J. Electrochem. Sci. Eng. **16** (2026) 3403; https://doi.org/10.5599/jese.3403

Open Access : : ISSN 1847-9286
https://pub.iapchem.org/ojs/index.php/JESE

Original scientific paper

Rapid single-molecule detection of *Salmonella* DNA based on an electrode surface-based polymerase chain reaction technique

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Received: April 27, 2026; Accepted: June 12, 2026; Published: June 25, 2026

Abstract

There is a current need for rapid, selective and sensitive specific detection of Salmonella in food matrices. We demonstrate a technique for rapid single-molecule detection of Salmonella DNA based on an electrode surface-based polymerase chain reaction (PCR) based sensing of 48-base target single-stranded DNA (ssDNA) (CP014358.1 GenBank), chicken-juice matrix under 20 minutes. This is based on the covalent attachment of a probe ssDNA to pseudo-graphite. This sensor can be used in two different modes, without and with PCR, for lower limits of detection (LOD). The pseudo-graphite electrode is prepared for ssDNA attachment through the functionalization of its surface with carboxylate groups, which form amide linkages with amine-modified ssDNA. Without PCR, the probe-modified electrode is immersed in a sample complementary target matrix for 10 minutes, removed, and then placed into a solution of the double-stranded DNA (dsDNA) intercalator, cobalt(III) tris-phenanthroline. Detection of dsDNA formation between the probe and target sequences is achieved via the SWV of the surface-bound cobalt complex. The LOD of this method is 2.0 aM for the target within 12 minutes. Lower LOD's are obtained by conducting PCR on the electrode surface. The on-electrode (oE) PCR process uses a 20-base primer attached to the pseudo-graphite. A chicken-juice sample matrix containing target ssDNA (48 bases of Salmonella typhimurium) was directly added to a PCR master mix with a reverse primer, placed in a standard PCR vial, and thermocycled 5 times. The electrode with surface-bound dsDNA was subjected to the cobalt(III) tris-phenanthroline protocol with a LOD of 35 zM. This is one DNA molecule within 17 minutes in a complex chicken-juice matrix.

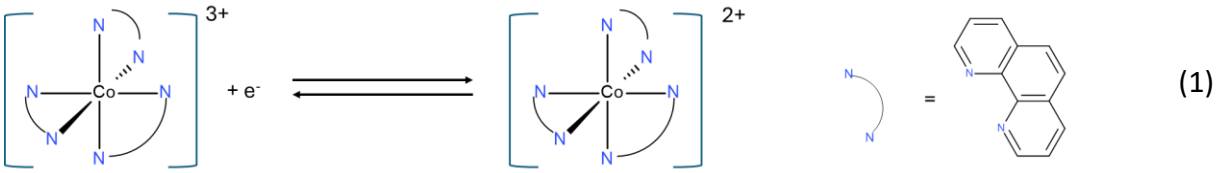
Keywords

DNA amplicons detection; electrochemical biosensor; *Salmonella* detection; polymerase chain reaction electrode; single DNA molecule detection

Introduction

There is an urgent need for rapid, selective and sensitive *Salmonella* detection in foodstuffs, especially in poultry [1-3]. The Centre for Disease Control and Prevention (CDC) estimates that about 1.35 million people contract *Salmonella* infections and that about 420 deaths occur *per* year [4,5]. Compounding this problem is the economic impact of *Salmonella* infections in chickens, which costs about $2.8 billion [6]. Trace analysis for *Salmonella* detection improves food safety and enables early outbreak surveillance. Nanomolar (nM) LOD has been reported to be adequate for *Salmonella* detection [7,8]. Traditional cell cultures can take up to 4 days [9]. More rapid techniques include quantitative polymerase chain reaction (qPCR), enzyme-linked immunosorbent assay (ELISA) and recombinase polymerase amplification (RPA), which take about 1-3 hours [10-12]. Detection based on current polymerase chain reaction (PCR) techniques significantly lowers the detection limit. However, the PCR sample processing steps make this process time-consuming, as this requires isolation of target DNA from complex matrices before detection. In a previous contribution, we demonstrated a DNA covalent linkage to a surface for PCR [13]. This allows for the rapid removal of single-stranded (ss) DNA from complex matrices and PCR solutions. This obviates the need for gel electrophoresis and speeds up the PCR process. Detection was based on the loss of optical absorbance in an aqueous ethidium bromide solution as it intercalated into the surface-immobilized double-stranded (ds) DNA. However, this approach gave a limit of detection (LOD) of 5.0 pM, which is still relatively high compared to other approaches. It is important to note that detecting DNA at femtomolar (fM) levels is crucial for monitoring pathogen outbreaks and clinical diagnosis [14,15].

