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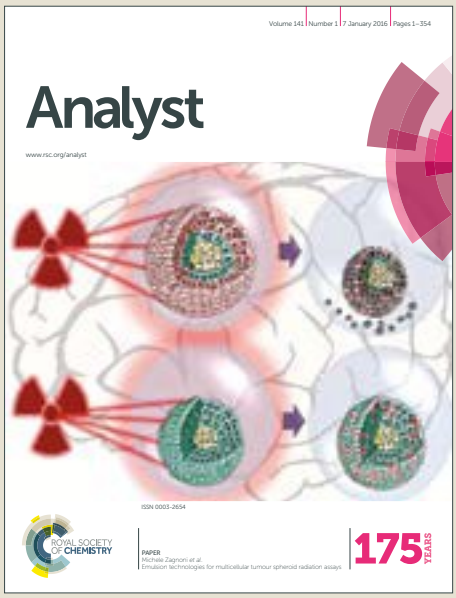

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Facile Signal-on Electrochemical DNA Sensing Platform for Ultrasensitive Detection of Pathogenic Bacteria Based on Exo III-assisted Autonomous Multiple-cycle Amplification

Qianqian Pei[a], Xiaolei Song[b],Su Liu[b], Jingfeng Wang[c], Xueqi Leng[b], Xuejun Cui[a], Jinghua Yu[a], Yu Wang[c],Jiadong Huang[a c]*

a Key Laboratory of Chemical Sensing & Analysis in Universities of Shandong, college of Chemistry and Chemical Engineering, University of Jinan, Jinan 250022, P.R. China.

b School of Water Conservancy and Environment, University of Jinan, Jinan 250022, P.R. China.

c College of Biological Sciences and Technology, University of Jinan, Jinan 250022, P.R. China.

* Corresponding author. E-mail: bio_wangy@ujn.edu.cn; Tel.: +86-531-89736122; Fax: +86-531-82769122.

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ABSTRACT

A facile signal-on electrochemical DNA biosensor has been developed for ultrasensitive detection of pathogenic bacteria using Exo III-assisted autonomous multiple-cycle amplification strategy. The strategy relies on pathogens and aptamer binding-initiated release of trigger, which combines to 3'-protruding terminus of the hairpin probe 1, leading to the formation of double-stranded DNA with a blunt 3' termini that start Exo III-assisted multiple signal amplification reaction. In addition, hairpin probe 2 labeled with electroactive reporter at the middle of the loop region is ingeniously designed to contain a short hairpin-embedded segment, which can fold into hairpin structure via Exo III-assisted cleavage reaction, thus bringing the redox molecule in proximity to the electrode surface for "signal-on" sensing. Under optimal conditions, this biosensor exhibits a very low detection limit as low as 8 cfu mL^{-1} and a wide linear range from 1.0×10^{1} to 1.0×10^{7} cfu mL^{-1} of target pathogenic bacteria. As far as we know, this is the first time that Exo III-assisted autonomous multiple-cycle amplification strategy has been used for signal-on electrochemical sensing of pathogenic bacteria. In addition, the proposed sensor can also be used for highly sensitive detection of other targets by changing the aptamer sequence, such as nucleic acids, proteins and small molecules. Therefore, the proposed signal-on electrochemical sensing strategy might provide a simple and practical new platform for detection of pathogenic bacteria and related biological analysis, food safety inspection and environmental monitoring.

Keywords: signal-on biosensing; electrochemical detection; aptamer; Exo III; pathogenic bacteria

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INTRODUCTION

According to the World Health Organization (WHO), there are 600 million (almost 1 in 10 people in the world) people fall ill after eating contaminated food and about 420 000 deaths each year [1]. Foodborne diseases are usually caused by bacteria entering the body through contaminated water or food [2]. Foodborne pathogens can cause severe gastroenteritis or debilitating infections including meningitis [3]. Thus, the development of simple and sensitive methods for detecting pathogenic bacteria in food and drinking water is essential in food safety inspection and environmental monitoring. Up to now, a myriad of analytical techniques of pathogen identification have been developed, including standard plate count (SPC) [4], enzyme-linked immunosorbent assay (ELISA) [5,6], quartz crystal microbalance (QCM) assay [7], protein microassay [8], flow cytometry assay [9]. Although each of these systems has its own appealing feature, they may suffer from some shortcomings such as the requirements of tedious culture and wash steps, expensive reagents and instruments, and trained technical personnels [10]. Therefore, it is necessary and urgent to construct rapid, low cost and convenient methods for highly sensitive detection of pathogen in foodstuffs and environmental samples.

