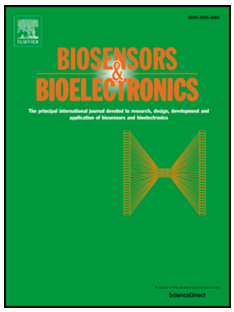

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PII: S0956-5663(19)30831-0

DOI: https://doi.org/10.1016/j.bios.2019.111752

Reference: BIOS 111752

To appear in: *Biosensors and Bioelectronics*

Received Date: 11 July 2019

Revised Date: 10 September 2019

Accepted Date: 30 September 2019

Please cite this article as: Li, Y., Liu, H., Huang, H., Deng, J., Fang, L., Luo, J., Zhang, S., Huang, J., Liang, W., Zheng, J., A sensitive electrochemical strategy *via* multiple amplification reactions for the detection of *E. coli* O157: H7, *Biosensors and Bioelectronics* (2019), doi: https://doi.org/10.1016/j.bios.2019.111752.

A sensitive electrochemical strategy *via* multiple amplification reactions for the detection of *E. coli* O157: H7

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Abstract: The sensitive and efficient strategy remains a central challenge for early diagnosis of pathogenic bacteria. Herein, an ultrasensitive electrochemical biosensor was proposed based on the multiple amplification strategy *via* the 3D DNA walker, rolling circle amplification (RCA) and hybridization chain reaction (HCR) for the accurate detection of Escherichiacoli O157:H7 (*E. coli* O157:H7). Firstly, the target sequence extracted from *E. coli* O157:H7 was transformed and amplified by the DNA walker firstly. Subsequently, a large number of transformed nucleic acid sequences were amplified by the RCA reaction. And then, the progress of HCR was triggered by every fragment in RCA products to form a long double-stranded DNA sequence to immobilize electrochemical indicators, generating a significantly enhanced electrochemical signal. As expected, a high sensitivity with a detection limit of 7 CFU/mL was achieved based on the proposed multiple amplification strategy, which is superior to most current methods for *E. coli* O157: H7 assay. The multiple amplification strategy could be readily expanded for the detection of various pathogenic bacteria, providing a new approach for early diagnosis of pathogenic microorganisms or other diseases.

Keywords: electrochemical biosensor; multiple amplification; *E. coli* O157: H7; early diagnosis

1. Introduction

Pathogenic bacteria were all around human living. Although some pathogenic bacteria are harmless, the pathogenic bacteria-inflicted infectious diseases resulted in millions of deaths and hospitalizations annually due to infection of the highly pathogenic bacteria, which are one of the leading causes of mortality throughout the world now. (Luanne et al., 2004; Stephen et al., 2001; Tauxe rt al., 1997) Specially, Escherichiacoli O157:H7 (*E. coli* O157:H7) as one of the highly pathogenic bacteria can be transmitted through food, drinking water, etc., which not only seriously threatens human health, but also causes serious economic losses, posing a serious threat to public health and financial stability. (Tryland et al., 1998; Varshney et al., 2007) It is significantly important to develop the sensitive methods for efficient detection of *E. coli* O157:H7 for clinical diagnosis and administration of the most effective treatment, which is not only significant for the patients, but also for all around them, because the inefficient identification of *E. coli* O157:H7 would result in delayed treatment, further spread of infectious disease, and even worse. Currently, the culture and colony counting method has been regarded as the standard method to identify *E. coli* O157:H7. In spite of its high sensitivity down to one colon, it is time-consuming with laborious processes. (Costello et al., 2002; Kwon et al., 2013) Most importantly, this conventional method requires a central laboratory with trained lab personnel and expensive equipments. In contrast, molecular methods could provide higher speed and specificity with equal sensitivity. The most commonly employed molecular method is the polymerase chain reaction (PCR). (Malorny et al.,

2004) Normally, the detection time could be shortened to about 24 h. It has been improved significantly compared with the culture and colony counting methods taking days for an accurate detection, but it is required essentially that extensive sample preparation and expensive instrumentation. Additionally, there is a serious problem of PCR methods associated with the sample matrix due to the PCR inhibitors in the biological samples, such as the blood, feces and urine. (Scheu et al., 1998; Maurer et al., 2011) Another important molecular method, immunoassay based on specific antigen/antibody binding, is rapid and cost-effective, but suffered from low sensitivity. (Xu et al., 2012; Belgrader et al., 1999; Gootenberg et al., 2017) Accordingly, development of ultrasensitive assays for pathogenic bacteria remains a central challenge today, despite its urgent need.

Electrochemical biosensor has attracted much attentions in the detection of pathogenic bacteria due to its distinct advantages in terms of size, cost, portability, sensitivity and rapidity, and it has been widely applied for the efficient determination of proteins, nucleic acids, cells, pathogens and so on. (Zhao et al., 2010; Zhuo et al., 2009; Wang et al., 2008) Whereas, in spite of its significant advantages, it is still on the way to improve its detection performances, especially sensitivity and efficiency. To this end, several strategies have been introduced in the development of electrochemical biosensors. Very recently, Kengo and co-workers reported an electrochemical strategy to sensitive detect the pathogenic bacteria. With the help of electrochemical reaction, the detection limit could be improved to 28 CFU/mL. (Ishiki et al., 2018) Meanwhile, we have also successfully developed a series of highly

