMENTAL SECTION

Reagents and Materials. BSA was obtained from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (China). The 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Enhanced K-blue substrate, H_2O_2 included) was purchased from Neogen (Lansing, USA). Streptavidin horseradish peroxidase (SA-HRP) was from Roche Diagnostics (Mannheim, Germany). Ampligase thermal stable ligase and 10 $\times$ reaction buffer were purchased from Epicenter Technologies (Madison, USA). The buffer solutions used in this study were as follows: the TE buffer for dissolving the synthetic oligonucleotides contained 10 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 M NaCl; the dilution buffer for DNA probes was 10 mM Tris-HCl (pH 8.0); the washing buffer was 10 mM PB buffer (pH 7.4); and the dilution buffer for SA-HRP was 10 mM PBS buffer (pH 7.4, 0.1 M NaCl) containing 0.25% BSA. All solutions were prepared with Milli-Q water (18 M Ω /cm resistivity) produced using a Millipore system. All oligonucleotides representing the specific sequence of genotype C from hepatitis B virus (HBV) were synthesized and purified by TaKaRa Inc. (Dalian, China), the base sequences of which are listed in Table S1. The clinical serum samples were supplied by the Mengchao Hepatobiliary Hospital of Fujian Medical University and approved by the medical ethics committee of the hospital.

Homogeneous Ligation Chain Reaction. A 50 μL volume of reaction solution containing 10 $\times$ reaction buffer, 2 U ampligase, 20 nM of the four types of probes (capture probe, biotinylated probe, half-target 1, and half-target 2), and target was vortexed thoroughly and then subjected to thermal cycling in a 2720 thermal cycler (Applied Biosystems). After a thorough optimization process, the LCR protocol was established as follows. The thermal cycle comprised 30 cycles of a 2 min ligation at 56 $^{\circ}\text{C}$ and a 1 min denaturation at 95 $^{\circ}\text{C}$. Thereafter, a 2.63 μL volume of 0.2 M PB buffer (pH 7.4) was added to the amplification products.

Preparation of the Electrochemical Biosensor. Initially, the gold electrodes of the biosensor were successively polished with 0.3 and 0.05 μm alumina slurries, after which the electrodes were sonicated sequentially in ethanol and ultrapure water to remove the residual alumina powder. The electrodes were further electrochemically cleaned in 0.5 M H_2SO_4 solution by potential scanning between 0 and 1.6 V until a reproducible cyclic voltammogram was obtained, followed by rinsing with ultrapure water and drying with nitrogen. Thereafter, the cleaned electrodes were incubated with 0.1% BSA at room temperature for 15 min. Following being washed and dried, the BSA-modified electrodes were exposed to the aforementioned LCR solution at room temperature for 1 h. Subsequently, the electrodes were rinsed with PB and dried, and a 3 μL volume of SA-HRP (0.5 U/mL) was spread on the electrode surface at room temperature for 15 min. Finally, the electrode was extensively rinsed and subjected to electrochemical measurements.

Scheme 1. Schematic Illustration of the Double-Stranded DNA-Assembled Electrochemical Biosensor Using Ligase Chain Reaction Amplification



Electrochemical Measurements. An electrochemical workstation (CHI 760d; CH Instruments, Austin, TX) was used for the amperometric experiment, in conjunction with a conventional Au disk working electrode (2 mm diameter), an Ag/AgCl reference electrode, and a Pt wire auxiliary electrode. Amperometric detection was performed in TMB substrate at room temperature with a fixed potential of 0.1 V, and the current was sampled at 100 s after the HRP redox reaction reached a steady state.