Currently, electrochemical-based biosensors have attracted great interests due to all kinds of intrinsic advantages, including high portability and affordability, relatively low cost, low power requirement, and amenability to miniaturization and multiplexing [11,12]. Although a range of electrochemical sensing platforms have been exploited, DNA-electrochemical (hereafter named E-DNA) sensor is more competent due mainly to its simple operation, small size, low cost, and flexible designing [13-15]. Many reported E-DNA sensors belong to “signal-off” sensor, in which a stem-loop DNA or redox-labeled linear probe immobilized on electrode surface, after hybridization

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with its complementary DNA, changes into a rigid, duplex DNA that brings the redox label far away the electrode surface, thus the observed electrochemical signal is suppressed [16,17]. For example, our class reported a rapid voltammetric method for simultaneous determination of the pathogens *E. coli* and *S. typhimurium* based on target recycling amplification [18]. However, these "signal-off" sensors suffer from limited signaling capacity owing to the intrinsic displacing and releasing of signal probes, in which a maximum of 100% signal suppression could be acquired under any experimental conditions [19]. To circumvent the limitations, many "signal-on" E-DNA sensors have been proposed. For instance, our group developed several signal-on electrochemical biosensors based on Exo III-assisted autonomous cascade signal amplification in the previous work [20,21]. Xiao's group reported a signal-on electronic DNA sensor based on target-induced strand displacement mechanism [22]. These methods rely on the addition of exogenous assisted DNA probes or the uncontrolled flexibility of redox-labeled single-stranded DNA close to the electrode surface to achieve signal transduction.

To further enhance the functionality of the E-DNA sensor, we here propose a facile signal-on E-DNA sensing platform for pathogenic bacteria detection. To meet the requirement of trace detection of pathogenic bacteria in environmental samples and foodstuffs, a signal amplification strategy that is flexible and efficient is strongly necessary. Typically, a variety of nucleic acid amplification techniques have been paid more and more attention to improve detection sensitivity, such as polymerase chain reaction (PCR) [23], rolling circle amplification (RCA) [24], hybridization chain reaction (HCR) [25], and nicking enzyme-assisted signal amplification (NESA) [26]. Although these strategies afford high detection sensitivity, they suffer from multistep thermal cycling, complex conjugation, or intricate probe design for amplification primer and specific recognition sequences.

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By contrast, Exo III is a kind of sequence-independent enzyme that does not require a specific recognition site, which is more attractive in constructing versatile biosensing platform [27-29]. For example, Ma's group developed a ratiometric electrochemical DNA biosensor based on Exo III-assisted target recycling amplification and one-step triggered dual-signal output [30].

Herein, we construct a facile signal-on electrochemical DNA biosensor for ultrasensitive detection of pathogenic bacteria based on Exo III-assisted autonomous multiple-cycle amplification. Aptamers, artificial single-stranded DNA or RNA molecules that can be selected in vitro, can bind to target molecules with high affinity. As recognition elements, aptamers can be folded into diversely defined tertiary structures, and offer remarkable flexibility in designing novel biosensors[31]. The strategy relies on pathogens and aptamer binding-initiated release of trigger, which combines to 3'-protruding terminus of the hairpin probe 1 (HAP1), leading to the formation of DNA duplex with a blunt 3' termini that start Exo III-assisted multiple amplification reaction. As far as we know, this is the first time that Exo III-assisted autonomous multiple-cycle amplification strategy has been used for signal-on electrochemical sensing of pathogenic bacteria. This strategy features with several significant advantages. First, only one tool enzyme, Exo III in this strategy, is adopted to achieve the signal amplification reaction, thus the reaction could be carried out in one-pot fashion under isothermal condition, allowing the proposed assay very simple and low cost to implement. Moreover, improved sensitivity can be achieved owing to the combination of Exo III-assisted autonomous multiple-cycle amplification strategy with the intrinsically high sensitivity of the electrochemical detection. Besides, hairpin probe 2 (HAP2) labeled with electroactive reporter at the middle of the loop region is designed to contain a short hairpin-embedded segment, which can fold into hairpin structure via Exo III-assisted cleavage reaction, thus bringing the redox molecule in proximity to the

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electrode surface for "signal-on" sensing. The ingenious design provides a facile signal transduction approach for "signal-on" sensing, which immensely simplifies interface reaction process compared to our previous work [18,20,32]. Therefore, the proposed signal-on electrochemical sensing strategy might create a facile and low cost platform for highly sensitive detection of pathogenic bacteria and related biological analysis, food safety inspection and environmental monitoring.