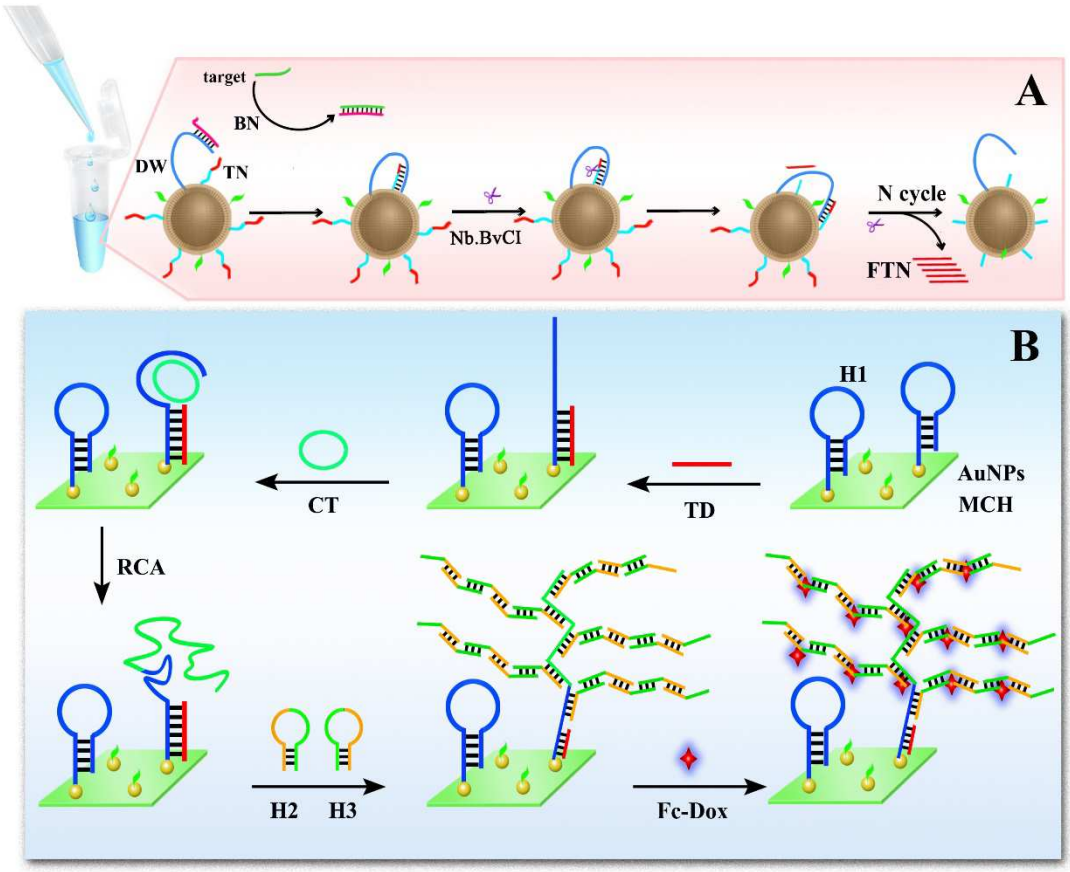
sensitive electrochemical biosensors for the efficient detection of pathogenic bacteria *via* specific immunological recognition with *E. coli* O157 as a model. (Li et al., 2012; Li et al., 2013; Li et al., 2015; Li et al., 2017) However, in spite of these great improvements, the detection performances of these proposed electrochemical biosensors were still far from the ultrasensitive detection of pathogenic bacteria in early stage of infection.

In this study, we focused on the study of multi-amplification techniques to further improve the detection sensitivity of electrochemical biosensors. As is well-known, proteins couldn't be amplified by itself, such as antibodies or antigens. On the contrary, the nucleic acids could be amplified simply based on various amplification strategies, such as rolling circle amplification (RCA) and hybridization chain reaction (HCR). (Georganopoulou et al., 2005; Yan et al., 2010; Zhao et al., 2013; Zhou et al., 2013; Yan et al., 2014; Wang et al., 2014; Wang et al., 2014; Liao et al., 2015) Based on these DNA amplification strategies, an initiator such as a short oligonucleotide could be extended to a long repeated DNA to generate significantly enhanced signal. Thus, it is possible to generate nucleic acids electrochemical biosensor with improved sensitivity based on the efficient amplification strategies for ultrasensitive detection of pathogenic bacteria. However, improvements were still needed to overcome of the limitation of single DNA amplification strategy. Recently, with the rapid development of nucleic acid amplification, various efficient DNA machine-based strategies have been introduced, in which the DNA walker has gained significant attentions due to its high efficiency. The DNA walker could act as a molecular machine to manipulate

specific DNA to move autonomously along programmed oligonucleotide trajectories with the generation of single strand nucleic acids or hybridization of two pre-locked nucleic acids for signal amplification. (Ding et al., 2013; Pan et al., 2015; Cai et al., 2016; Ji et al., 2017; Liu et al., 2017; Feng et al., 2018; Qing et al., 2018; Yan et al., 2018) However, conventional DNA walkers are mostly limited to one-dimensional or two-dimensional spaces with a limited amplification efficiency due to the influence of steric hindrance. If combined with magnetic separation technology to generate the DNA walker in the three-dimensional space on the surface of the magnetic microspheres, it could be expected that the target nucleic acid sequence based on the DNA walker can be efficiently transformed and amplified, and the detection signal can be greatly enhanced based on the improved amplification efficiency. Additionally, the magnetic microspheres could not only improve the sensitivity of the detection, but also frees template of the possible inhibitors for nucleic acid-based reactions. Furthermore, RCA as a kind of constant temperature nucleic acid amplification method could transform a short nucleic acid to a repetitive single-stranded long nucleic acid with the help of cyclic template and deoxynucleotides (dNTPs). Thus, hundreds of duplicate template complementary fragments could be synthesized in a long nucleic acid. Whereas, the single strand nucleic acid was hard to immobilize electrochemical indicators for signal amplification. As contrast, another useful nucleic acid amplification method, HCR, could be triggered by a small single strand nucleic acid to form a double strand nucleic acid-based copolymer with multiple repeating units, which could immobilize electrochemical indicators simply based on the

interaction between the electrochemical indicator and double strand nucleic acid. Thus, it could be expected that a highly improved amplification efficiency could be achieved by combining RCA and HCR reactions.

Taking the advantages and challenges into consideration, an ultrasensitive electrochemical biosensor was proposed based on a multiple amplification strategy *via* the 3D DNA walker, RCA and HCR amplification reactions for the accurate detection of *E. coli* O157: H7 simply, in which the *E. coli* O157: H7 specific target gene generated significant enhancement on the electrochemical signals based on three main amplification processes: 1) the target gene could trigger the 3D DNA walker, generating the first amplification by producing many special DNA sequences; 2) every produced DNA sequence could open the hairpin DNA 1 (H1) on the biosensor surface, exposing new terminus to generate the second amplification, RCA reaction, forming a long single-stranded DNA sequence with many duplicate fragments; 3) every fragment could react with hairpin DNA 2 (H2) and hairpin DNA 3 (H3) to generate the third amplification, HCR reaction, forming a long double-stranded DNA sequence to immobilize electrochemical indicators. Thus, it could be achieved that a significantly enhanced electrochemical signal, and therefore, a highly improved sensitivity. The proposed electrochemical bioassay based on the multiple amplification strategy *via* the 3D DNA walker, RCA and HCR amplification reactions simultaneously provides a sensitive approach for efficient determination of specific genes for early diagnosis of pathogenic bacteria in early stage of infection.