RESULTS AND DISCUSSION

Design Principle of the Sensor. The principle of the proposed dsDNA-assembled electrochemical biosensor for DNA detection using LCR amplification is illustrated in Scheme 1. The system consists of four short ssDNA probes (capture probe, biotinylated probe, half-target 1, and half-target 2), target DNA, and ampligase. Half-target 1 and half-target 2 are similarly designed as each half of the target; whereas the capture probe and the biotinylated probe are

complementary to each half of the target, thus generating two short dsDNAs (capture probe/half-target 1 and biotinylated probe/half-target 2). In the presence of the target, the capture and biotinylated probes are hybridized to adjacent positions on the target template at 56 °C and are subsequently covalently joined by ampligase to form the ligated product. When heated to 95 °C, all duplexes are denatured, releasing target DNA and the ligated product. In this way, when the temperature is reduced to 56 °C, the DNA target is recycled to undergo target-recycled ligation; whereas the ligated product serves as a new template for half-target 1 and half-target 2 to form a new ligation product that also serves as the secondary target for subsequent hybridization/ligation with the capture probe and biotinylated probe to generate the biotinyl-ligated capture probe. Thus, from the second thermal cycle, the amount of ligated products theoretically doubles after each cycle, resulting in an exponential amplification of the ligated probes (secondary target and biotinyl-ligated capture probe) through repeating the thermal cycling of hybridization/ligation at 56 °C

and denaturation at 95 °C. In general, two short dsDNAs are successfully transformed into a single integrated dsDNA through LCR, which we named “probe lengthening” vividly.

The LCR procedure generates a large number of duplex products of the secondary target/biotinyl-ligated capture probe. Here, the capture probe was designed with a thiol label at its 5' end, and the biotinylated probe was modified with biotin at its 3' end. Thus, the duplex products with the capture probe can be readily immobilized on the gold surface in the gaps between BSA molecules via Au–S chemistry. SA-HRPs were then specifically bound to the biotin labels via biotin–streptavidin interaction, which generates the catalytic amperometric readout. Notably, the lengthened dsDNAs containing the biotin labels could be formed only in the presence of target DNA, so that nonspecific currents could be effectively eliminated.

Optimization of Conditions. In order to obtain high amplification efficiency, key factors in the LCR were examined and optimized. Taking into consideration the time requirements and the specificity of the amplification, we fixed the thermal cycle number of the LCR at 30 cycles. Initially, we optimized the temperature of hybridization/ligation. As indicated in Figure 1A, temperature is a key factor for optimal

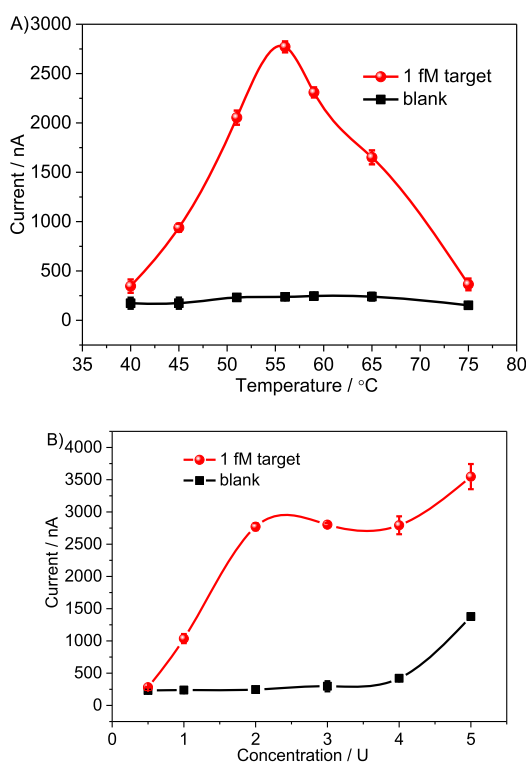


Figure 1. Effects of the hybridization/ligation temperature (A) and the quantity of ampligase (B) on the signal of the 1 fM target DNA and blank (without target). The error bars represent the standard deviation of triplicate samples.

