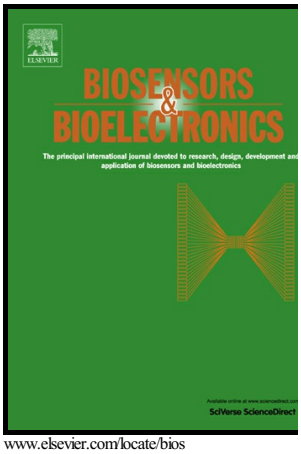

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Label-Free and Highly Sensitive Electrochemical Detection of *E. coli* based on Rolling Circle Amplifications Coupled Peroxidase-Mimicking DNAzyme Amplification

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Abstract

In this work, a simple, label-free, low cost electrochemical biosensor for highly sensitive and selective detection of *E. coli* has been developed on the basis of rolling circle amplification (RCA) coupled peroxidase-mimicking DNAzyme amplification. A aptamer-primer probe (APP) containing anti-*E. coli* aptamer and a primer sequence complementary to a circular probe, which includes two G-quadruplex units, is used for recognizing target and triggering RCA-based polymerase elongation. Due to RCA coupled DNAzyme amplification strategy, the presence of target *E. coli* leads to the formation of numerous G-quadruplex oligomers on electrode, which folds into G-quadruplex/hemin complexs with the help of K^+ and hemin, thus generating extremely strong catalytic activity toward

H_2O_2 and giving a remarkably strong electrochemical response. As far as we know, this work is the first time that RCA coupled peroxidase-mimicking DNAzyme amplification technique have been integrated into electrochemical assay for detecting pathogenic bacteria. Under optimal conditions, the proposed biosensor exhibits ultrahigh sensitivity toward *E. coli* with detection limits of 8 cfu mL^{-1} and a detection range of 5 orders of magnitude. Besides, our biosensor also shows high selectivity toward target *E. coli* and has the advantages in its rapidness, low cost, simplified operations without the need of electrochemical labeling steps and additional labile reagents. Hence, the RCA coupled peroxidase-mimicking DNAzyme amplification-based electrochemical method might create a useful and practical platform for detecting *E. coli* and related food safety analysis and clinical diagnosis.

Keywords: label-free electrochemical assay; RCA; peroxidase-mimicking DNAzyme amplification; *E. coli* detection

1. Introduction

Safety concerns about food, drinking water, health surveillance, and clinical diagnosis related to microbial pathogens have attracted increasing attention worldwide (Hamula et al., 2011, Li et al.; 2010). Among all pathogens, *Escherichia coli* (*E. coli*) is one of the most dangerous pathogens (Ichi et al., 2014), which can cause hemorrhagic colitis with symptoms such as bloody diarrhea, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (Shen et al., 2011). Therefore, the development of simple and sensitive assay methods for the detection of *E. coli* is essential in clinical diagnostics, food safety inspection, and environmental monitoring.

The conventional culturing-based method for *E. coli* identification is simple and cost-efficient,

however, it usually takes 2 to 3 days to obtain a result, which is rather time-consuming and does not permit on-site detection (Fournier-Wirth et al., 2006). Therefore, many new techniques, such as enzyme-linked immunosorbent assay (ELISA) (Nazemi et al., 2015), polymerase chain reaction (PCR) (Dharmasiri et al., 2010; Patel et al., 2012), protein microassay (Maddalo et al., 2011), quartz crystal microbalance (QCM) system (Jiang et al., 2011), surface plasmon resonance (SPR) (Yazgan et al., 2014), flow cytometry (FC) (Pinero et al., 2015), and chemiluminescence (CL) (Zhang, et al., 2014), have been combined to develop rapid and sensitive *E. coli* assay methods to monitor and control its contamination in food and water. Although these techniques own some appealing features such as multiplexing ability, excellent sensitivity, or rapid detection, they may require laborious pretreatment of samples including cell lysis, expensive instruments and reagents, and trained technical personnel. Thus, there is great demand to develop convenient, highly sensitive, and low cost assay methods for the determination of *E. coli*.