Scheme 1 Schematic representation of the multiple sensitizing electrochemical biosensor for the detection of *E. coli* O157:H7. A, the schematic diagram of the 3D DNA walker-based amplification reactions triggered by the target gene to produce many fragment of TN (FTN); B, the schematic diagram of HCR and RCA-based amplification reactions on the electrode surface to generate to long double-stranded DNA sequences to immobilize many electrochemical indicators related with the concentration of target gene. (AuNPs, gold nanoparticles; BN, Blocking DNA; CT, Circular template; DW, DNA walker; FTN, fragment of TN; H1, hairpin DNA 1; H2, hairpin DNA 2; H3, hairpin DNA 3; HCR, hybridization chain reaction; MCH, 6-mercapto ethanol; RCA, rolling circle amplification; TN, transfer oligonucleotide).

2. Experiment

2.1 Chemicals and materials

Streptavidin coated magnetic beads, 6-mercapto ethanol (MCH), gold chloride

tetrahydrate ($HAuCl_4 \cdot 4H_2O$), 1, 1'-dicarboxyferrocene (Fc), doxorubicin (Dox) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). T4 DNA ligase and phi29 DNA polymerase were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). dNTPs were obtained from Genview Scientific Inc. (El Monte, CA, U.S.A.). Exonuclease III (Exo III) and exonuclease I (Exo I) were obtained from Thermo Fisher Scientific Inc. (Shanghai, China). Tris buffer (pH 7.4, 10 mM, with 1.5 mM $MgCl_2$) was obtained by dissolving Tris-HCl and $MgCl_2$ with ultrapure water. Tris-EDTA buffer (pH 8.0, 10 mM, with 1.0 mM EDTA) was prepared by 10× Tris buffer and EDTA. Phosphate buffered saline (PBS, 0.1 M, pH 7.0) was prepared by dissolving Na_2HPO_4, KH_2PO_4 and KCl with ultrapure water. Ferricyanide/ferrocyanide mixed solution ($[Fe(CN)_6]^{3-/4-}$, pH 7.0, 5.0 mM) was obtained by dissolving $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$ and with PBS solution. The ultrapure water (specific resistance ≥ 18 MΩ cm^{-1}) was used throughout the study.

The oligonucleotides were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) with the sequences listed as follows (Table 1). Before using the hairpin DNA, they were prepared to form stem-loop structure by heating to 95 ºC for 2 min, and then cooling to 25 ºC. For the RCA amplification, the mixture of circular templates and associated primers was heated to 65 ºC for 10 min firstly. After cooling to room temperature, the mixture was reacted with T4 ligase to link 3' and 5' end of the circular templates. And then, Exco I and Exo III was added into the mixture, heating to 37 ºC for 30 min to clean the primers and nonreacted circular templates. Finally, the

Exo I and Exo III were deactivated by heating on 90 ºC for 15 min.

Table 1. The DNA oligonucleotides employed in the experiment and RCA products, in which the matched sequences among them were highlighted with different type, color or underline.

Name	Sequence (5'→3')	Length
DNA walker (DW)	Biotin-T10-T TGC ATC GCT TTC AAT CCT CAG C	33 aa
transfer nucleotide (TN)	Biotin-T10-T GC*T GAG GAT CTG AC	25 aa
blocking DNA (BN)	*TGA GGT GAA AGC GAT GCA GCT ATT A*	25 aa
Circular template (CT)	PO_4-GTC AGA ATC AGA TCC TAA GCC CCA CCC AAT TCT AGC T	37 aa
hairpin DNA1 (H1)	TCT GAC AGC TAG CTA CCG GTC AGA TCC TCA T5-T-SH	36 aa
hairpin DNA 2 (H2)	ATC AGA TCC TAA GCC CCA CCC AAT TTC AAA GTG ATT CAA TTG GGT GGG *GC*	50 aa
hairpin DNA 3 (H3)	AAT TGG GTG GGG CTT AGG ATC TGA TGC CCC ACC CAA TTG AAT CAC TTT GA	50 aa
Target	*TAA TAG CTG CAT CGC TTT CA*	20 aa
Associated primer (AP)	TCT GAC AGC TAG	12 aa
RCA Product	(TCT GAC AGC TAG AAT TGG GTG GGG CTT AGG ATC TGA T)$_n$ TCT GAC AGC TAG CTA CCG GTC AGA TCC TCA T5-T	n/a

Notes: The matched sequence of Target *vs* BN was highlight in italic type, the matched sequence of DW *vs* BN was highlight with wave underline, the matched sequence of DW *vs* TN was highlight with wave underline, the matched sequence of FTN *vs* H1 was highlight in yellow color, the matched sequence of H1 *vs* CT was highlight in blue color, the matched sequence of RCA produce *vs* H2 was highlight in green color, the matched sequence of H2 *vs* H3 was highlight in pink color and the matched sequence of H3 *vs* H2 was highlight in green color.

2.2 Apparatus

Cyclic voltammetric (CV), electrochemical impedance spectroscopy (EIS), differential pulse voltammetry (DPV) and electrodeposition were carried out on a CHI660e electrochemical workstation (Shanghai Chenhua Instrument, Shanghai,

China) with a conventional three-electrode system *via* a Ag/AgCl electrode as the reference electrode, a platinum wire as the auxiliary electrode, and an unmodified or modified glassy carbon electrode (GCE) as the working electrode, respectively. The CV measurements were invited in 5 mM $[Fe(CN)_6]^{3-/4-}$ in the potential range from -0.2 V to 0.6 V with a scan rate of 50 mV/s and the EIS measurements were obtained in a frequency range from 5×10^{-2} to 1×10^{6} Hz with a given open circuit potential of 10 mV.

