


Rolling circle amplification based amperometric aptamer/immuno hybrid biosensor for ultrasensitive detection of *Vibrio parahaemolyticus*

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Abstract The authors describe an antibody-aptamer based hetero-sandwich amperometric biosensor for the foodborne pathogen *Vibrio parahaemolyticus*. Antibody on the surface of a gold electrode first captures the target pathogen, and then the immunocomplex is incubated with an aptamer consisting of an ssDNA probe. A hetero-sandwich structure is formed which allows for signal enhancement by rolling circle amplification. Following addition of Methylene Blue as an electrochemical DNA probe, the amperometric signal, best measured at 0.28 V (vs. Ag/AgCl), covers the 2.2 to 2.2*10⁸ cfu per mL concentration range, and the limit of detection is as low as 2 cfu per mL. The method was successfully applied to the quantification of *V. parahaemolyticus* in spiked fish samples. In our perception, this hetero-sandwich electrochemical

biosensor provides an ultrasensitive tool for the quantitation of a variety of pathogen if appropriate antibodies and aptamers are available.

Keywords Food safety · Bacteria detection · *Vibrio parahaemolyticus* · Aptamer · Antibody · Hetero-sandwich · RCA · Signal amplification · Gold nanoparticles

Introduction

The presence of pathogen microorganisms, such as *Escherichia coli*, *Salmonella*, *Listeria*, *Vibrio* and *Staphylococcus aureus* in foods can be serious threatens to public health [1, 2]. *V. parahaemolyticus* is a Gram-negative halophilic microorganism and a major food-borne pathogen that causes diarrheal illness in humans through consumption of various seafood throughout the world [3–5]. Undoubtedly, effective and rapid detection, identification and quantification of microbial pathogens including *V. parahaemolyticus* are crucial for food safety and human wellness.

Common and traditional diagnostic and surveillance methods including the culture-based methods, enzyme-linked immunosorbent assays (ELISA), and molecular biological methods have been widely used [6–8]; and such methods meet or partially meet the urgent needs for sensitive and specific detection of *V. parahaemolyticus*. Unfortunately, limitations of these traditional and molecular methods, including the labor-intensive and time-consuming properties still hamper their applications [9]. The inability to differentiate viable and dead microorganisms and the false-positive-prone results of molecular and biological methods require additional means to complete the detection process. Also, molecular methods base on the detection of specific genetic markers of pathogens meticulously require specialized instrumentation and highly

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trained personnel [10]. Finally, despite the immuno methods have been widely used, the availability of antibody and the sensitivity of these methods indicate a need for rapid and sensitive assay protocols for foodborne pathogen detection as well.

Aptamers are artificial nucleic acids or peptides screened by systematic evolution of ligands by exponential enrichment (SELEX) [11–13], which can bind to their molecular targets with high affinity and specificity, similar to antibodies. Aptamers exhibit distinct advantages over antibodies such as easy large-scale preparation, simple chemical modification, low cost, target versatility and thermal stability [14, 15]. Aptamers have been used for the detection of microbes including *L. acidophilus* [16], *S. aureus* [17], *V. parahaemolyticus* [18], *S. typhimurium* [19], and *S. dysenteriae* [20, 21]. To remedy the hurdle raised due to the scarcity of antibody and to improve the sensitivity and specificity of the detection scheme, aptamers to *V. parahaemolyticus* were adopted as one of the recognition probes for signal reporting.

In addition, to improve the sensitivity of the detection methods, RCA can be adopted for signal amplification [22, 23]. RCA is an isothermal amplification technology that generates long ssDNA with repetitive sequence units complementary to the circle template. This technology possesses the advantages as a rapid and isothermal amplification process without requiring special laboratory instruments, which makes it a useful and economic tool over other amplification methodologies. Zhou et al. have constructed an aptamer-based RCA platform for electrochemical detection of protein [24]. In addition, Bi et al. developed a chemiluminescence imaging array for detection of cancer cells by using dual-aptamer recognition and bio bar-code nano-probe-based RCA [25]. Also, our group has adopted a similar approach by using aptamer-based probe and RCA for signal enhancement [23]. Together with RCA, protocols based on gold nanoparticles (AuNPs) labeling have also been extensively used as ideal candidates for both the electron transfer and probe immobilization substrate due to their excellent affinity with biomolecules, large surface/volume ratio and good conductivity [26].