MATERIALS AND METHODS

Reagents and materials.

We purchased exonuclease III (Exo III) from New England Biolabs Inc. (Beijing, China). All oligonucleotide sequences used in this experiment were synthesized and HPLC-Purified by Sangon Biotechnology Co. Ltd. (Shanghai, China). The sequences of oligonucleotides are shown in Table S1. *Salmonella typhimurium* (*S. typhimurium*, CMCC50115), *Bacillus subtilis* (KCTC 1028), *E. coli* (KCTC 2571), and *Listeria* (KCTC 3569) were purchased from the Institute of Microbiology Chinese Academy (Beijing, China). 6-Mercapto-l-hexanol (MCH) was purchased from Aladdin Chemistry Co., Ltd (Shanghai, China). Peptone, bacto-tryptone, beef extract powder, and bacto-yeast extract were purchased from Qingdao Biological Technology Co. Ltd (Qingdao, China). All other chemicals obtained from Sinopharm Chemical Reagent Co. Ltd (Beijing, China) were analytical grade. All solutions were dissolved using ultrapure water with an electric resistance >18.25 MΩ cm, which was acquired through a Millipore Milli-Q water purification system (Billerica, MA, USA).

Apparatus.

All electrochemical signal detection, including cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), and differential pulse voltammetry (DPV) were performed on a CHI 660D electrochemical workstation (Shanghai Chen Hua Instrument Co. Ltd, China) at room

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temperature. The conventional three-electrode system consists of three pars, in which a planar gold electrode (2 mm in diameter) as the working electrode, a Ag/AgCl electrode with a saturated KCl solution as the reference electrode, and a platinum wire as the counter electrode. Fluorescence spectra were recorded using an Agilent Cary Eclipse spectrofluorometer (Agilent, USA). The excitation/emission wavelengths are 494 nm/ 519 nm.

Electrochemical detection of target *S. typhimurium*.

Prior to the experiment, the aptamer and trigger with a molar ratio of 1:1 were added to the solution containing 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4). The mixed solution was heated to 90 °C for 5 minutes and then slowly cooled to room temperature to obtain the arched probe before use. The detailed steps for the Exo III-assisted multiple signal amplification was as follows. First, 2 μL of the arched probe (10 μM) was added to 16 μL reaction buffer containing 10 μM HAP1, 10 U Exo III, 50 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$. Second, 2 μL of the sample solution of *S. typhimurium* with a final concentration of 0 to 1.0×10^8 cfu mL^{-1} was added to the above reaction solution to perform the enzymatic reactions and incubated at 37 °C for 2 hours. Then 10 μL of the final reaction solution was added dropwise onto the MCH/HAP2/gold electrode surface and incubated at 37°C for 2 hours in a humid environment. Finally, the obtained electrode was thoroughly rinsed with PBS for three times and investigated by DPV in 10 mM PBS (containing 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl and 1 mM $CaCl_2$, pH 7.4) at room temperature. DPV was measured within the potential range from 0.1 to -0.5 V under modulation amplitude of 50 mV, a step potential of 0.83 mV, and a scan rate of 50 mV s^{-1}.

The culture of bacteria and preparation of the MCH/HAP2/gold electrode are given in Electronic Supplementary Information.

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RESULTS AND DISCUSSION

Design principle of the proposed signal-on electrochemical sensing strategy.