2.3 Construction and characterization of electrochemical biosensor

Before the construction of the electrochemical biosensor, the GCE was firstly burnished entirely using 0.3 and 0.05 μm alumina slurry and then rinsed ultrasonically three time in ultrapure water and ethanol, respectively. And then, the gold nanoparticles (AuNPs) were modified onto the electrode surface *via* electrodeposition in 1 % $HAuCl_4$ solution for 30 s at 0.2 V. Subsequently, the prepared electrode (AuNPs/GCE) was incubated in 0.1 μM H1 for 12 h at 4 ºC to immobilize H1 on the electrode surface through Au-S interaction between the AuNPs on the electrode surface and SH groups on H1. Finally, the modified electrode was incubated with 10 μL 0.3 μM MCH for 30 min to block the nonspecific binding sites. All of the constructions of the electrochemical biosensors were characterized based on the CV and EIS in 5 mM $[Fe(CN)_6]^{3-/4-}$.

2.4 Preparation of 3D DNA Walker amplification system

The 3D DNA walker amplification system was operated based on the DNA walker reaction on the magnetic beads. Typically, the DNA walker (DW) was firstly blocked with blocking DNA (BN) based on the hybridization between the DW (1.5 μM) and BN (1.5 μM) in TE buffer with Zn^{2+} for 2 h at 37 °C. And then, the prepared DW-BN and transfer oligonucleotide (TN, 1.5 μM) were mixed with the magnetic beads on continuous oscillation for 1 h to immobilize DW-BN and TN onto the magnetic beads based on the specific interaction between the streptavidin on the magnetic beads and biotin on DW and TN. The unreacted DW-BN and TN were removed by magnetic separations and the obtained magnetic beads with DW-BN and TN were re-suspended in PBS with tween 20. For the electrochemical assay, the *E. coli* O157: H7 specific target gene was added into the reaction solution with Nb. BbvC I (20 U) and the prepared magnetic beads. The mixture was hold at 37 °C for 30 min to generate the DNA walker-based amplification reaction *via* the hybridization between the target gene and BN to release DW for the further hybridization between DW and TN to form DW-TN. With the help of Nb. BbvC I, the hybridized TN could be nicked to a fragment (FTN) and then the released DW would hybridize with another TN. Finally, the FTN was concentrated by magnetic separation. The circularly nicking and hybridizing processes amplified one target to many FTN with a significantly high efficiency.

2.5 Amplification based on the RCA and HCR

The multiple amplification based on RCA and HCR was operated on the electrode

surface. Typically, the prepared biosensor was immersed in the reaction solution with the generated FTN in the 3D DNA walker amplification at 37 °C for 40 min, which could open H1 on the electrode surface to expose the terminal to react with the prepared circular template (CT). After cleaning with water, the as-prepared electrode was immersed in the solution with CT (1.5 μM), phi29 (20 U) and dNTPs, the opened H1 could form long single-stranded DNA sequence based on RCA reactions. Subsequently, the reaction solution with H2, H3 and Dox modified Fc (Fc-Dox) was dropped onto the biosensor surface, and the biosensors were kept at 37 °C for 40 min to generate HCR amplification to form a long double-stranded DNA sequence. At the same time, the Fc-Dox could be embedded into the double-stranded DNA sequence to generate the electrochemical signal. Thus, a big dendrimer with Fc-Dox could be obtained for the sensitive assay.

2.6 Native polyacrylamide gel electrophoresis (PAGE) characterization of the related nucleic acid reactions. Briefly, 10 μL of each sample was firstly mixed with 2 μL of 6 × loading buffer, and the mixture was transferred into the gel electrophoresis system, respectively. And then, the electrophoresis was operated at a 120 V constant voltage. At last, the gels were photographed with the Gel Doc XR$^+$ System after staining with Gen Green for 20 min.

3. Results and discussion

3.1 Electrochemical characterizations for the construction of the electrochemical

biosensor

In order to characterize the construction of the electrochemical biosensor, the assembly steps of the sensing interface were investigated by CV measurements and EIS measurements respectively. As shown in Figure 1, the bare GCE showed a well-defined redox peak of $[Fe(CN)_6]^{3-/4-}$ (curve a). After AuNPs were electrodeposited onto the surface of the GCE, the redox peak increased obviously because of the improved electron transfer and enlarged surface area (curve b). When the H1 was immobilized onto the electrode surface, a significantly decreased redox peak could be observed due to the low conductive property of oligonucleotide (curve c). When the electrode was blocked with MCH, a decline was exhibited because of the inhibited electron transfer *via* MCH (curve d). Furthermore, the redox peak decreased significantly after RCA reaction and generation of dendrimer *via* RCA and HCR amplification (curve e and f).

The characterizations based on EIS with 5 mV amplitude were showed in Figure 1B. As one of the key parameter in EIS, the electron transfer resistance (R_{et}) was used to quantitatively characterize the electron transfer kinetics on the electrode surface, which was accurately computed based on the model impedance data *via* the equivalent circuit as shown in the insert figure of Figure 1B. The R_{et} of the bare GCE (curve a) was 55 Ω. When AuNPs was modified onto the GCE surface, the R_{et} decreased obviously to 23 Ω (curve b). After the modification of H1 onto the electrode surface, an increased R_{et} (113 Ω) was obtained because of the low conductive property of oligonucleotide (curve c). After blocking with MCH (curve d),

R_{et} increased to 149 Ω. In order to characterize the RCA reaction and multiple amplification strategy *via* RCA and HCR on the electrode surface, 1 nM FTN was employed to unfold some of the modified H1 firstly. And then, the R_{et} was increased to 190 Ω after RCA reaction and 334 Ω after the generation of dendrimer *via* RCA and HCR amplification, respectively (curve e and f). These EIS results quantified the electron transfer kinetics with accurate R_{et} to characterize the electron transfer tendency in the fabrication of the electrochemical biosensor. All of these results confirmed the successful construction of the electrochemical biosensor.