LCR amplification efficiency due to its influence on the hybridization between target and partial probes. The highest amperometric current was generated at 56 °C, which was therefore used in the following experiments. Thereafter, the effect of the quantity of ampligase on the signal response was examined. As shown in Figure 1B, for the detection of the 1 fM target DNA, the current intensity increased markedly with an increase in the quantity of ampligase used up to 2 U and then

changed more solely thereafter. For a blank sample without target DNA, an increasing tendency of amperometric current was observed above 3 U. Thus, to obtain the maximum current signal difference between target DNA and the blank, 2 U of ampligase was used in subsequent experiments.

Sensitivity of the Sensor. Under the optimized experimental conditions, the target DNA at various concentrations was analyzed using the proposed LCR-based electrochemical biosensor. As shown in Figure 2A, the current

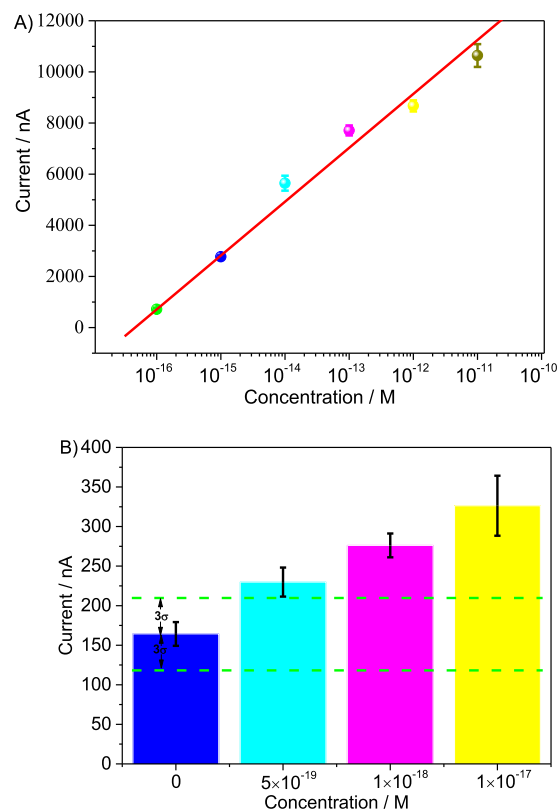


Figure 2. (A) Calibration plot of the amperometric current versus logarithmical concentration of target DNA (from 1.0×10^{-16} to 1.0×10^{-11} M). (B) Amperometric response of the biosensor in the presence of 0, 5×10^{-19} , 1×10^{-18} , and 1×10^{-17} M target DNA.

increased linearly with the target concentration ranging from 1.0×10^{-16} to 1.0×10^{-11} M, extending over 6 orders of magnitude with a regression equation of $I = 34412.68 + 2106.32 \lg C$ ($R = 0.9957$), in which I is the amperometric signal value in nA, and C is the concentration of target in M. As shown in Figure 2B, the detection limit was estimated to be 0.5 aM ($>3\sigma$), indicating that as few as 15 copies of DNA molecule in a 50 μ L volume can be detected. A comparison of the proposed new strategy with a range of different nucleic acid amplification techniques is shown in Table S2.²⁸ The proposed method not only matches these nucleic acid amplification-based methods in detection performance but also has notable advantages in terms of design simplicity and operational ease.

Notably, the sensitivity of this protocol was considerably greater than that required for direct gene expression profiling, which is attributed to (i) a substantial increase of the amount of the biotinyl-ligated capture probe during LCR, (ii) the direct assembly of dsDNA on the electrode surface without hybridization on a heterogeneous surface, (iii) the high resistance to nonspecific adsorption of the nucleic acid and

protein of the BSA-based DNA probe carrier platform, and (iv) the enzyme-catalyzed amplification in the TMB-H₂O₂-HRP system. In addition, on the basis of five repeated measurements of the 1 fM target DNA, the relative standard deviation (RSD) was estimated to be 2.0%, indicating the good precision of this assay.