In this research, we present a hetero-sandwich recognition model based aptamer/immuno hybrid electrochemical biosensor for rapid and sensitive detection of *V. parahaemolyticus* with RCA as the signal enhancement strategy. The immobilized antibody on surface of the electrode acts as the capture probe of *V. parahaemolyticus* while the designed aptamers serve as the signal reporting probe. Aptamer induced RCA products produce extraordinary electrochemical sensing currents for ultrasensitive and accurate detection of *V. parahaemolyticus*. Additionally, the aptamer/immuno hybrid electrochemical biosensor was successfully employed to evaluate its applicability in the determination of *V. parahaemolyticus* in food samples.

Experimental section

Chemicals and materials

All the oligonucleotides were synthesized and purified by Sangon Inc., (<http://www.sangon.com/>, Shanghai, China). Phi29 DNA polymerase, T4 DNA ligase, dNTPs and cysteamine hydrochloride were also ordered from Sangon Inc. Gold (III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, (www.sigmaaldrich.com, St. Louis, MO, USA). Sodium citrate dehydrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$) was obtained from Alfa Aesar, USA. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were from Thermo Scientific (<https://www.thermofisher.com>, USA). Antibody against *V. parahaemolyticus* and *V. parahaemolyticus* ATCC 17802 were both purchased from Prajna Biology Tech. Ltd. (<http://www.sangon.com/>, Shanghai, China). *V. parahaemolyticus* ATCC 17802 samples were supplied by Jiangsu Entry-Exit Inspection and Quarantine Bureau (Nanjing, China). Typically, *V. parahaemolyticus* ATCC 17802 was cultured in alkaline peptone with 3% (w/v) NaCl overnight past the logarithmic phase. Then, 100 μL bacteria was diluted with medium and coated on the agar plates and culture at 37 for 24 h to determine the colony-forming units (cfu mL^{-1}). *V. parahaemolyticus* ATCC 17802 solutions at different concentrations was used as received without further cultivation. The detailed sequences of the ssDNA probes used in this research are as follows:

Vp-aptamer included primer: 5'-TCT AAA AAT GGG CAA AGA AAC AGT GAC TCG TTG AGA TAC TAA AAA AAA ACA GGG CTG GGC ATA GAA GTC AGG GCA GA-3' (under lined sequence is the aptamer sequence of Vp);

circle template: 5'-P-TAT GCC CAG CCC TGT AAG ATG AAG ATA GCG CAG AAT GGT CGG ATT CTC AAC TCG TAT CTG CCC TGA CTT C-3';

labeling ssDNA probe (ssDNA): 5'-SH-(CH_2)₆-GCG CAG AAT GGT-3';

complementary ssDNA probe (csDNA): 5'-ACC ATT CTG CGC-3'.

All other chemicals were of analytical grade and used as received without further purifications. The water used in the experiments was deionized water. The antibody to *V. parahaemolyticus* was diluted with 0.1 M phosphate-buffered saline (PBS). *V. parahaemolyticus* powder was dissolved in the binding buffer of 50 mM Tris-HCl buffer (pH 7.4) including 100 mM NaCl, 5 mM KCl, and 1 mM MgCl_2 .

Cyclic voltammetry (CV), differential pulse voltammetry (DPV), and electrochemical impedance spectroscopy (EIS)

were conducted with a CHI 650D electrochemical workstation (Chen Hua CH Instruments, China), which contains a conventional three-electrode system: platinum wire as auxiliary, Ag/AgCl electrode as the reference, and a 3 mm-diameter gold electrode as the working electrode. The morphology of Au nanoparticles was tracked by a transmission electron microscope (TEM) (JEOL2100 HR, JEOL Instrument, Japan).