Electrochemical methods, which have potential advantages over other analytical techniques such as high portability and affordability, low power requirement, rapid response, and low cost, are particularly attractive for *E. coli* identification (Dou et al., 2013). Several electrochemical-based strategies for *E. coli* detection have been reported by using methylene blue-labeled DNA probe (Li et al., 2015), or glucoseoxidase (GOD)-labeled antibody (Li et al., 2013) as signal reporters. However, such approaches may suffer from some drawbacks such as the requirements of labeling electro-active substances, or adding additional labile reagents. Therefore, it is highly desirable to develop simple and label-free electrochemical biosensing strategies for quantifying *E. coli* with high sensitivity and selectivity. In order to satisfy the requirements for profiling low abundance of *E. coli* in water or foodstuffs, the development of electrochemical biosensing strategies with a signal enhancement

capability is extremely attractive (Walter et al., 2011; Zhang et al., 2015). RCA is one of the most widely used isothermal amplification techniques, which is carried out with a short DNA primer and a circular probe with the help of a special DNA polymerase with strong strand-displacement ability. It is noteworthy that RCA can achieve extremely long single-stranded DNA with repeating sequences that linked to DNA primer, signifying it is a site-anchored amplification method and fit for solid phase formats (Liu et al., 1996; Dahl et al., 2004; Li et al., 2013).

Herein, we construct a novel label-free electrochemical biosensor for highly sensitive and specific detection of *E. coli* based on RCA coupled peroxidase-mimicking DNAzyme amplification. DNAzymes, which are selected by SELEX (systematic evolution of ligands by exponential enrichment), have the ability to catalyze a chemical reaction (Ellington and Szostak., 1990; Bi et al., 2010). G-quadruplex/hemin complex is a typical DNAzyme that possess peroxidase activity similar to that of horseradish peroxidase (HRP) to catalyze the reduction of H_2O_2. As far as we know, this work is the first time that RCA coupled peroxidase-mimicking DNAzyme amplification technique have been integrated into electrochemical assay for detecting pathogenic bacteria. Our method features with several significant aspects. First, improved sensitivity and widen dynamic range for *E. coli* quantification is achieved by the utilization of the above amplification strategy. Second, the HRP-mimicking DNAzyme is used as the catalytic tag, which makes our approach technically label-free, and avoids electrochemical labeling steps and the addition of additional labile reagents. Third, our electrochemical-based detection method has the advantages of high detection speed, minimal sample consumption, easy of miniaturization, and low cost detection. Hence, our biosenosr might provide a useful and practical platform for highly sensitive and selective pathogenic bacteria determination and related food safety analysis and clinical diagnosis.