Specificity of the Sensor. In addition, DNAs with single-base mutation at the ligation sites, including C (C-Mut), T (T-Mut), and G (G-Mut), were used to assess the ability of the proposed assay for differentiating between the single-base mismatched DNAs and the target DNA (A-Full). All of the assay conditions, including the thermal cycling conditions (denaturation at 95 °C for 60 s and hybridization/ligation at 56 °C for 120 s), the number of thermal cycles (30), concentration of the single-base mismatched target DNA (1 fM), and the detection procedures, were identical to those used for assaying complementary target DNA. As shown in Figure 3, the signal intensity from A-Full was nearly five times

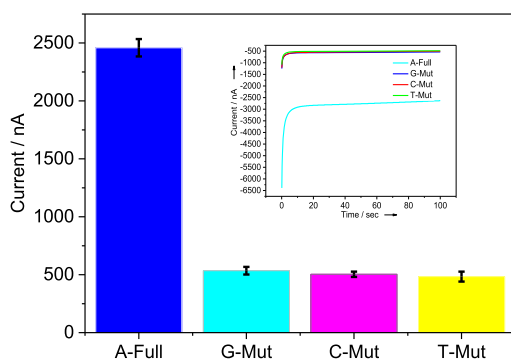


Figure 3. Selectivity of the biosensor in analyzing the full-match target DNA (A-Full) and single-base mismatched target DNAs (T-Mut, C-Mut, and G-Mut) at 1 fM. The inset shows the current–time curves of different targets. Mean values and standard deviations are obtained from three independent experiments.

stronger than that from the other three mutated DNAs (T-Mut, C-Mut, and G-Mut), which clearly demonstrated that single-base mismatched DNAs could be readily distinguished using the proposed method. The excellent specificity of this LCR-based electrochemical assay is largely attributed to the fidelity of the thermal-stable ampligase during the thermal cycles, which in turn results in the fabrication of ligated probes on the electrode surface to produce an amperometric signal. Therefore, these results clearly demonstrate that the proposed method can be used for analysis of single nucleotide polymorphisms (SNPs), which plays an important role in gene expression.

Real Sample Assay. To determine the feasibility of practical application of the proposed method, we further examined its direct detection of genomic DNA extracts from serum samples. Herein, we selected genotype C of the HBV as the detection target on the basis of its practical significance. HBV infection is prevalent worldwide, and the dominant genotypes in China are B and C.^{29,30} Moreover, an increasing number of studies have documented that patients with genotype C infection are more likely to progress to chronic hepatitis, cirrhosis, or liver cancer.^{31–33} Thus, distinguishing genotype C from genotype B in HBV infection plays a critical role in predicting the risk of progression of HBV infection, as well as in the selection of an appropriate treatment regimen.

The serum samples used in the current study were obtained from the Mengchao Hepatobiliary Hospital of Fujian Medical University, where they had been collected from patients infected with genotype B or C of HBV, as determined using a diagnostic kit for Hepatitis B Virus Gene Type (PCR Fluorescence). Nine genotype-B samples and nine genotype-C samples were randomly selected, and nine serum samples from healthy individuals were used as controls for comparison. Genomic DNA was extracted from a 200 μ L volume of each serum sample using an automatic magnetic bead extraction and purification system (Thermo Fisher Scientific) according to the manufacturer's instructions and resuspended in 100 μ L of sterile ultrapure water. Thereafter, the extracts were added to the reaction system for LCR amplification and subsequently electrochemical analysis. As shown in Figure 4, the

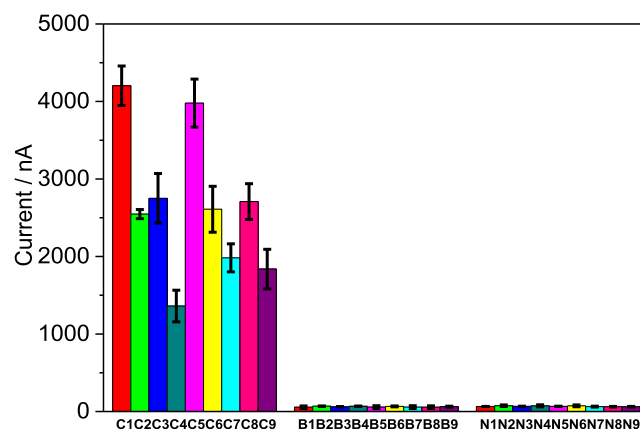


Figure 4. Amperometric response of the biosensor when used to analyze real clinical samples. Error bars show the standard deviations of measurements taken from three independent experiments for each sample.