Preparation of AuNPs and AuNP-detection probe (AuNP-ssDNA) conjugates

Gold nanoparticles with the uniform diameters were prepared based on our previously reported method [26, 27]. Briefly, 850 μL of 5 g L^{-1} HAuCl₄ solution was added into 60 mL of ultrapure water, and then heated to boiling under magnetic stirring. After quickly injecting 850 μL of 1% trisodium citrate solution, the mixture was stirred for 10 min until the solution became wine red and kept stirring for another 5 min. After gradual cooling to room temperature, the colloidal Au NPs with the size of 15 nm was stored in a refrigerator at 4 °C until needed. For preparation of AuNP-ssDNA conjugates, the disulfide bond of the ssDNA was initially reduced by incubating with 15 μL of 5 μM aptamer with 5 μL of 1 mM TCEP and 3 mL of acetate buffer for 1 h in the dark at room temperature. Then, 100 μL of AuNPs solution was added to the activated aptamers and kept for 1 h for assembling the aptamer onto the surface of AuNPs. Twenty μL of 100 μM dATP was added to the mixture and reacted for 30 min, followed by adding of 20 μL of NaCl (0.1 M) solution. Afterward, the AuNP-ssDNA conjugates were centrifuged at 10000 g for 7 min and washed twice with suspension buffer. The sediment was suspended in 100 μL of suspension buffer and ready for use or stored at 4 °C.

Preparation of circularization mixture and RCA

RCA was carried out based on our previous studies with few modifications [23]. Briefly, 10 μL of phosphorylated padlock DNA probe (5 μM) was mixed with 10 μL of aptamers (5 μM). Then, 2 μL of PEG [50%(w/v)] polyethylene glycol 4000] and 10 μL of 1 \times ligation buffer [40 mM Tris-HCl, 10 mM MgCl₂, 10 mM dithiothreitol (DTT), and 0.5 mM pH 7.8 ATP at 25 °C] were added into the above mentioned mixture, and incubated at 37 °C for 1 h. Ligation was performed by adding 3 μL of T4 DNA ligase (5 U per μL) to the mixture and incubating at 22 °C for 1 h. Then, the T4 ligase was inactivated by heating at 65 °C for 10 min. The resulting mixture was ready for use or stored at 4 °C.

Fabrication of the sensing interface of the electrochemical biosensor

Gold electrode was polished to mirror-like surface with wet alumina power (0.1 and 0.03 μm respectively) and rinsed thoroughly with DI water. Then, the polished electrode was successively cleaned ultrasonically in ethanol and DI water, followed by immersing it in a piranha solution [concentrated sulfuric acid to 30% hydrogen peroxide (3:1 volume ratio)] for 5 min and rinsed with DI water. Then, the electrode was electrochemically cleaned by performing CV in 0.5 M H₂SO₄ solution between -0.3 V and 1.5 V until the curve was stable. The electrode was rinsed again and ready for use.

The cleaned gold electrode was placed into a 30 mM cystamine dihydrochloride solution overnight and then rinsed with PBS to remove physically adsorbed dithiols followed by reacting with 2.5% glutaraldehyde aqueous solution at 37 °C for 1 h. Then the electrode was incubated with 10 μL of antibody (Ab) solution (0.05 $\mu\text{g } \mu\text{L}^{-1}$) in PBS (pH 7.4) at 37 °C for 1 h in a humidity chamber. After sufficiently rinsing with PBS, the electrode was dipped into a 0.1% BSA solution at 37 °C for 30 min to block the remaining adsorption reactive sites and to avoid the nonspecific adsorption effect during the sensing process.

The antibody modified electrode was incubated with the target pathogen *V. parahaemolyticus* at 37 °C for 0.5 h and thoroughly rinsed with PBS. In order to investigate the concentration-dependent *V. parahaemolyticus* sensing, the modified electrode was incubated with different concentrations of bacteria (10-time serial dilution 2.2×10^0 – 10^8 cfu mL⁻¹) in borate buffer (pH 7.4) at 37 °C for 0.5 h followed by PBS rinsing. The reference bacterial strains used as controls were also processed in parallel with *V. parahaemolyticus* to test the specificity of the assay.