2. Experimental

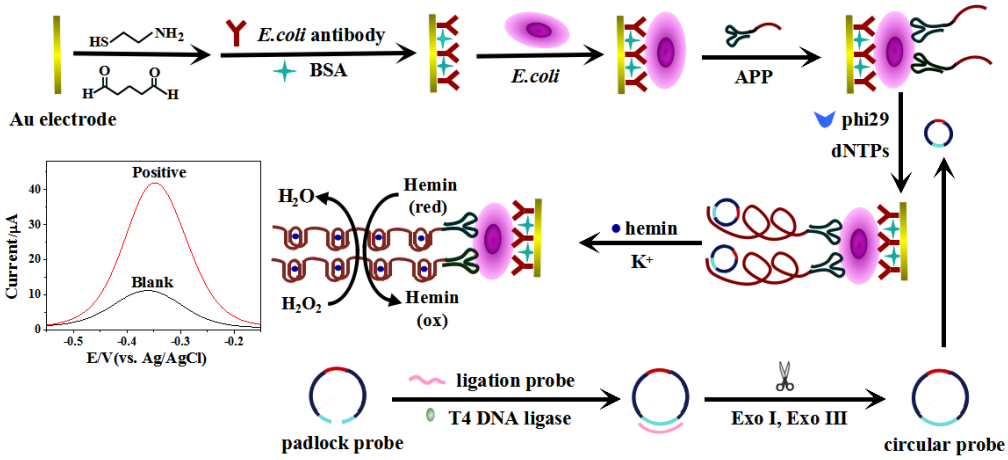
See Supplementary Material

3. Results and discussion

3.1. Design principle of the proposed biosensing strategy

Scheme 1 illustrates the analytical principle of the proposed biosensing strategy for highly sensitive detection of *E. coli*. A aptamer-primer probe (APP) is designed to contain anti-*E. coli* aptamer and a universal primer sequence complementary to circular DNA probe. The circular probe was first prepared by using a ligation probe and a padlock probe via T4 DNA ligase-catalyzed ligase reaction. Initially, anti-*E. coli* polyclonal antibody was immobilized on electrode surface via self-assembling of cysteamine and the bifunctional linker of glutaraldehyde (Zhou et al., 2007). Then BSA is assembled on electrode surface to block extra active sites and reduce non-specific adsorption. In the presence of target *E. coli*, *E. coli* is captured on electrode surface via specific recognition. Followed by the addition of APP, APP is bound to the surface-captured *E. coli*, resulting in the primer sequence regions of APP available for subsequent RCA reaction. Then, an *in situ* RCA reaction is initiated after the addition of circular probe, phi29 DNA polymerase and dNTPs, producing a long tandem repeated DNA sequence. As the specially designed circular probe contains two G-quadruplex units, the long DNA molecule with numerous G-quadruplex units is therefore generated. Subsequently, these G-quadruplex units combine with the hemin molecules and fold into the active HRP-mimicking DNAzyme sturcture with the help of K^+. As a result, a dramatically strong current signal is achieved owing to the electro-reduction of H_2O_2 reaction catalyzed by HRP-mimicking DNAzyme. The use of RCA coupled DNAzyme amplification techniques contributes significantly to the amplified current signal output, which improves substantially the

sensitivity of the biosensor compared with traditional electrochemical methods. Furthermore, this approach does not involve any labeling steps and additional labile reagents, which is simple and cost-effective.



Scheme 1. Illustration of the label-free electrochemical assay for highly sensitive detection of *E. coli* based on RCA and DNAzyme amplification.

3.2. Electrochemical characterization of the biosensor

To test the feasibility of the proposed approach, a series of control experiments were performed by DPV measurements. As shown in Fig. 1A, when the electrode was incubated in the presence of *E. coli*, a significantly strong peak current around -0.35 V appeared in the DPV curves, which was attributed to the electrochemical reduction of hemin incorporated in the G-quadruplex (Liu et al., 2013). The active center hemin (Red) in the HRP-mimicking DNAzyme could reduce H_2O_2 to produce H_2O, at the same time hemin (Red) was oxidized to hemin (Ox), which then was reduced to hemin (Red) (Wu et al., 2015). In contrast, it was observed that a very weak peak current was obtained after incubation without *E. coli* (curve b). This clearly revealed the remarkably enhanced DPV signal was indeed caused by target *E. coli*-triggered *in situ* RCA reaction and formation of G-quadruplex/hemin complex. Moreover, several proof-of-concept experiments were performed to

validate the specificity of the biosensor. When IgG polyclonal antibody was immobilized on electrode surface in place of *E. coli* antibody, a negligible peak current was observed in the DPV curves (curve c). Similarly, almost no obvious peak current was achieved after incubation with only primer sequence-contained probe in place of APP (curve d). Additionally, after incubation with non-target pathogenic bacteria *Salmonella* instead of *E. coli*, there was a very weak peak current similar to that of blank sample in the DPV curves (curve e). These results implied the RCA reaction and resulted formation of DNAzyme was target *E. coli* dependent, rather than induced by other non-specific interactions. Furthermore, the above control experiments were also performed by CV measurements and the results were shown in Fig. 1B. In the presence of target *E. coli*, an obviously reduction peak was obtained in the CV curves (curve a). This indicated a substantial number of G-quadruplex/hemin complex was formed on electrode surface, thus generating extremely strong catalytic ability toward the reduction of H_2O_2 to give an amplified current signal. In contrast, there was a very weak reduction peak for the blank sample (curve b). Additionally, the reduction peak for other proof-of-concept experiments (curve c, d, e) was similar to the blank sample, which was ascribed to the nonspecific adsorption of hemin on electrode surface (Yu et al., 2014). On the basis of the above results, it was reasonably concluded that the proposed approach can be potentially employed to detect low abundance of *E. coli*.