amperometric responses from all C-type samples (from 1200 to 4200 nA) were significantly higher than those of all B-type and normal samples (from 40 to 50 nA), which is in line with our expectation. For further confirmation, we selected four samples (C1, C2, B1, and B2) for direct DNA sequencing. As indicated in Figure 5, the sequence of the two C-type samples was fully consistent with that of the target DNA fragment detected; whereas the two B-type samples had eight mutational sites in the corresponding position from 96 to 143 nt. Furthermore, native polyacrylamide gel electrophoresis (PAGE) (Figure 6) was performed to confirm successful LCR amplification of the C-type sample (the concentration of partial probes was 2.0 μ M). As shown in lane 1 of Figure 6, the slowest mobile species was observed, indicating the formation of the ligated duplex of the secondary target/biotinylated capture probe. Although the capture probe/half-target 1 comprised the same base pairs as the biotinylated probe/half-target 2, a T_{10} overhang decreased its mobility. We further conducted PAGE analysis using a series of diluted LCR-amplified products of C1 samples (3\9\27\81\243). From lane 2 to lane 5, the band became gradually weaker and disappeared in lane 5 when diluted 81 times. We calculated that our electrochemical DNA biosensor was at least 3 orders of magnitude more sensitive than PAGE analysis of the amplified products, which clearly demonstrates its promising application to the detection of genomic DNA.