Then, the electrode was placed into an aptamer-included primer solution at room temperature for 45 min and washed for subsequent RCA. For the RCA process, basically, it was followed our previous studies [22]. Briefly, 1.5 μL of Phi29 DNA polymerase (10 U μL^{-1}), 5 μL of dNTPs (10 mM) and 10 μL of 1 \times Phi29 buffer [33 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 66 mM KCl, 0.1%(v/v) Tween 20, and 1 mM DTT] were sequentially added into the polymerization mixture. The polymerization reaction was carried out at 37 °C for 1 h. Finally, the detection probe (DNA-AuNP) was added to the mixture to hybridize with the RCA products at 37 °C for 30 min. Then, csDNA probe at 0.5 μM was added and hybridized with labeling ssDNA probe on the surface of AuNPs at 37 °C for 30 min to form the numerous double stranded DNA structure for MB labeling. After hybridization, the electrode was cultured in MB solution for 5 min for electrochemical measurement. After washing, the electrochemical signal was measured in 0.1 M PBS as the working buffer including 0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄, 2 mM MgCl₂·6H₂O,

and 10 mM KCl (0.1 M, pH 7.4) by using DPV (−0.6 V to 0.1 V) with 4 mV of potential incremental, 50 mV of amplitude, 0.05 s of pulse width, and 0.0167 s of sample width.

Results and discussions

Design of hetero-sandwich aptamer/immuno hybrid electrochemical biosensor

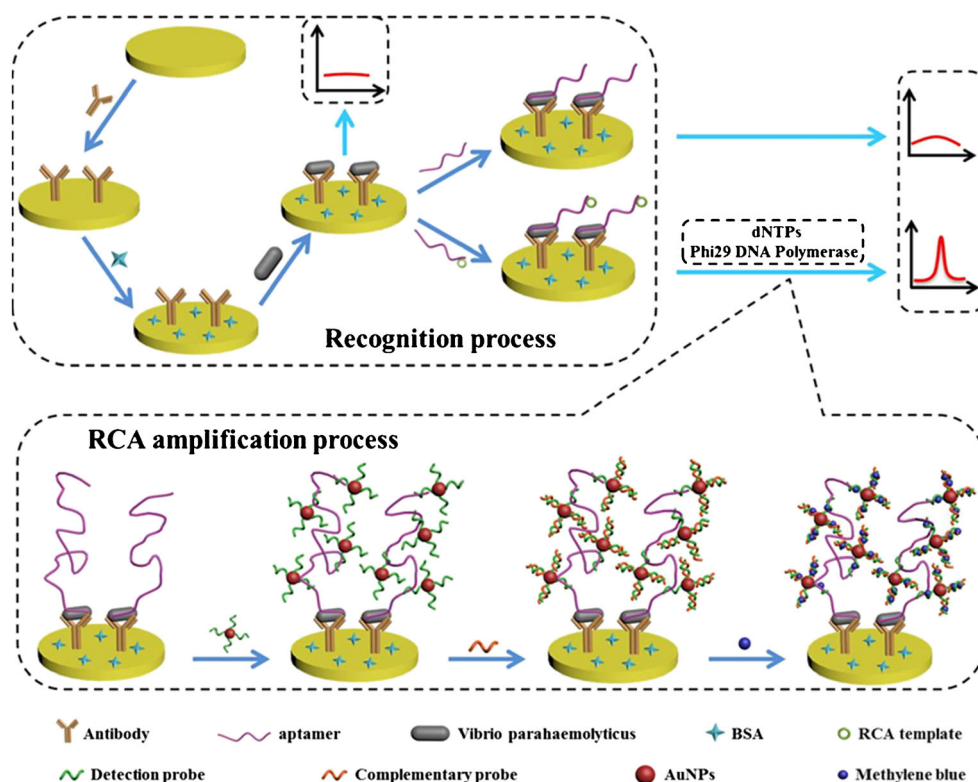
Our detection protocol initially is designed with two types of recognition probes targeting *V. parahaemolyticus* as shown in Scheme 1. It depicts the complete detection process and the sensing principle of this hetero-sandwich based electrochemical biosensor. First, the Ab is immobilized onto the electrode chemically, and the functionalized electrode acts as the capture probe for *V. parahaemolyticus*. Second, the aptamer probe of *V. parahaemolyticus* recognizes and binds to *V. parahaemolyticus* to form a HSS. The reasons for using Ab and aptamers as the capture probe and signal reporting probe are as follows: i) using Ab as the capture probe on the electrode can minimize the background current signal generated from the nonspecific adsorption between methylene blue (MB) and DNA probe; ii) using aptamer as the signal reporting probe provides the base for further signal amplification by in vitro isothermal nucleic acid amplification protocol, e.g., RCA was adopted to increase the sensitivity; and iii) using

aptamer enables the gold nanoparticle-ssDNA conjugates to label the RCA products, so that the amplified electrochemical signal of MB can be measured for quantitative detection of *V. parahaemolyticus*. Of note, due to the duplex DNA formed as the amplification products, MB is adopted as the electrochemical redox probe for its prior affinity to dsDNA. Thus, the hetero-sandwich recognition model based signal amplification protocol provides a novel sensing platform for ultrasensitive and accurate detection of *V. parahaemolyticus*.