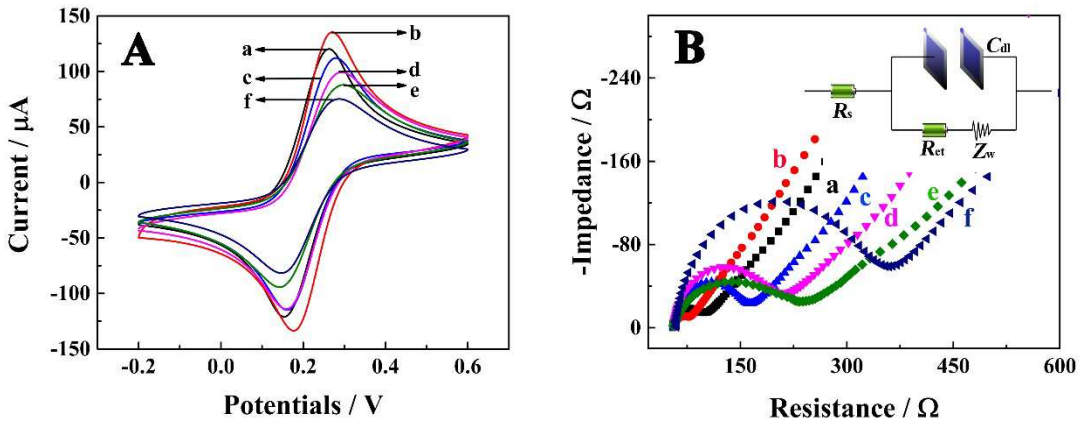


Figure 1 The characterizations for construction of the electrochemical biosensor based on CV in the potential range from -0.2 to 0.6 V with a scan rate of 50 mV/s (A) and EIS measurements in a frequency range from 5×10^{-2} to 1×10^{6} Hz (B): a, bare GCE; b, AuNPs modified GCE; c, H1/AuNPs/GCE; d, H1/AuNPs/GCE after blocking with MCH; e, the proposed electrochemical biosensor after RCA amplification; f, the proposed electrochemical biosensor after the generation of dendrimer *via* RCA and HCR amplification. Insert figure of B, the scheme for the equivalent circuit used to model impedance data on the electrode surface, in which C_{dl} was the double-layer capacitance, R_{et} was the electron transfer resistance, R_s was the electrolyte resistance and Z_w was the Warburg impedance, respectively.

3.2 Native polyacrylamide gel electrophoresis characterizations of the DNA

amplification strategy.

All of the oligonucleotides and products based on the proposed amplification were characterized by the native polyacrylamide gel electrophoresis, in which the UV band from the notch indicated the molecular weight of the oligonucleotides or the oligonucleotide structures. As can be seen in the Figure 2, the lane a and b represented the DW and BN, respectively. After the hybridization between the DW and BN to form DW-BN, a bright band with higher molecular weight was observed (Figure 2A lane c). In the presence of target gene (lane e), it could reacted with the DW-BN to form the new hybridized product of BN-target with releasing of DW, performing two obvious bands for these oligonucleotide. When the released DW reacted with the TN in the presence of Nb. BvCI, the TN could be spliced specially by Nb. BvCI to fragment of TN (FTN) with a lower molecular weight (lane g). Lane 1 showed the UV band of CT. When it reacted with the AP, a higher molecular weight was obtained with a close UV band from the notch (lane 2). After the specific enzymatic splicing *via* Exo I and Exo III for the products of CT-AP, an obvious band for the circular linked CT was showed in the lane 3. When it was mixed with the H1 without TN, there was no hybridization among them (lane 5). In the presence of TN, it could open the hairpin structure of H1 and then hybridize with the circular linked CT to generate RCA reactions with the help of phi 29 and dNTPs, forming long oligonucleotide sequence with massive repeated sequences (lane 6). Lane 7 and lane 8 showed the PAGE results for H2 and H3, respectively. After the hybridization among RCA products, H2 and H3 (lane 9), it could initiate the HCR amplification to form a long

double-stranded DNA sequence with higher molecular weight. These results indicated the successful transformation from the target gene to FTN *via* 3D DNA walker and then generated DNA dendrimer *via* RCA and HCR amplification.

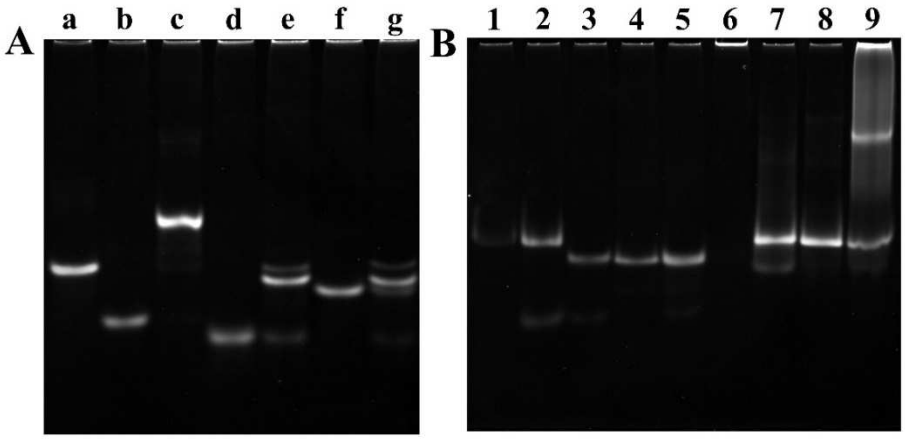


Figure 2 The polyacrylamide gel electrophoresis characterizations (10%) for oligonucleotides employed for the signal amplification: lane a, DW; lane b, BN; lane c, DW reacted with BN; lane d, target gene; lane e, the hybridized products of DW-BN reacted with the target gene; lane f, TN; lane g, the hybridized products of DW-BN reacted with the target gene, TN in presence of Nb. BvCI; lane 1, CT; lane 2, the hybridized products of CT reacted with AP; lane 3, the hybridized products of CT reacted with AP in presence of Exo I and Exo III; lane 4, H1; lane 5, H1 reacted with the products of lane 3; lane 6, H 1 reacted with the products of lane 3 in presence of TN, dNTPs and phi29; lane 7, H2; lane 8, H3; lane 9, the products of lane 6 reacted with H2 and H3.