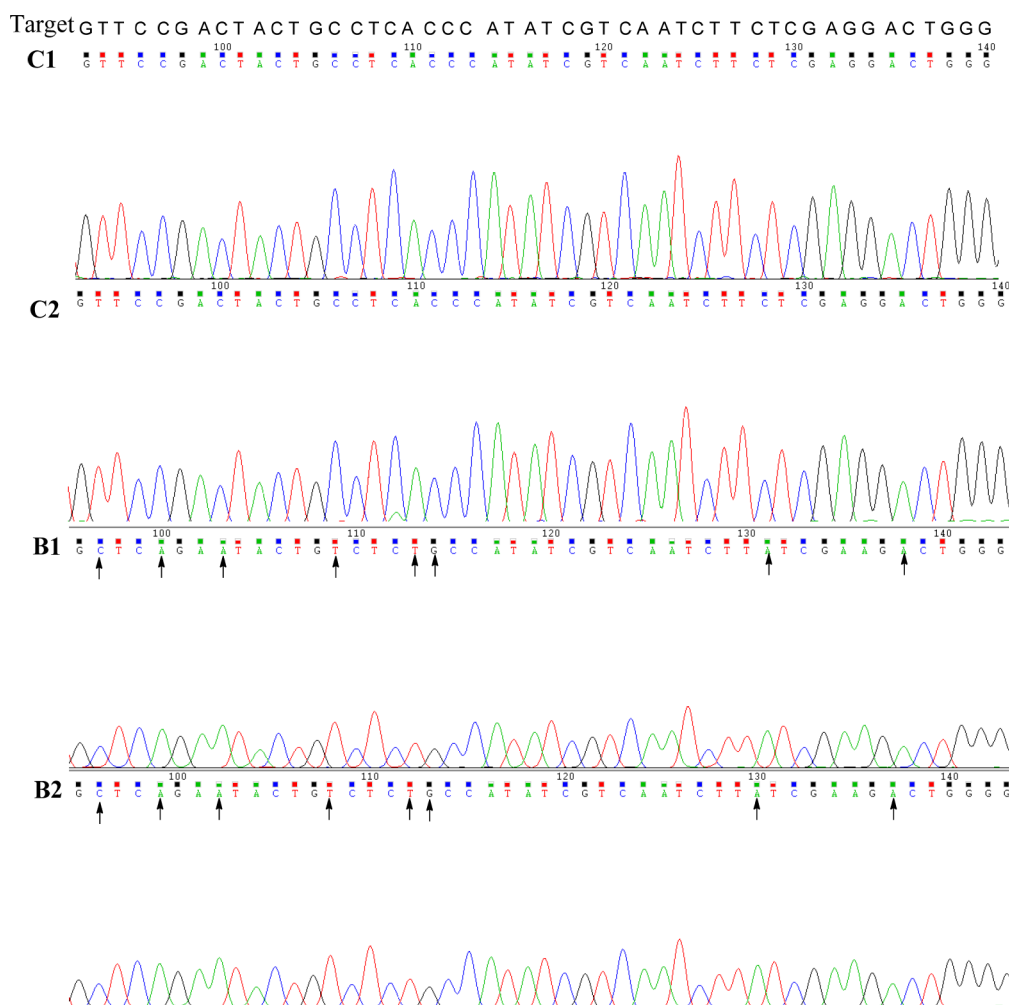


Figure 5. Graph matching gene sequencing of the designed target with that of four samples (C1, C2, B1, and B2). Arrows indicate the mutational sites.

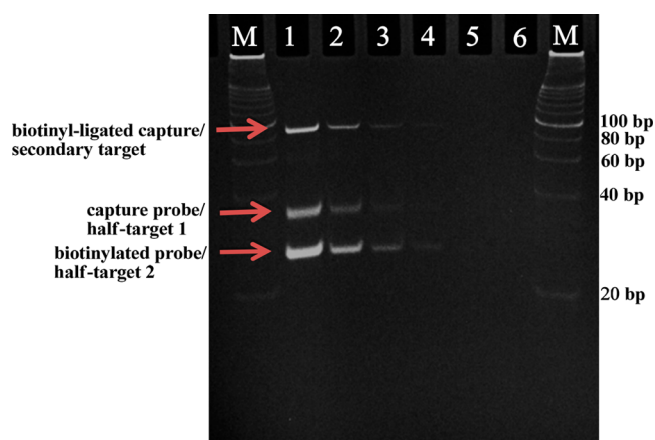


Figure 6. Native PAGE (20% native gel) of the LCR products amplified from sample C1. Lane M, 500 base pair (bp) DNA ladder; lane 1, final products of the LCR; lane 2, final products diluted 3 times; lane 3, final products diluted 9 times; lane 4, final products diluted 27 times; lane 5, final products diluted 81 times; and lane 6, final products diluted 243 times. The concentrations of the partial probes are 2.0 μ M.

Collectively, these results provide comprehensive evidence that the proposed dsDNA-assembled electrochemical bio-

sensor can be applied to the detection of genomic DNA in real clinical samples with high sensitivity and selectivity. Most importantly, this biosensor can be extended to assay other DNA biomarkers merely by altering the DNA sequence, thereby demonstrating its considerable potential in fields ranging from virus detection to the diagnosis of genetic diseases.

CONCLUSIONS

In summary, we have developed an ultrasensitive electrochemical biosensor through probe lengthening to detect trace levels of genomic DNA, which is based on the novel strategy of lengthening probes without producing large numbers of new DNA copies that can readily lead to nonspecific results. Under the optimum conditions, our approach can be used to detect as few as 15 copies of target DNA over a wide linear concentration range from 1.0×10^{-16} to 1.0×10^{-11} M and also shows a high specificity with regards to the analysis of SNPs. Furthermore, the developed biosensor successfully detected the C genotype of HBV in real clinical samples, clearly differentiating these from B-type and normal samples, thereby fully demonstrating its excellent sensitivity and specificity. Moreover, this method is universal, meeting the demands for ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and delivered to

those who need it) diagnosis recommended by WHO, merely by altering the sequences for detection of other DNA biomarkers. Therefore, this is a simple, ultrasensitive, selective, and effective assay that has considerable potential for clinical application in the early diagnosis and prognosis of DNA-related diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b05692.