Characterization of the development and optimization of the electrochemical aptasensor for *V. parahaemolyticus* detection

The RCA-based signal amplification strategy is confirmed before the development of hetero-sandwich electrochemical aptasensor for *V. parahaemolyticus* detection. In comparison of the images in the dark and bright fields of the agarose gel electrophoresis, it is clearly demonstrated that RCA was successful, which can be verified by the presence of the product of high molecular weight in lane 3 in the dark field of the gel (Fig. 1a); the RCA products are well labeled with the Au-ssDNA conjugates in lane 4 in the bright field of the gel (Fig. 1b). Also, the RCA products and the product labeling are confirmed by the TEM results (Fig. 1c-e). A critical assessment is made from the TEM results as shown in Fig. 1e that AuNPs with the diameter of about 18 ± 3 nm (Fig. 1c) are

Scheme 1 The schematic diagram of the RCA based hetero-sandwich electrochemical biosensor for ultrasensitive detection of *V. parahaemolyticus*



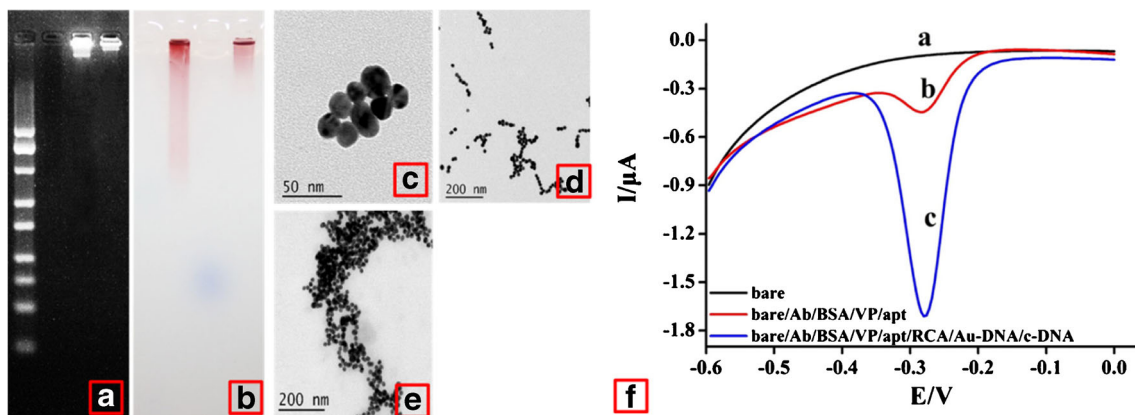


Fig. 1 The confirmation results of using RCA for signal amplification. The results of the RCA products in the dark field (**a**) and bright field (**b**) of an agarose gel electrophoresis; (**c**), the TEM result of AuNPs; (**d**), the

TEM result of the detection probe-AuNPs conjugates; (**e**), the TEM result of the RCA products labeled with AuNPs; (**f**), DPV results of RCA based signal amplification

well assembled into a linear structure in high density after the hybridization induced labeling with the Au-ssDNA conjugates (Fig. 1d), which greatly increases the amount of the electrochemical redox tag of MB, and thus improves the electrochemical sensing signal. Due to the macro gold electrode adopted, therefore, SEM is not applied for direct characterization of electrode surface. Besides, the RCA time is also considered and 60 min is adopted as the optimal time for amplification (See details in Fig. S3). Consequentially, excellent signal amplification is achieved for *V. parahaemolyticus* detection, which is the core mechanism for the signal amplification in this detection scheme.