Electrochemical sensing of double-stranded DNA (dsDNA) through a redox-active tag presents a strong possibility for lower LOD than UV absorbance. These tags may include covalent linkages or form a strong binding action with strands. The DNA intercalator, cobalt (III/II) tris-phenanthroline ($Co(phen)_3^{3+/2+}$) offers an excellent possibility as it gives a set of reversible voltammetric waves at a half-wave ($E_{1/2}$) of 0.12 V *vs*. Ag/AgCl (Equation 1) [16-18].



(1)

The binding constant of $Co(phen)_3^{3+/2+}$ to dsDNA is 5.8×10^3 M^{-1} [19]. This complex was used by Liu *et al*. [17] for the electrochemical sensing of dsDNA on a modified gold surface, with an LOD of 1.0 pM. It is important to note that the thiol linkage of immobilized DNA on Au is not robust enough to withstand the thermocycling (95 to ~55 °C) required for PCR [20,21]. In that previous study by Koirala and coworkers [13], the attachment of single-stranded DNA (ssDNA) to the pseudo-graphitic material proved stable to thermocycling. This enables surface-bound PCR and the rapid removal of dsDNA from the master mix and/or the sample matrix, thereby avoiding more time-consuming separation processes. The basal material for this is graphite from the University of Idaho thermo-lyzed asphalt reaction (GUITAR), a pseudo-graphite consisting of 85/15 % sp^2/sp^3 carbon [22]. This material has excellent electrode properties t, including fast heterogeneous electron transfer, a wide aqueous potential window and resistance to fouling.

In this contribution, we examine the sensing abilities of single-strand (ss)DNA tethered to GUITAR electrodes to recognize targets. The formation of the corresponding duplex DNA is detected by a voltammetric wave at the surface-bound $Co(phen)_3^{3+}$. In cases where the capture of target ssDNA does not fully saturate the surface-bound complementary sequences, we employ rapid PCR directly

on the electrode (on-electrode PCR). We demonstrate that this technique has a low detection limit of 2.0 attomolar (aM) for DNA hybridization and 35 zeptomolar (zM) for PCR amplification of 48 base pairs of *Salmonella typhimurium* with high specificity. Under the experimental conditions of this study, this is the detection of one strand of target DNA. This is performed in a challenging matrix that typically inhibits Taq polymerase [23,24]. In other protocols, this requires careful sample preparation and isolation of the DNA before PCR. In this work, that requirement is excluded in the on-electrode (oE) PCR technique.