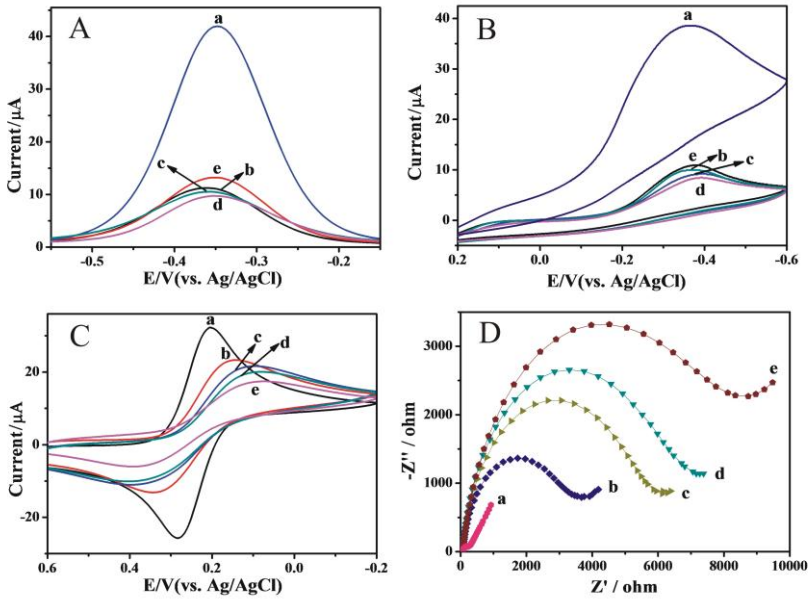


Fig. 1 Typical DPV (A) and CV (B) responses of the biosensor obtained upon analyzing 9.4×10^5 cfu mL^{-1} *E. coli* (a). Curves b to e are for the control experiments which are performed with no target *E. coli* (b), IgG polyclonal antibody in place of *E. coli* antibody (c), only primer sequence-contained probe in place of APP (d), and non-target pathogenic bacteria *Salmonella* in place of *E. coli* (e). CVs (C) and EIS (D) obtained for the bare Au electrode (a), antibody/Au electrode (b), *E. coli*/BSA/antibody/Au electrode (c), APP/*E. coli*/BSA/antibody/Au (d), RCA products-linked APP/*E. coli*/BSA/antibody/Au electrode (e). The sweeping direction of DPV was from -0.13 V to -0.55 V. DPV measurements were performed in 10 mM PBS (pH 7.4) containing 2.5 mM H_2O_2 and 100 mM K^+. CV and EIS measurements were performed in 10 mM PBS (pH 7.4) containing 0.25 mM KCl and 5.0 mM $K_3[Fe(CN)_6]$.

3.3. Electrochemical characterization of the modified electrodes

The modification of the sensing interface was monitored by CV and electrochemical impedance spectra (EIS), and the results were shown in Fig. 1C and D. With the bare Au electrode, a pair of well-defined redox peaks appeared at 0.17 mV and 0.26 mV, a typical redox peak range of

$K_3[Fe(CN)_6]$ (curve a). After the immobilization of *E. coli* antibody on electrode, an obviously weaker CV signal was observed, indicating the electron transfer between electrode and the solution was largely hindered by the isolated antibody macromolecules (curve b). Followed by the addition of target *E. coli*, the current signal was decreased compared with that of curve b, indicating *E. coli* was captured on electrode and further hindered the electron transfer (curve c). With the electrode modified by APP, there was a weaker peak current in the CV curves (curve d). This implied APP was combined with the surface-captured *E. coli* by the specific recognition. After adding the circular probe, phi29 and dNTPs, it was observed that a dramatically decreased CV signal appeared compared with that of curve d, which might be attributed that the negatively charged phosphate skeleton of the produced extremely long single-stranded DNA could block negatively charged $[Fe(CN)_6]^{3-/4-}$ close to the electrode surface (curve e) (Wang et al., 2014). Fig. 1D depicted Nyquist plots obtained in the fabrication process of the modified electrodes. It was found that the impedance plot for bare gold electrode displayed an almost straight line (curve a). However, the diameter of the semicircle (Ret) increased with the stepwise modification of antibody (curve b), *E. coli* (curve c), APP (curve d), and RCA products (curve e) respectively on electrode surface. This was due to the increase of the electron-transfer resistance by isolated biomolecules on electrode surface, which correspondently resulted in the decreased redox peak current in the CV measurements. All above results suggested the construction of the sensing interface for *E. coli* and the specific target recognition were successfully achieved.

3.4. Validation of Signal Amplification of RCA products

We first validated whether APP could serve as a primer to initiate the RCA reaction. A series of different samples were analyzed by gel electrophoresis, and the results were shown in Fig. S1A.