The step-by-step modification of the sensing interface of the electrode is characterized by EIS and CV tests, respectively. Compared with the bare electrode (curve a in Fig. 2), the Ab immobilized electrode shows a larger electron transfer impedance R_{et} (curve b) due to the poor conductivity of antibody. Similarly, blocking with BSA and recognition of *V. parahaemolyticus* leads to an increase of R_{et} due to the insulativity of protein (curves c and d). Meanwhile, the amount of the aptamer-included DNA probe, the recognition time of antibody, the incubation time of MB labeling are also confirmed to get the best sensing results. And 1.4 M of aptamer-included DNA probe, 15 min of antibody recognition and 5 min for MB labeling are adopted as the optimal conditions for the final determination (See detailed results in Fig. S4–S6).

For signal reporting and amplification, the binding of aptamer to the target *V. parahaemolyticus* further increases the R_{et} for the negative charge of the DNA (curve e in Fig. 2). Based on this immobilized aptamers, RCA is carried out and the elongated RCA products made the repulsion of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ stronger and R_{et} larger (curve f). Finally, both the labeling of RCA products with AuNP-ssDNA conjugate and hybridization of complementary DNA probe onto AuNPs conversely and obviously decreases the R_{et} (curves g and h in

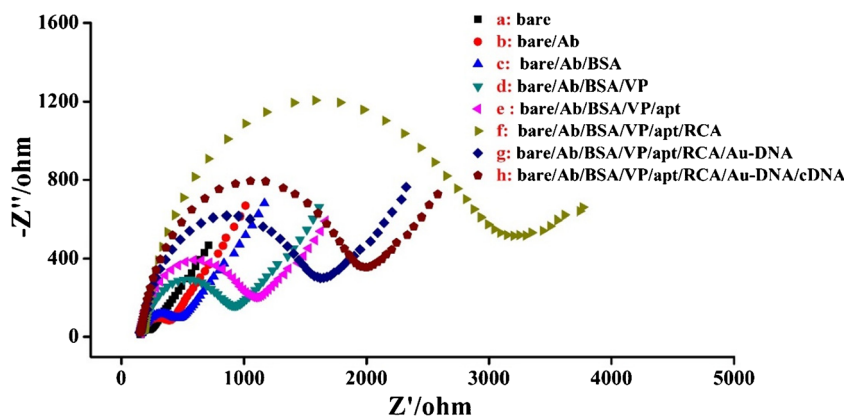
Fig. 2). The reason for this noticeable decrease of R_{et} is the superior conductivity of AuNPs, which can greatly enhance the transfer of electron. In addition, the CV tests are also performed to characterize the development of the electrode and sensing system. Similar results are achieved as those of EIS (detailed CV results in Fig. S1). Overall, the results of EIS and CV tests well document a successful modification and development of the hetero-sandwich structure based electrochemical aptasensor for sensitive detection of *V. parahaemolyticus*.

Another important aspect of this research is to improve the sensitivity of the hetero-sandwich recognition electrochemical aptasensor for accurate detection of *V. parahaemolyticus* and to deepen the insight into the amplified electrochemical signal with RCA. The amplified electrochemical signal with RCA is characterized and the comparison analysis is performed with different settings. In comparison with the blank signal of the bare electrode control (curve A in Fig. 1f), the traditional measuring signal of *V. parahaemolyticus* without signal amplification treatment (curve B in Fig. 1f) is easily distinguished from the blank one with the characteristic peak at -0.27 V. Of great importance, is the great enhancement in measuring signal of *V. parahaemolyticus* with RCA (curve C in Fig. 1f) compared with the signal derived from the control (bare electrode) and traditional testing without RCA (curves A and B in Fig. 1f), further demonstrating the extraordinary signal amplification performance of our designed hetero-sandwich aptasensor.

Analytical performance of *V. parahaemolyticus* with the hetero-sandwich recognition electrochemical aptasensor

Under optimal conditions (detailed optimization results were not shown here), the analytical performance of the our aptasensor is evaluated by measuring different concentrations of *V. parahaemolyticus* in 0.1 M PBS (pH 7.4) containing

Fig. 2 EIS results of the modification process of the electrode and the sensing process



10 mM KCl and 2 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. The results in Fig. 3a demonstrate that the characteristic peak currents increases in proportion with the increase of the concentrations of *V. parahaemolyticus* ranging from 2.2 to 2.2×10^8 cfu/mL compared the current from the bare electrode control. In addition, a corresponding calibration curve is constructed (Fig. 3b), which showed a good linear relationship between the peak currents and the concentrations of *V. parahaemolyticus*. Not only is sensing performance of the hetero-sandwich recognition based electrochemical aptasensor excellent, but also the LOD achieved as low as 2 cfu/mL, indicating that *V. parahaemolyticus* even can be detected at this concentration.