3.3 The performances of the signal amplification strategies

The efficiency of different signal amplification strategy was investigated by the comparative experiments on the similar biosensor platform including the following three groups: (1) control without any amplification method, in which the transferred oligonucleotides from DNA walkers could open the H 1 and then hybridized with the signal probes labeled with the electronic mediator, Fc, to generate electrochemical responses; (2) HCR, in which the transferred oligonucleotides from DNA walkers

could open the H 1 and then hybridized hairpin DNA 4 (H4) and hairpin DNA 5 (H5), respectively, generating HCR amplification reaction to form massive double-stranded DNA for the immobilization of Fc-Dox as electronic mediator; (3) RCA, in which the transferred oligonucleotides from DNA walkers could open the H 1 and then hybridized with the prepared circular template 2 to generate RCA reaction forming long single-stranded DNA sequence, which further reacted with the associated oligonucleotide to form double-stranded DNA to immobilize of the Fc-Dox to generate electrochemical responses; (4) the proposed multiple amplification strategy *via* RCA and HCR, in which the transferred oligonucleotides from DNA walkers could generate the RCA amplification and then the produced auxiliary sequences could trigger HCR amplification, generating double-stranded DNA structures to immobilize the electrochemical mediator, Fc-Dox, to generate electrochemical responses. As shown in Figure 3A, under the optimum experimental conditions, the electrochemical response signal was only 2.48 μA for the electrochemical biosensor without amplification. For the HCR and RCA amplification only, electrochemical responses of 2.95 μA and 3.14 μA was obtained, respectively (Figure 3B and C). Based on the proposed multiple amplification strategy *via* RCA and HCR (Figure 3D), the electrochemical response signal was significantly enhanced to 5.25 μA, which indicated the good amplification efficiency of the proposed amplification strategy due to the improved immobilization of electronic mediator on the electrochemical biosensor surface.

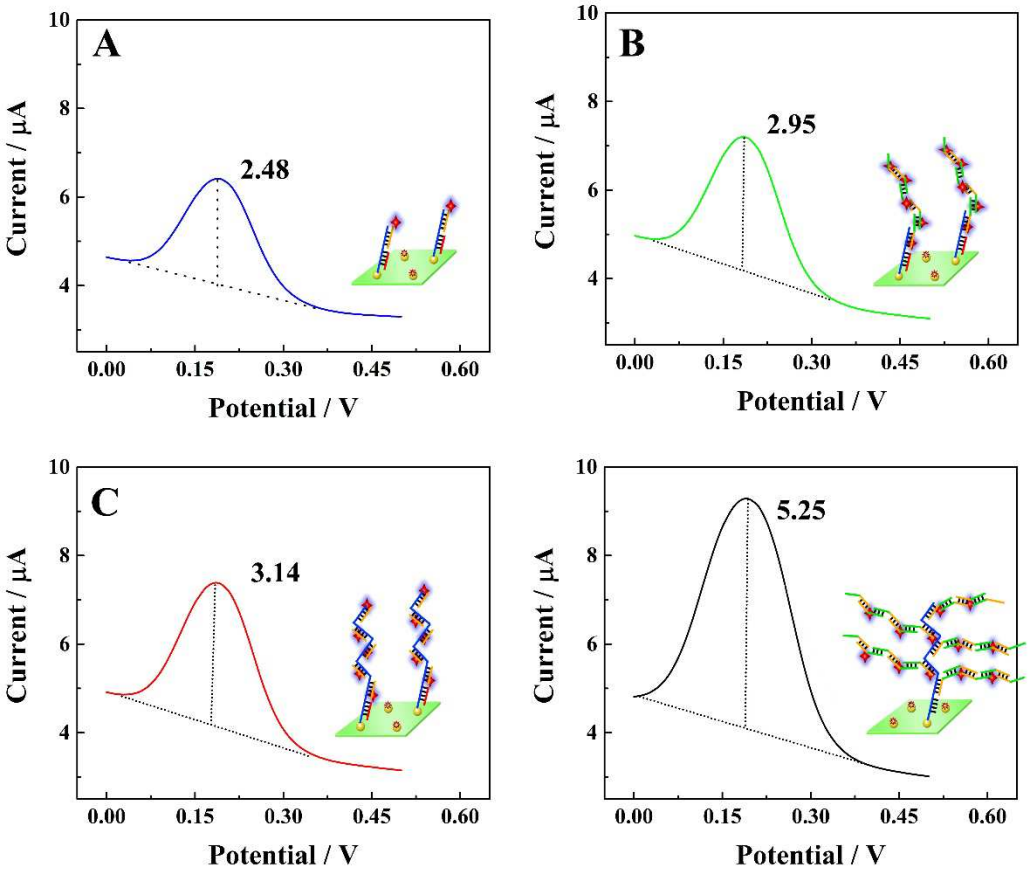


Figure 3 Comparative study of signal amplification strategies based on DPV electrochemical detection technology: (A) without amplification; (B) HCR; (C) RCA; (D) the multiple amplification strategy *via* RCA and HCR.

3.4 Optimal Conditions for the electrochemical bioassay

Herein, the important parameters of the electrochemical assay were optimized based on the electrochemical determination of *E. coli* O157:H7, including the concentration of hairpin DNA and the reaction time of nucleic acid amplification. As shown in Figure 4A, the current signal increased with the concentration of hairpin DNA and then reached a plateau when the concentration of hairpin DNA increased to 1.0 μM. Thus, 1.0 μM was selected as the optimized concentration for hairpin DNA. As can be seen in Figure 4B, the current signal increased with the reaction time of HCR, and then reached a plateau after 40 min. Thus, 40 min was chosen as the

optimal reaction time of HCR amplification. The concentration of CT for RCA reaction was optimized based on the electrochemical tests (Figure 4C), and the results indicated that the electrochemical signals increased with the concentration of CT in the range from 0.1 to 1.5 μM, and the tended to a platform. Thus, 1.5 μM was used as the optimal concentration of CT for RCA reaction. In Figure 4D with the optimization study of the RCA reaction time, the electrochemical current responses increased with the RCA reaction time and tended to a steady state after 40 min. Therefore, the RCA reaction time of 40 min was employed for RCA reaction.