The working principle of the proposed signal-on electrochemical DNA sensing platform is shown in Figure 1, and we choose *S. typhimurium* as target organism. An arched probe is designed to contain *S. typhimurium* aptamer (denoted in brown) and trigger DNA segment (denoted in red), which is complementary to 3'-protruding terminus of the hairpin probe 1 (HAP1). HAP1 consists of three functional regions: Region I (denoted in purple) is the trigger-annealing region with the protruding fragment at 3' termini of HAP1, which can resist the digestion because Exo III only hydrolyzes the blunt or recessed 3' termini of double-stranded DNA. Region II (denoted in green) is complementary with part of region III, functioning as a stem to stabilize the hairpin configuration. Region III (at 5' termini) consists of the same sequence as trigger DNA (called the secondary trigger, ST), which can combine to region I and initiate new cycle of cleavage amplification with the aid of Exo III. A ingeniously hairpin probe 2 (HAP2) with the protruding 3' terminus is designed to contain ST-annealing region at 3' termini (denoted in blue) and a short hairpin-embedded region at 5' terminus (denoted in pink), which itself can fold into hairpin structure. HAP2 labeled with electroactive reporter methylene blue (MB) in the loop region is immobilized on the gold electrode surface via thiol-gold chemistry, which would exhibit only a negligible electrochemical signal due to the remote distance between MB and the electrode surface. In the presence of target *S. typhimurium*, the arched probe specifically combines to *S. typhimurium*, leading to the release of trigger DNA. Immediately, the hybridization of trigger with region I of the HAP1 induces the formation of a blunt 3' terminus, thus Exo III catalyzes the gradual hydrolysis of mononucleotides in the direction from 3' to 5', which results in the release of trigger and ST. Both the released trigger and ST again

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hybridizes with other HAP1 and activate new cycles of Exo III-assisted hydrolysis reaction, respectively. These constitute cycle I and cycle II, respectively. Consequently, after a two-step cycles of amplification reaction, one target can generate a large number of ST in the system. Immediately after the above reaction solution is dropped onto the electrode surface, ST hybridizes with HAP2 to form duplex DNA with a blunt 3' terminus, thus Exo III specifically hydrolyzes the duplex from 3' terminus, resulting in the production of ST for a continuous cleavage process. Meanwhile, the remaining part of HAP2 after digestion by Exo III (that is short hairpin-embedded region, denoted in pink) transforms quickly into hairpin structure, bringing MB close to the electrode surface, thus a very strong current signal can be obtained, which forms cycle III. On the basis of multiple cycle of cleavage reaction aided by Exo III, numerous hairpin structures can be exponentially produced, leading to significant amplification of the current output signal. Therefore, the proposed signal-on electrochemical strategy has a excellent potential for providing a convenient and ultrasensitive biosensing platform for *S. typhimurium* assay.

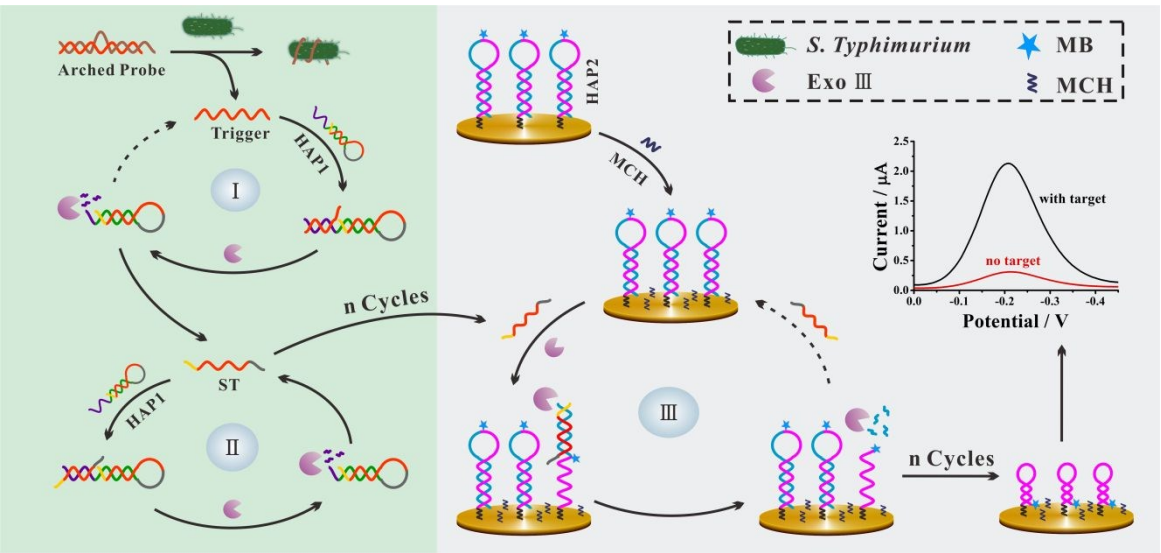

Figure 1. Schematic of the signal-on electrochemical sensing of *S. typhimurium* based on Exo III-assisted multiple signal amplification.