To assess the specificity of this hetero-sandwich recognition electrochemical aptasensor for *V. parahaemolyticus* detection, exclusivity experiments are conducted with reference strains consisting of common foodborne pathogens, *S. aureus*, *Shigella sonnie*, *E. coli* O157:H7, *Salmonella*, *C. jejuni* and

V. mimicus. The exclusivity test results clearly indicate that only the *V. parahaemolyticus* samples and the *V. parahaemolyticus* mixtures induced obvious redox current response (in Fig. 3c and d), whereas all the reference strains did not induce any redox current response. The excellent specificity of the electrochemical aptasensor can be attributed to the designed hetero-sandwich recognition structure which is composed of both antibody and aptamers. The reproducibility of the hetero-sandwich recognition electrochemical aptasensor is further evaluated by using the electrodes from different manufacture batches and a given concentration (2.2×10^5 cfu per mL) of *V. parahaemolyticus*. The results show that a variation coefficient (under 4.3%) is achieved among the detection replicates done with the electrodes of different manufacture batches. The stability of the developed hetero-sandwich recognition electrochemical aptasensor is assessed by the long-term storage test. After two-week storage

Fig. 3 DPV results of different concentrations (10-time serial dilutions from 2.2 to 2.2×10^8 cfu/mL) of target *V. parahaemolyticus* (a) and a quantitative calibration curve of peak currents vs. logarithm of the concentrations of *V. parahaemolyticus* (b); the specificity confirmation results of the hetero-sandwich electrochemical biosensor for *V. parahaemolyticus* detection (c) the DPV results of the specificity testing and (d) the quantitative results of the specificity testing

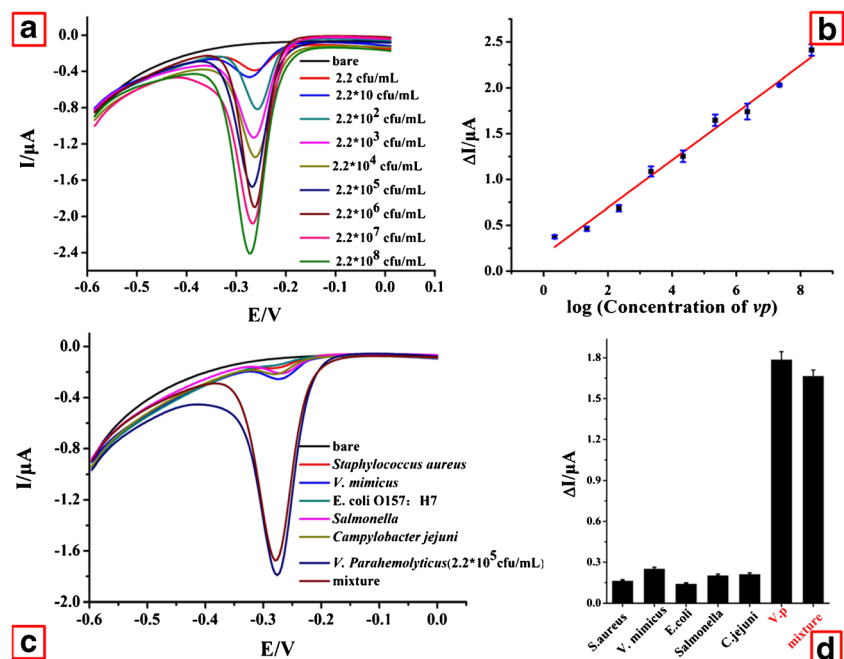


Table 1 Detection of *V. parahaemolyticus* in a spiked fish samples by this method ($n = 5$)

Sample	Spiked concentration (cfu/mL)	Measured concentration (cfu/mL)	Recovery (%)
1	2.2×10^2	$(2.14 \pm 0.09) \times 10^2$	97 ± 4.1
2	2.8×10^5	$(3.03 \pm 0.07) \times 10^5$	108.3 ± 2.5
3	4.1×10^4	$(3.84 \pm 0.04) \times 10^4$	93.7 ± 0.97
4	3.5×10^6	$(3.54 \pm 0.03) \times 10^6$	101 ± 0.86

at 4 °C, the fabricated electrochemical aptasensor retains the sensing current response to about 95.2% of its initial level. Therefore, the hetero-sandwich recognition electrochemical aptasensor demonstrates excellent reproducibility and stability, suggesting great potential for practical detection of *V. parahaemolyticus* in food (Table 1).