After incubation the padlock probe with the ligation probe, and T4 DNA ligase, two bright bands with the same mobility and intensity was observed on the image before (lane 2) and after (lane 3) the treatment with Exo I and Exo III, indicating the successful preparation of the padlock probe and its resistance to enzymatic digestion. In contrast, it was observed that no band appeared on the image after incubation the padlock probe, the ligation probe in the absence of T4 DNA ligase (lane 4). After incubation the padlock probe with APP, dNTPs, and phi29 DNA polymerase, a bright band for the large-molecule-weight product was observed in the image, which suggested APP could act as the primer to initiate RCA rection (lane 5).

It was known that G-quadruplex/hemin DNAzyme can catalyze the oxidation of the colorless $ABTS^{2-}$ to colored $ABTS^{\cdot-}$. To further assess whether the generated RCA products could efficiently combine with hemin and form DNAzyme thus exhibit strong catalytic ability, we introduced ABTS-H_2O_2 system and analyzed color change of ABTS by UV-Vis adsorption technique. An extra aptamer-G-quadruplex probe was synthesized, which was designed to contain *E. coli* aptamer and only one G-quadruplex unit sequence. As shown in Fig. S1B, after the incubation aptamer-G-quadruplex probe with hemin, ABTS, and H_2O_2, a very weak absorption peak appeared at 415 nm, typically for $ABTS^{\cdot-}$ with blue-green color (curve a). In contrast, dramatic increase in absorption intensity was observed after incubating RCA products with hemin, ABTS, and H_2O_2 (curve b). This demonstrated numerous repeated DNA sequences coupling with hemin to form G-quadruplex/hemin complex were generated.

The signal amplification of the RCA coupled DNAzyme amplification approach was further proved by DPV measurements. As shown in Fig. S1C, with the electrode incubated with aptamer-G-quadruplex probe in the presence of *E. coli*, it was observed that a small current peak was

appeared in the DPV curves (curve a). In contrast, after incubating with APP and undergoing the followed RCA reaction in the presence of *E. coli*, we clearly observed the peak current was significantly higher than that without RCA (curve b). These results gave immediate evidence that the proposed RCA-based approach could provide enormous signal amplification in our assay.

3.5. Analytical performance of the biosensor

To achieve an optimal analytical performance of the biosensor for *E. coli* detection, some key experimental parameters, *e.g.* the reaction time of RCA-based polymerase elongation (80 min), the concentration of hemin (100 nM) and H_2O_2 (2.5 mM), and the incubation time of target *E. coli* (60 min), were investigated in detail in Fig. S2. Under the optimal experimental conditions, the constructed biosensor was adopted for the determination of a series of different concentrations of *E. coli*. Fig. 2A depicted typical DPV responses of the biosensor to *E. coli* of varying concentration. It was observed that the DPV signal increased with the increasing concentration of *E. coli*. The plot of the peak current intensity versus the logarithm value of *E. coli* concentration displayed a good linear relationship in the range from 9.4 to 9.4×10^5 cfu mL^{-1} (Fig. 2B), and the limit of detection (LOD) was calculated to be 8 cfu mL^{-1} in terms of the rule of three times standard deviation over the blank response. Furthermore, the proposed biosensor showed very desirable reproducibility. The relative standard deviations (RSDs) of peak intensity at -0.35 V were 3.89%, 3.84%, 3.12%, and 3.28%, respectively, in four repetitive assay of 9.4, 9.4×10^1, 9.4×10^3, and 9.4×10^5 cfu mL^{-1} of *E. coli*. These data signified our biosensor held great potential for the determination of *E. coli* with high sensitivity and desirable reproducibility. Besides, the analytical performance of our biosensor in the quantitative assay of *E. coli* was compared with that of some previously reported methods. As shown in Table S2, it was found that the proposed RCA coupled DNAzyme amplification-based electrochemical method

displayed improved sensitivity and widen dynamic range compared with these methods (Zhang, et al., 2014;Nazemi et al., 2015; Jiang et al., 2011; Yazgan et al., 2014; Pinero et al., 2015; Li et al., 2013). As a result, our biosensor might satisfy the requirements for the profiling low abundance of *E. coli* in water or foodstuffs due to its very low detection limit.