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Electrochemical characterization of the modified electrodes.

Electrochemical impedance spectroscopy (EIS), one of the most effective methods for interfacial analysis, is often used to monitor the fabrication process of the working electrode. In the EIS plots, the semicircle portion observed at higher frequencies represents the charge-transfer limited process, and the increase of the semicircle diameter is a reflex of the increasing of the interfacial charge-transfer resistance (Ret). The EIS results of the modified electrodes at different stages were shown in Figure 2A. It was discovered that the impedance plot of the bare gold electrode was almost a straight line, indicating the process of electron transfer was very rapid (curve a). The self-assembly layer of HAP2 on the electrode surface was negatively charged and effectively repels $[Fe(CN)_6]^{3-/4-}$ anions, thereby resulting in the enhancement of the resistance of the electron transport (curve b). Followed by the surface blocking with MCH, the Ret value of the electrode was obviously enhanced (curve c). After treatment with the reaction solution containing 1.0×10^7 cfu mL^{-1} *S. typhimurium* and Exo III, the semicircle diameter decreased significantly (curve d), which was due to the large amount of HAP2 were digested and the electroactive reporters were in proximity to the electrode surfaces. In order to further confirm the gradual electrode modification process, the modified electrode was also measured by CV. As shown in Figure 2B, compared to the bare gold electrode (curve a), the current signal of the HAP2/gold electrode decreased and the redox peak separation increased obviously (curve b). After the assembly of MCH on the surface of the HAP2/gold electrode, the output peak current value was reduced accordingly (curve c). Followed by the incubation with the reaction solution containing 1.0×10^7 cfu mL^{-1} *S. typhimurium* and Exo III, it was found that the peak current value increased and the redox peak distance decreased (curve d), certifying the Exo III-assisted multiple cyclic amplification process would be able to implement. These results were consistent with

the results obtained by EIS measurement. Based on the results gained by the above electrochemical measurements, the construction of the electrochemical biosensor had been successfully achieved.

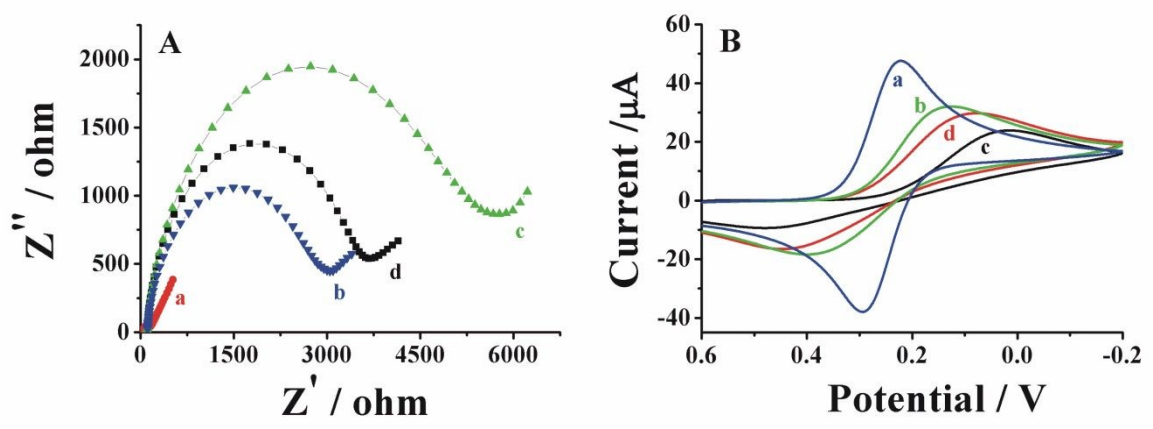

Figure 2. (A) Electrochemical impedance spectra (Nyquist plots) of the different modified electrodes obtained for the bare gold electrode (a), HAP2/gold electrode (b), MCH/HAP2/gold electrode (c), MCH/HAP2/gold electrode after incubation with the reaction solution containing 1.0×10^7 cfu mL^{-1} *S. typhimurium* and Exo III (d). (B) Cyclic voltammograms of the different modified electrodes obtained for the bare gold electrode (a), HAP2/gold electrode (b), MCH/HAP2/gold electrode (c), MCH/HAP2/gold electrode after incubation with the reaction solution containing 1.0×10^7 cfu mL^{-1} *S. typhimurium* and Exo III (d).