Moreover, the suitability of this hetero-sandwich recognition electrochemical aptasensor for foodborne pathogen detection is evaluated with *V. parahaemolyticus* spiked fish samples. All *V. parahaemolyticus* samples with known concentrations are all prepared and determined with the classic culture method, which is supplied by the Jiangsu Entry-Exit Inspection Guarantee. Different amount of *V. parahaemolyticus* are then added into the fish samples and determined with the fabricated electrochemical aptasensor. The detection results are used to assess the accuracy achieved with the fabricated electrochemical aptasensor. The recoveries of *V. parahaemolyticus* at different spiked concentrations reach from 93.7% to 108.3%, indicating a good accuracy for this hetero-sandwich recognition electrochemical aptasensor in *V. parahaemolyticus* detection. Besides, five different biosensors are used to determine the same sample at the concentration of and the related peak current are compared. The calculated relative standard deviation was about 4.92%, which reveals the good repeatability of the biosensor. Furthermore, the sensing performance of this amperometric aptamer/immuno hybrid biosensor is compared to other similar reported methods. Results in Table 2 demonstrate that the method reported in this research is comparable to other method in sensing performance and has the superior properties in sensitivity

[28–35]. All these research indicate that this aptamer/immuno hybrid biosensor is a useful platform with potential for sensitive and accurate analysis of general food pathogens.

Conclusions

In summary, a hetero-sandwich recognition electrochemical aptasensor was reported for sensitive and accurate detection of *V. parahaemolyticus*. Both antibody and aptamers to *V. parahaemolyticus* were adopted as the recognition probes to form an antibody-analyte-aptamer hetero-sandwich structure. The antibody in the hetero-sandwich structure acted as the immobilized probe; while the aptamers served as the signal reporting probe. In addition, to further improve the sensing performance of the strategy, RCA was employed in the system to amplify the current signal response. The data in the present study clearly demonstrated that the sensing performance reached the LOD of $2 \text{ cfu} \cdot \text{mL}^{-1}$ in detection, representing a huge improvement. Using both antibody and aptamers in the sensor may have improved the specificity of the strategy; while the adaptation of RCA into the strategy may have increased the sensitivity of the assay. Moreover, we have successfully applied the detection strategy to spiked food samples with satisfactory sensitivity and specificity. This hetero-sandwich recognition electrochemical aptasensor technology not only provides a specific and ultrasensitive protocol for detection of *V. parahaemolyticus* in food, but also offers a powerful and versatile platform with the potential for rapid

Table 2 An overview and comparison on recently reported nanomaterial-based methods for detection of *V. parahaemolyticus*

Methods applied	Materials used	Merits of the methods (LOD)	Ref
Electrochemical method	Grapheme oxide	$1.69 \times 10^{-13} \text{ M}$	[28]
Electrochemical DNA sensor	DNA probes	$0.025 \text{ } \mu\text{M}$	[29]
Amperometric immunosensor	Agarose/AuNP	$7.374 \text{ cfu mL}^{-1}$	[30]
Fluorescent method	DNAzyme & AuPd	300 cfu mL^{-1}	[31]
Voltammetric	LAMP	20 fM	[32]
Microfluidic	AuNP	1 pg mL^{-1}	[33]
Colorimetric	peroxidase-mimicking	3.4 nM	[34]
Colorimetric	recombinase PCR	0.8 pM	[35]
Hetero-sandwich electrochemical biosensor	AuNPs	2 cfu mL^{-1}	This research

and sensitive detection of a variety of pathogens in the clinical diagnosis, food safety, and bio-threat detections. Further research to find a more friendly antibody modification protocol and highly simplified fabrication procedures of the sensing electrode is ongoing in our group.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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