Corresponding illustration of oligonucleotides used in this study, and the comparison of the proposed method with recently reported nucleic acid amplification-based electrochemical biosensors (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: ailingliu@fjmu.edu.cn.

*E-mail: chenandhu@163.com.

*E-mail: 13906909638@163.com.

ORCID

Ai-Lin Liu: 0000-0002-0996-6632

Wei Chen: 0000-0003-3233-8877

Author Contributions

†J.-Y.C., Z.-J.L., and X.-W.W. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (21775023); the Medical Elite Cultivation Program of Fujian Province (2018-ZQN-49); Joint Funds for the Innovation of Science and Technology of Fujian Province (2017Y9124 and 2016Y9055); and the Startup Fund for Scientific Research, Fujian Medical University (2017XQ1057).

■ REFERENCES

- (1) He, L.; Musick, M. D.; Nicewarner, S. R.; Salinas, F. G.; Benkovic, S. J.; Natan, M. J.; Keating, C. D. *J. Am. Chem. Soc.* **2000**, *122*, 9071–9077.
- (2) Caruso, F.; Rodda, E.; Furlong, D. F.; Niikura, K.; Okahata, Y. *Anal. Chem.* **1997**, *69*, 2043–2049.
- (3) He, S. J.; Song, B.; Li, D.; Zhu, C. F.; Qi, W. P.; Wen, Y. Q.; Wang, L. H.; Song, S. P.; Fang, H. P.; Fan, C. H. *Adv. Funct. Mater.* **2010**, *20*, 453–459.
- (4) Ge, L.; Sun, X. M.; Hong, Q.; Li, F. *ACS Appl. Mater. Interfaces* **2017**, *9*, 13102–13110.
- (5) Li, H. X.; Rothberg, L. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 14036–14039.
- (6) Hsieh, K.; White, R. J.; Ferguson, B. S.; Plaxco, K. W.; Xiao, Y.; Soh, H. T. *Angew. Chem., Int. Ed.* **2011**, *50*, 11176–11180.
- (7) Xuan, F.; Fan, T. W.; Hsing, I. M. *ACS Nano* **2015**, *9*, 5027–5033.
- (8) Jambrec, D.; Gebala, M.; La Mantia, F.; Schuhmann, W. *Angew. Chem., Int. Ed.* **2015**, *54*, 15064–15068.
- (9) Duprey, J.-L. H. A.; Carr-Smith, J.; Horswell, S. L.; Kowalski, J.; Tucker, J. H. R. *J. Am. Chem. Soc.* **2016**, *138*, 746–749.
- (10) Daggumati, P.; Appelt, S.; Matharu, Z.; Marco, M. L.; Seker, E. *J. Am. Chem. Soc.* **2016**, *138*, 7711–7717.
- (11) Liu, G.; Wan, Y.; Gau, V.; Zhang, J.; Wang, L. H.; Song, S. P.; Fan, C. H. *J. Am. Chem. Soc.* **2008**, *130*, 6820–6825.
- (12) Wang, K.; Lei, Y.; Zhong, G. X.; Zheng, Y. J.; Sun, Z. L.; Peng, H. P.; Chen, W.; Liu, A. L.; Chen, Y. Z.; Lin, X. H. *Biosens. Bioelectron.* **2015**, *71*, 463–469.
- (13) Kerman, K.; Vestergaard, M.; Nagatani, N.; Takamura, Y.; Tamiya, E. *Anal. Chem.* **2006**, *78*, 2182–2189.