Feasibility analysis of the electrochemical biosensor.

In order to verify the feasibility of the proposed strategy for *S. typhimurium* detection, DPV measurements for a series of control experiments were performed, and the results were shown in Figure 3. When the MCH/HAP2/Au electrode was incubated with the positive sample, a dramatically strong DPV signal at around -0.22 V was obtained (curve a), indicating that the specific interaction of *S. typhimurium* and aptamer activated Exo III-assisted multiple amplification reaction, thus numerous electroactive reporters were in close proximity to the electrode surface for efficient electron transfer. In contrast, the blank sample displayed a negligible current signal, implying there

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was no change for HAP2 on electrode surface (curve b). These results clearly suggested that the obviously enhanced current signal was attributed to the specific target and aptamer interaction-triggered enzymatic amplification reaction. For the proof-of-concept experiment, the other experiments were carried out under the control of enzymes and targets. When Exo III is absent in the system, a negligible DPV peak similar to the DPV curve of the blank sample was observed (curve c). This indicated that the change of current signal was originated from Exo III-assisted multiple amplification process. Likewise, when non-target bacteria *E. coli* substituted for *S. typhimurium*, a very weak DPV response was observed (curve d). This proved that the specific recognition of target *S. typhimurium* and aptamer caused the significant enhancement of DPV signal, rather than other uncontrolled factors. Furthermore, we perform further control experiment by challenging the system with the dead *S. typhimurium*, and the results were shown in Figure S1. The dead *S. typhimurium* was prepared by heating at 95℃ for 30 min. It was observed that the DPV response incubating with heated-killed *S. typhimurium* was similar with that of the blank sample. These results indicated the dead bacteria can't create the false positive signal. On the basis of the above mentioned results, it was clearly demonstrated that the proposed strategy could be feasibly used to detect trace levels of *S. typhimurium*. Additionally, the characterization of Exo III-assisted enzymatic amplification in solution was performed by fluorescence measurements, and the results were shown in Figure S2. A fluorescence-quenched hairpin probe (FQHP) with the same sequence with that of HAP1was introduced. After the incubation of *S. typhimurium* with arched probe, FQHP and Exo III, a very strong fluorescent signal was observed, implying the target-aptamer binding activated Exo III-assisted cyclic cleavage of FQHP (curve a). In contrast, we observed a negligible fluorescent response when incubated arched probe, FQHP with Exo III in the absence of *S.*

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typhimurium (curve b). Moreover, when incubated *S. typhimurium* with arched probe and FQHP in the absence of Exo III, a very weak fluorescent signal similar with that of the blank sample was obtained (curve c). This gave immediate evidence that the significantly enhanced fluorescent signal was attributed to Exo III-aided cyclic cleavage reaction.

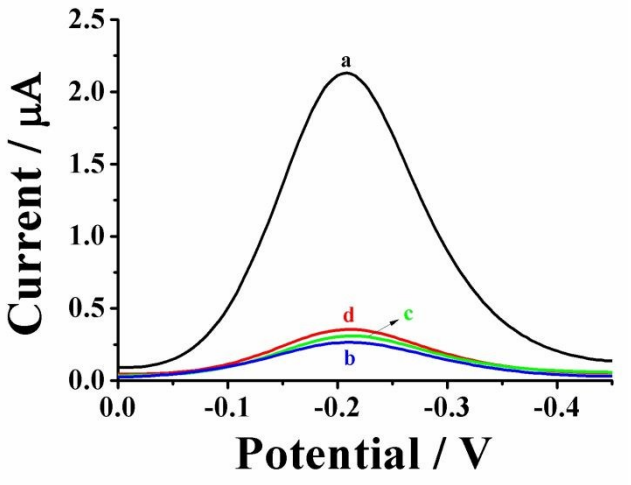

Figure 3. Typical DPV responses of the biosensor obtained upon analyzing 1.0×10^{7} cfu mL^{-1} *S. typhimurium* (curve a). Other three curves are for the control experiments which are performed with no target *S. typhimurium* (curve b), without Exo III (curve c), *E. coli* substitute for *S. typhimurium* (curve d).