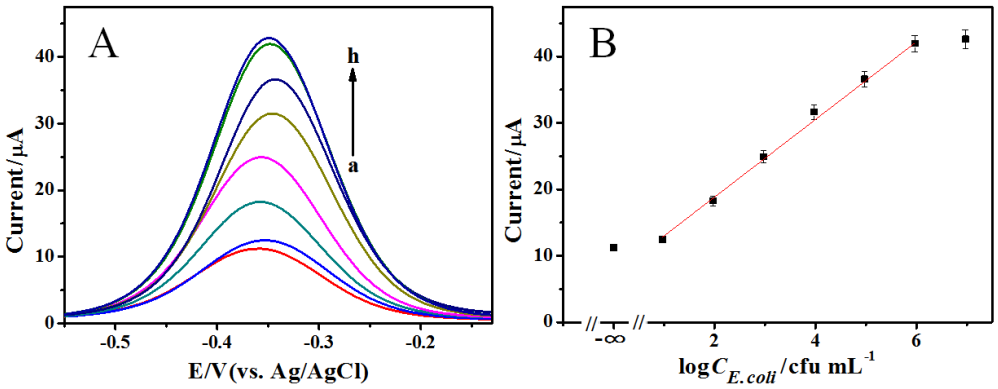


Fig. 2 (A) Typical DPV responses of the biosensor to different concentrations of *E. coli* (from curve a to h: 0, 9.4, 9.4×10, 9.4×10^2, 9.4×10^3, 9.4×10^4, 9.4×10^5, 9.4×10^6 cfu mL^{-1}). (B) The calibration curve of DPV peak current for different *E. coli* concentrations. Error bars are standard deviations across three repetitive experiments.

The selectivity of the proposed biosensor for *E. coli* assay was also examined by using three non-target bacteria, including *Salmonella, Bacillus subtilis and Listeria*, respectively. The results were shown in Fig. S3. The presence of even 10-fold excess of the non-target bacteria caused minimal current responses, which were closed to that of the blank sample. These results implied that our biosensing strategy could be used for the assay of *E. coli*.

3.6. Real sample analysis

To demonstrate the validity of our biosensor to real samples, the quantitative assay of spiked milk samples was considered. Different concentrations of *E. coli* were spiked into the milk samples and measured directly without any pretreatments except for dilution with a buffer solution. Table S3

depicted the data in the assay for the spiked samples. It was observed that the recovery of our method was in the range of 93.7-102.7%. In addition, the spiked samples were further quantified by a classic plate count method and compared with the results obtained by our method. We found the data obtained by our method were in good agreement with those obtained using plate count method, and the discrepancies between two methods were all smaller than 9.76%. These data clearly demonstrated our biosensor could be applied to complex samples.

4. Conclusion

In conclusion, we have developed a novel label-free electrochemical biosensor for highly sensitive and selective detection of *E. coli* based on RCA coupled peroxidase-mimicking DNAzyme amplification. A aptamer-primer probe is designed to contain anti-*E. coli* aptamer and a primer sequence complementary to a circular probe, which includes two G-quadruplex units. The strategy uses APP as the probe for recognizing target and triggering RCA-based polymerase elongation and the circular probe for *in situ* hybridization with APP. Due to RCA coupled DNAzyme amplification strategy, the presence of target *E. coli* leads to the formation of numerous G-quadruplex/hemin DNAzymes on electrode, thus generating extremely strong catalytic activity toward H_2O_2 and giving a remarkably strong electrochemical response. Under optimal conditions, the detection of *E. coli* with the signal-amplified electrochemical strategy can achieve an extraordinary LOD of 8 cfu mL^{-1}, which is better than those previous reported methods. The proposed biosensor also exhibits high selectivity toward *E. coli* against other interfering molecules. Moreover, it is noteworthy that the proposed electrochemical biosensing strategy has the advantages of rapidness, low cost, simplified operations without the need of electrochemical labeling steps and additional labile reagents. Thus, these distinct advantages of our proposed biosensing strategy make it a potential platform for

detecting *E. coli* and related food safety analysis and clinical diagnosis.

Acknowledgements

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Research Highlights

(1) A simple, label-free, low cost electrochemical biosensor for highly sensitive and selective detection of *E.coli* has been developed.
(2) This work is the first time that rolling circle amplification coupled peroxidase-mimicking DNAzyme amplification technique have been integrated into electrochemical assay for pathogenic bacteria.
(3) HRP-mimicking DNAzyme is used as catalytic tag, which makes our approach technically label-free.