- (14) Lai, R. Y.; Lagally, E. T.; Lee, S. H.; Soh, H. T.; Plaxco, K. W.; Heeger, A. J. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 4017–4021.
- (15) Xu, X. W.; Weng, X. H.; Wang, C. L.; Lin, W. W.; Liu, A. L.; Chen, W.; Lin, X. H. *Biosens. Bioelectron.* **2016**, *80*, 411–417.
- (16) Defever, T.; Druet, M.; Evrard, D.; Marchal, D.; Limoges, B. *Anal. Chem.* **2011**, *83*, 1815–1821.
- (17) Hsieh, K. W.; Patterson, A. S.; Ferguson, B. S.; Plaxco, K. W.; Soh, H. T. *Angew. Chem., Int. Ed.* **2012**, *51*, 4896–4900.
- (18) Barreda-Garcia, S.; Miranda-Castro, R.; de-los-Santos-Alvarez, N.; Miranda-Ordieres, A.; Lobo-Castanon, M. *Anal. Bioanal. Chem.* **2016**, *408*, 8603–8610.
- (19) Li, J. L.; Chen, Z. P.; Xiang, Y.; Zhou, L. L.; Wang, T.; Zhang, Z.; Sun, K. X.; Yin, D.; Li, Y.; Xie, G. M. *Biosens. Bioelectron.* **2016**, *86*, 75–82.
- (20) Yuan, R.; Ding, S. J.; Yan, Y. R.; Zhang, Y.; Zhang, Y. H.; Cheng, W. *Biosens. Bioelectron.* **2016**, *77*, 19–25.
- (21) Pei, H.; Lu, N.; Wen, Y.; Song, S.; Liu, Y.; Yan, H.; Fan, C. *Adv. Mater.* **2010**, *22*, 4754–4758.
- (22) Zeng, D. D.; Zhang, H.; Zhu, D.; Li, J.; San, L. L.; Wang, Z. H.; Wang, C. G.; Wang, Y. S.; Wang, L. H.; Zuo, X. L.; Mi, X. Q. *Biosens. Bioelectron.* **2015**, *71*, 434–438.
- (23) Lin, M. H.; Wang, J. J.; Zhou, G. B.; Wang, J. B.; Wu, N.; Lu, J. X.; Gao, J. M.; Chen, X. Q.; Shi, J. Y.; Zuo, X. L.; Fan, C. H. *Angew. Chem., Int. Ed.* **2015**, *54*, 2151–2155.
- (24) Liu, Y. H.; Li, H. N.; Chen, W.; Liu, A. L.; Lin, X. H.; Chen, Y. Z. *Anal. Chem.* **2013**, *85*, 273–277.
- (25) Li, C.; Wu, D.; Hu, X. L.; Xiang, Y.; Shu, Y. Q.; Li, G. X. *Anal. Chem.* **2016**, *88*, 7583–7590.
- (26) Liu, Y. H.; Deng, H. H.; Li, H. N.; Shi, T. F.; Peng, H. P.; Liu, A. L.; Chen, W.; Hong, G. L. *J. Electroanal. Chem.* **2018**, *827*, 27–33.
- (27) Marx, V. *Nat. Methods* **2015**, *12*, 393–397.
- (28) Ji, H. X.; Yan, F.; Lei, J. P.; Ju, H. X. *Anal. Chem.* **2012**, *84*, 7166–7171.
- (29) Kao, J. H.; Chen, D. S. *Lancet Infect. Dis.* **2002**, *2*, 395–403.
- (30) Zeng, G.; Wang, Z.; Wen, S.; Jiang, J.; Wang, L.; Cheng, J.; Tan, D.; Xiao, F.; Ma, S.; Li, W.; Luo, K.; Naoumov, N. V.; Hou, J. J. *Viral Hepatitis* **2005**, *12*, 609–617.
- (31) Lin, C. L.; Kao, J. H. J. *Gastroenterol. Hepatol.* **2011**, *26*, 123–130.
- (32) Chu, C. J.; Hussain, M.; Lok, A. S. F. *Gastroenterology* **2002**, *122*, 1756–1762.
- (33) Wai, C. T.; Chu, C. J.; Hussain, M.; Lok, A. S. F. *Hepatology* **2002**, *36*, 1425–1430.