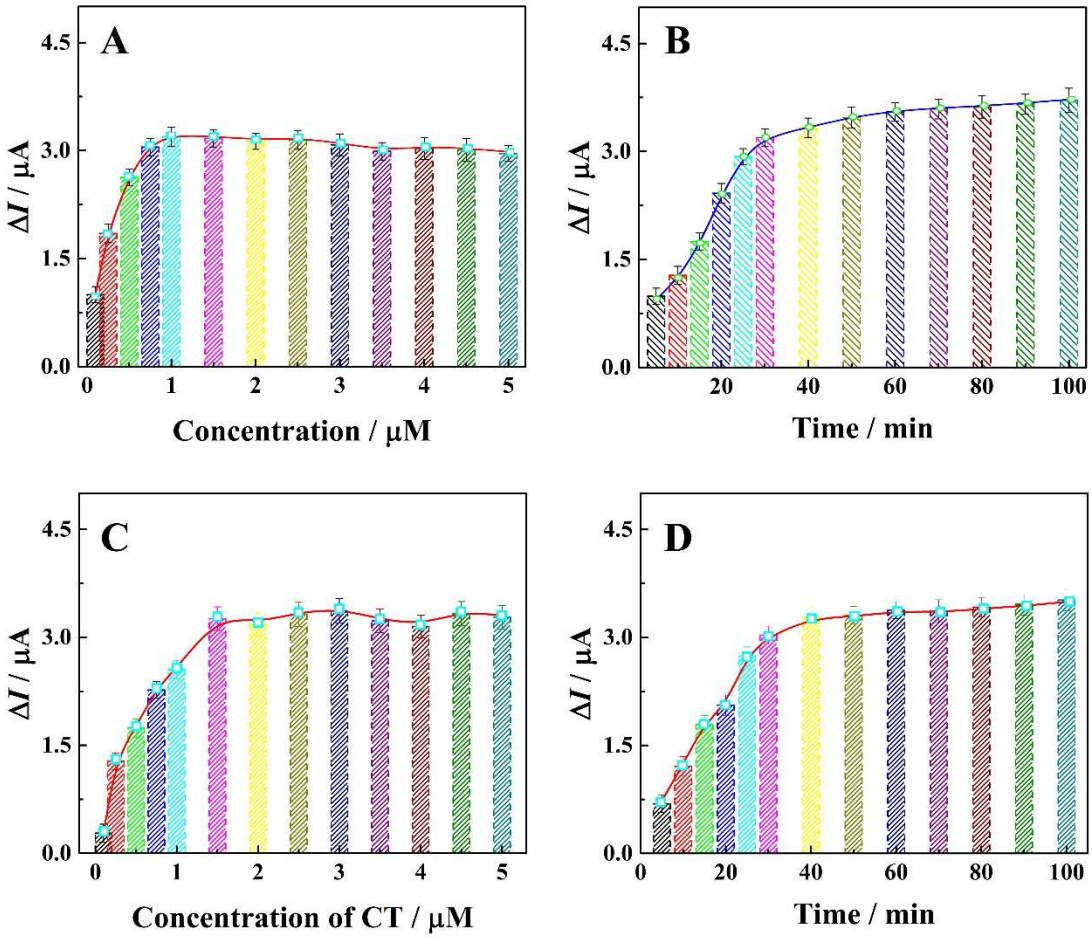


Figure 4 Optimization for the detection conditions based on the electrochemical determination of *E.coli* O157: H7 with the concentration of 5.0×10^3 CFU/mL, including (A) the concentration of hairpin DNA, (B) the reaction time of HCR amplification, (C) the concentration of CT and (D) the reaction time of RCA reactions.

3.5 Analytical Performances of the electrochemical biosensor

The analytical performances of the proposed electrochemical biosensor were examined based on the correspondence between the current responses *via* differential pulse voltammetry technique and the concentrations of *E. coli* O157: H7 (Figure 5A). In this experiment, the *E. coli* O157: H7 was cultured to an initial concentration of 10^9 CFU/mL and then diluted to various concentrations ranged from 0 to 10^4 CFU/mL. The DNA of *E. coli* O157: H7 was extracted using the TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit Ver 3.0 according to the operation instruction, which was further employed to investigate the analytical performances of the proposed electrochemical biosensor for the detection of *E. coli* O157: H7. As shown in Figure 5B, the electrochemical signals of the proposed electrochemical biosensor showed a good logarithmic relationship to the concentrations of *E. coli* O157: H7 in the range of 10 ~ 1.0×10^4 CFU/mL with the linear equation of $y = 0.9803 \lg c_{E.\ coli\ \mathrm{O157:\ H7}} - 1.34$ and the detection limit of 7 CFU/mL, which was obtained with 3 times standard deviation (Mean + 3SD, in which the Mean was the average and SD was the standard deviation of ten times detection, respectively). Compared with the detection strategies in some previous literatures (Table 2), the proposed electrochemical biosensor showed a wide linear range and good sensitivity. These results indicated the proposed electrochemical biosensors with good analytical performances could be further applied to the actual sample analysis.

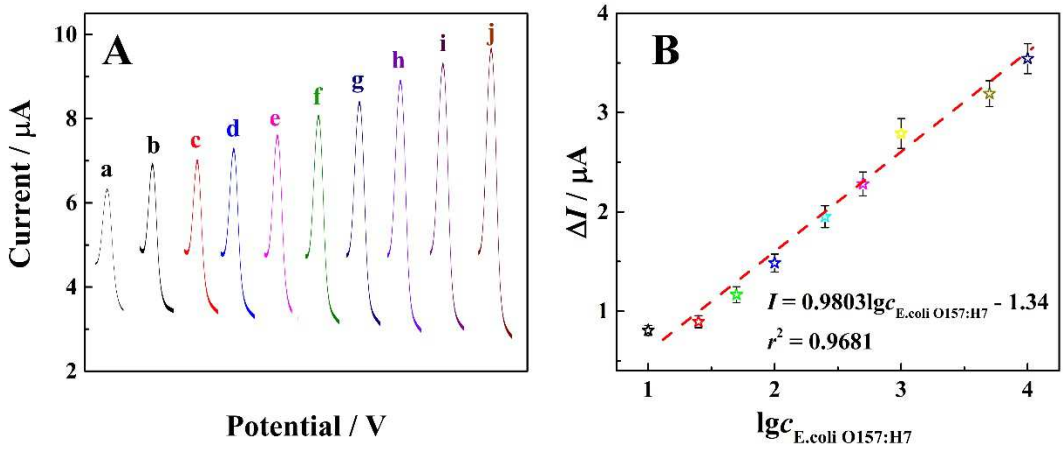


Figure 5 Relationship between electrochemical responses and concentrations of *E. coli* O157:H7: (A) Electrochemical signals of the proposed biosensor for different concentrations of *E. coli* O157:H7 (curve a-j, the concentration of *E. coli* O157:H7 was 0, 1.0×10^1, 2.5×10^1, 5.0×10^1, 1.0×10^2, 2.5×10^2, 5.0×10^2, 1.0×10^3, 5.0×10^3, 1.0×10^4 CFU/mL); (B) Calibration curve between electrochemical signals and concentrations of *E. coli* O157:H7.