Analytical performance of the biosensor.

Under the optimized experimental conditions (optimization experiments were given in Figure S3 in Electronic Supplementary Information), the sensitivity and the dynamic range of the constructed biosensor was investigated by determining a series of different concentrations of *S. typhimurium*. As shown in Figure 4A, the current intensity increased with the increasing concentration of *S. typhimurium* in the range of 0 to 1.0×10^{8} cfu mL^{-1}. The plot of current intensity at -0.22 V had a good linear relationship with the logarithm value of *S. typhimurium* concentration in the range from 1.0×10^{1} to 1.0×10^{7} cfu mL^{-1} (Figure 4B), and the linear regression equation was *I*

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(10^{-6} A) = 0.099+0.289×lg($C_{S.\ typhimurium}$/cfu mL^{-1}) with a correlation coefficient of 0.9985. According to the rule of three times standard deviation of the blank response, the limit of detection (LOD) was calculated to be 8 cfu mL^{-1}. Besides, the analytical performance of the constructed biosensor in the detection of *S. typhimurium* was compared with the previously reported methods. The results were shown in Table S2, these data manifested that our strategy showed improved detection limit and widened the detection range. Therefore, the proposed biosensor held great potential for quantitative assay of *S. typhimurium* with excellent sensitivity.

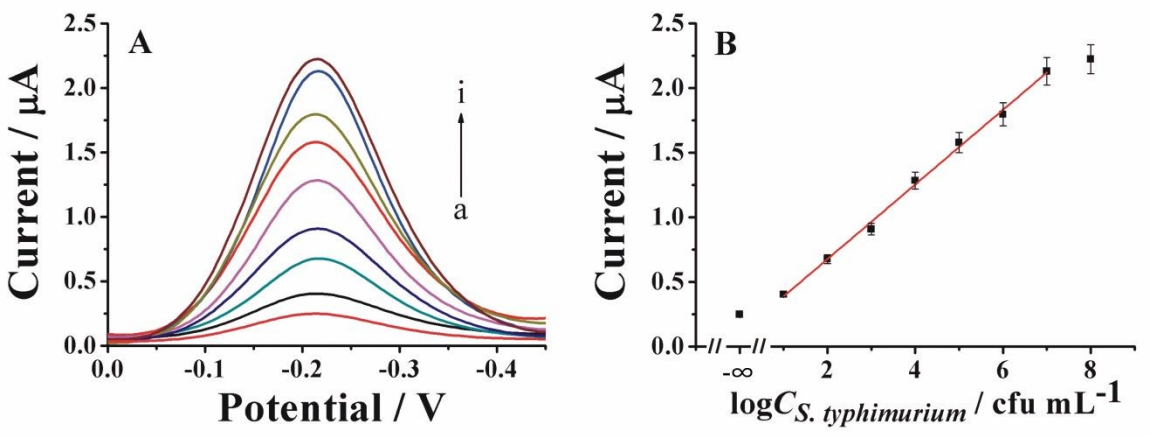

Figure 4. (A) Typical DPV responses of the biosensor to different concentrations of *S. typhimurium* (from curve a to i: 0, 1.0×10^1, 1.0×10^2, 1.0×10^3, 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7, and 1.0×10^8 cfu mL^{-1}). (B) The calibration curve of the current intensity at -0.22 V versus the logarithm of *S. typhimurium* with various concentrations. Error bars are standard deviations across three repetitive experiments.

The selectivity of the proposed biosensor for *S. typhimurium* assay was also studied by incubation the modified electrode with three non-target bacteria including *E. coil*, *Bacillus subtilis* and *Listeria*. As shown in Figure 5, the non-target bacteria with an excess of 10 times had similar current values as the blank sample, proving that our biosensor had an excellent selectivity for *S. typhimurium* detection.

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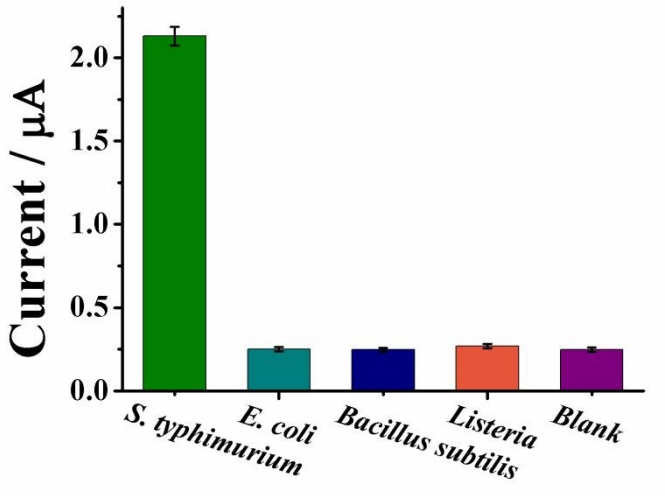

Figure 5. Plot of the current intensity at -0.22 V obtained with different bacteria. The concentration of *S. typhimurium* is 1.0×10^{7} cfu mL^{-1}. The concentration of the non-target bacteria is 1.0×10^{8} cfu mL^{-1}. Error bars are standard deviations across three repetitive experiments.

Real sample analysis.

To further confirm that the proposed biosensor had a good effect on real sample analysis, we considered quantitative analysis of spiked milk samples. 2 μL of the milk was added to 8 μL of PBS (10 mM, pH 7.4) and completely mixed for 2 min. Then 10 μL of different concentrations of *S. typhimurium* were spiked into the diluted milk samples and detected directly without any pretreatments. The milk samples were spiked with different concentrations of *S. typhimurium* and tested directly without any additional treatment besides dilution with buffer solution. The spiked samples were further detected using the classic plate count method and compared with the results achieved by our biosensor. The test results were shown in Table S3. It was perceived that these data obtained by our biosensor was very similar to the results obtained by the plate count method, and the differences between the two methods were less than 7.2%. This clearly demonstrated that the proposed biosensor could be applied to the analysis of complex samples with high reliability and accuracy.

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CONCLUSION

In summary, we constructed a facile "signal-on" electrochemical DNA biosensor for ultrasensitive detection of pathogenic bacteria based on Exo III-assisted multiple-cycle amplification. The amplification reaction was activated specifically by target pathogens-aptamer binding and attempted to convert pathogenic bacteria detection into DNA sensing. Due to the inherent high sensitivity of electrochemical detection and significantly enhanced signal amplification, the sensor showed a wide detection range of 1.0×10^{1}-1.0×10^{7} cfu mL^{-1} and a very low detection limit of 8 cfu mL^{-1} under the optimal conditions for the detection of *S. typhimurium*, which was better than the results of the previously reported methods [10,33,34]. In addition, the proposed biosensor could be combined with other detection methods, such as fluorescence analysis, to further expand the scope of application of nuclease-based isothermal signal amplification strategies. Moreover, the sensor could also be used for highly sensitive detection of other targets by changing the aptamer sequence, such as nucleic acids, proteins and small molecules. Therefore, the biosensor constructed in this study provided a simple and practical platform for detecting trace amounts of molecules in environmental analysis, food safety monitoring, and bioanalysis.

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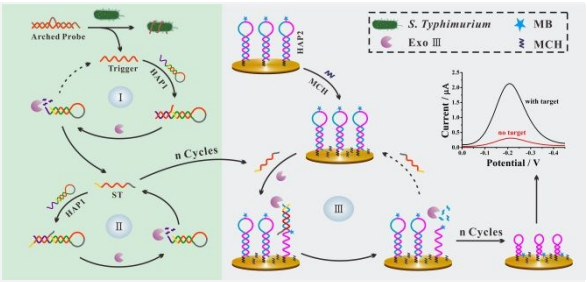
Title Text

Facile Signal-on Electrochemical DNA Sensing Platform for Ultrasensitive Detection of Pathogenic Bacteria based on Exo III-assisted Autonomous Multiple-cycle Amplification

Qianqian Pei, Xiaolei Song, Su Liu, Jingfeng Wang, Xueqi Leng, Xuejun Cui, Jinghua Yu, Yu Wang, Jiadong Huang*



A facile signal-on electrochemical DNA biosensor for ultrasensitive detection of pathogenic bacteria based on Exo III-assisted autonomous multiple-cycle amplification.