Table 2 Comparative study between the analytical performances of the proposed electrochemical biosensor with that in some previous literatures.

Detection method	Linear range (CFU/mL)	Detection limit (CFU/mL)	References
Polymerase chain reaction	10^4 ~ 10^8	5.8×10^3	Zhong et al., 2018
Polymerase chain reaction	10^2 ~ 10^5	37	Kim et al., 2019
Polymerase chain reaction	10 ~ 10^6	10	Park et al., 2019
Microfluidic chip	10 ~ 10^4	1	Li et al., 2017
Microfluidic chip	10^2	n/a	Jiang et al., 2017
Piezoelectric sensor	6.0×10 ~ 6.0×10^4	n/a	Han et al., 2019
Two-phase system	10^2 ~ 10^7	10^2	Cheung et al., 2018
Colorimetric method	10^2 ~ 10^5	10^2	Trinh et al., 2019
Electrochemical sensor	1.0×10 ~ 1.0×10^4	7	This work

3.5 Selectivity, reproducibility and stability of electrochemical biosensor

Selectivity as a key factor of the biosensor was evaluated with different interference, including single-base mismatch (SBM); adjacent double-base mismatch (ADBM); interval double-base mismatch (IDBM); adjacent three-base mismatch (ATBM); interval three-base mismatch (ITBM), microRNA-141 and microRNA-27. As depicted in Figure S2A, an obvious current response was achieved for the E.coli O157:H7 specific target sequence (1 nM), and no significant electrochemical responses were observed for the SBM, ADBM, IDBM, ATBM, ITBM, microRNA-141 and microRNA-27, respectively, suggesting the good selectivity of the proposed electrochemical biosensor.

The reproducibility as another important factor to evaluate the performance of the biosensor was investigated by intra-assay and inter-assay coefficients of variation, which was evaluated by determination of 10^3 CFU/mL *E. coli* O157: H7 with the same protocol *via* ten proposed electrochemical biosensors prepared in the same batch or different batch for intra- or inter-assay, respectively. A coefficient of variation of 7.2% for intra-assay (Figure S2B), and 5.8% for the inter-assay (Figure S2C) were obtained, respectively, which indicated the acceptable reproducibility of the developed strategy for *E. coli* O157:H7 assay.

At the same time, the stability of the proposed electrochemical biosensor was showed in Figure S2D with long-term storage assay. After a storage period of 30 days at 4 ºC, the responses of the proposed electrochemical biosensor still remained 94.3% of its initial response, indicating the good stability of the electrochemical biosensor.

Additionally, the specificity of the proposed electrochemical bioassay was

investigated by detection of both target *E. coli* O157: H7 and non-target pathogens, including *Vibrio cholera* O1, *Samonella spp*, *S. aureus* and *Listeria innocua*, at concentrations of 10^2 and 10^3 CFU/mL, respectively. As shown in Figure S3, for the target *E. coli* O157: H7 with both concentrations, there were obvious electrochemical responses. Whereas, the electrochemical responses for all of the non-target samples were minimal, which demonstrated the acceptable specificities of the proposed electrochemical bioassay against *E. coli* O157: H7.

3.6 Preliminary application of electrochemical biosensor

To assess the feasibility of the electrochemical biosensor, the *E. coli* O157: H7 was detected by the proposed electrochemical biosensor and plate culture counting technique, and the detection results were compared with each other based on the linear regression analysis. As shown in Figure S3, the correlation coefficient and slope were close to 1, respectively, which indicated the good applicability of the proposed biosensor for *E. coli* O157:H7 assay.

4. Conclusion

In summary, a simple, ultrasensitive and efficient electrochemical bioassay was proposed based on the multiple amplification strategy *via* the 3D DNA walker, RCA and HCR amplification reactions simultaneously, in which the generated dendrimer-like double stranded nucleic acid could efficiently immobilization the electronic medium Fc-Dox to enhance the electrochemical responses and improve the analytical performances of electrochemical biosensor. The successful approach of the

multiple amplification strategy could be readily expanded to estimate various pathogenic bacteria, providing a new approach for early diagnosis of pathogenic microorganisms or other diseases.

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Notes

The authors declare no competing financial interest.

■ACKNOWLEDGMENT

Financial support for this work was provided by the National Natural Science Foundation of China (No. 81401722 and 81572078).

Abbreviation: adjacent double-base mismatch (ADBM); AP, Associated primer; AuNPs, gold nanoparticles; adjacent three-base mismatch (ATBM); BN, blocking DNA; C_{dl}, the double-layer capacitance; CT, Circular template; CV, Cyclic voltammetric; dNTPs, deoxynucleotides; DW, DNA walker; EDTA, ethylenediaminetetraacetic acid; ECA, Electrochemical assay; EIS, electrochemical impedance spectroscopy; Exo I, exonuclease I; Exo III, exonuclease III; $[Fe(CN)_6]^{3-/4-}$, ferricyanide/ferrocyanide mixed solution; FTN, fragment of TN; GCE, glassy carbon electrode; H1, hairpin DNA 1; H2, hairpin DNA 2; H3, hairpin DNA 3; $HAuCl_4 \cdot 4H_2O$,

gold chloride tetrahydrate; HCR, hybridization chain reaction; interval double-base mismatch (IDBM); interval three-base mismatch (ITBM); MCH, 6-mercapto ethanol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RCA, rolling circle amplification; R_{et}, electron transfer resistance; R_s, the electrolyte resistance; SBM, single-base mismatch; SD, standard deviation; TN, transfer oligonucleotide; Z_w, the Warburg impedance.

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(1) A novel electrochemical biosensor was proposed based on the multiple amplification strategy for ultrasensitive detection of pathogenic bacteria.

(2) The sensitive strategy *via* the 3D DNA walker, rolling circle amplification and hybridization chain reaction possessed excellent performances, superior to most current methods.

(3) The electrochemical strategy could be readily expanded for the estimation of a variety of other pathogenic bacteria.

(4) The proposed method provide a new avenue for early diagnosis of pathogenic microorganisms or other diseases.

A sensitive electrochemical strategy *via* multiple amplification reactions for the detection of *E. coli* O157: H7

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Neither the entire paper nor any part of its content has been submitted, accepted, published or appeared in any print or in electronic online systems.

All the authors listed have approved the manuscript that is enclosed.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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