Experimental

Chemicals

Potassium monophosphate (99.6 %) and potassium diphosphate (99.8 %) were obtained from Fisher Scientific (Waltham, NJ, USA). Magnesium chloride (99 %), sodium nitrite (98 %) and cobalt chloride were purchased from Fisher Scientific, USA. *N*-ethyl-*N*'-(3-(dimethylamino) propyl) carbodiimide hydrochloride (EDC) was purchased from Chemimpex (Wood Dale, IL). Hydrogen peroxide was from Macron Fine Chemicals (Radnor, Pennsylvania). Hydrochloric acid ACS grade, 4-aminobenzoic acid (99 %) and 1,10 phenanthroline (phen) monohydrate (99 %) were obtained from Sigma Aldrich (Milwaukee, WI). All aqueous solutions were prepared with deionized water purified by passing through an activated carbon purification cartridge (Barnstead, model D8922, IA, USA). Titanium foil, 1 mm thick (99.8 % purity), and carbon cloth were obtained from Amazon (Seattle, WA), and vegetable oil was sourced from Winco Foods (Moscow ID, USA). Carbon fibre (diameter = 1.48 mm) was isolated from carbon cloth. The sensing area of the electrode was isolated with GE Advanced Silicon Sealant obtained from (MA, USA). DNA was purchased from Integrated DNA Technology Inc. (Coralville, IA). The $Co(phen)_3^{3+}$ complex was prepared using the procedure reported in the literature [25,26]. The sequences for DNA hybridization and PCR study are shown in Tables S1 and S2 of the Supplementary Material. These are from *Salmonella typhimurium* and are used for its detection [27-30]. The sequence was from gene bank CP014358.1.

Instruments and materials

GUITAR coating was performed on a Thermo Scientific tube furnace (MA, USA) and is described in detail in previous publications [22]. PCR was conducted using a Chai QPCR (Santa Clara, CA, USA), and voltammetric studies were conducted using a Bioanalytical Systems CV-50 W (West Lafayette, IN, USA). Ag/AgCl reference electrode from BASi (West Lafayette, IN, USA), glassy carbon electrode from Aliexpress (Hangzhou Shi, China). UV-Vis absorbance measurements were performed using a SpectraMax QuickDrop spectrophotometer (San Jose, CA). The square-wave voltammetric parameters were a 1.0 mV step potential, 10 mV amplitude, and a frequency of 10 Hz.

Chemical vapor deposition GUITAR

Electrodes consisted of GUITAR deposited onto carbon fiber for the PCR-based studies or on Ti foil for non-PCR detection. The chemical vapor deposition (CVD) process is described in detail in previous studies [22]. In brief, N_2 gas flows through a hot-tube furnace for about 4 min before the precursor is pumped into the furnace at a flow rate of 7 mL min^{-1}. After coating for 25 minutes, the furnace is cooled to 500 °C, and the quartz tube is removed, allowed to cool to room temperature. This process is repeated one more time for coating carbon fibres and the coated GUITAR is then tested and used for specific applications.

Electrochemical modification of GUITAR and glassy carbon electrodes

The electrochemical modification step is listed in a previous publication [31]. XPS analysis shows 6.7 % -COOH on the surface of the GUITAR electrode [31].

Immobilization of ssDNA on functionalized GUITAR

Surface-modified GUITAR and glassy carbon disk electrodes from above were covalently linked to a probe ssDNA 52 base (Table S1, Supplementary material) or to 20 base *Salmonella* right primer (Table S2) *via* an amide linkage [31]. This was produced by adding a solution of 5 µM $H_2NC_6H_{12}$-5'ssDNA in 0.1 M MES Buffer pH 5.5 and 0.1 M *N*-ethyl-*N*'-(3-(dimethylamino) propyl) carbodiimide hydrochloride (EDC) with the modified electrode immersed for 4 hours. About ~22 %, 1.1 nmol of ssDNA was immobilized on modified GUITAR fibres (16 fibres) estimated using the UV-vis nanodrop method [32]. The electrode was then removed, rinsed with 0.1 M phosphate buffer (PBS) at pH 7.10, and used for further analysis. A previous study found the surface density of DNA on GUITAR's surface to be 3.81×10^{14} molecules cm^{-2} through the cyclic voltammetry (CV) of $Ru(NH_3)_6^{3+}$ [31]. Also, another study found a similar surface density of covalently attached amino-ferrocene through the same coupling (6×10^{14} molecules cm^{-2}) [33].

Results and discussion

DNA hybridization and electrochemical detection for non-PCR route

In this work, we focus on oligonucleotides as proof of principle using a random sequence of 52 bases. The steps taken for DNA hybridization are highlighted in Figure 1.

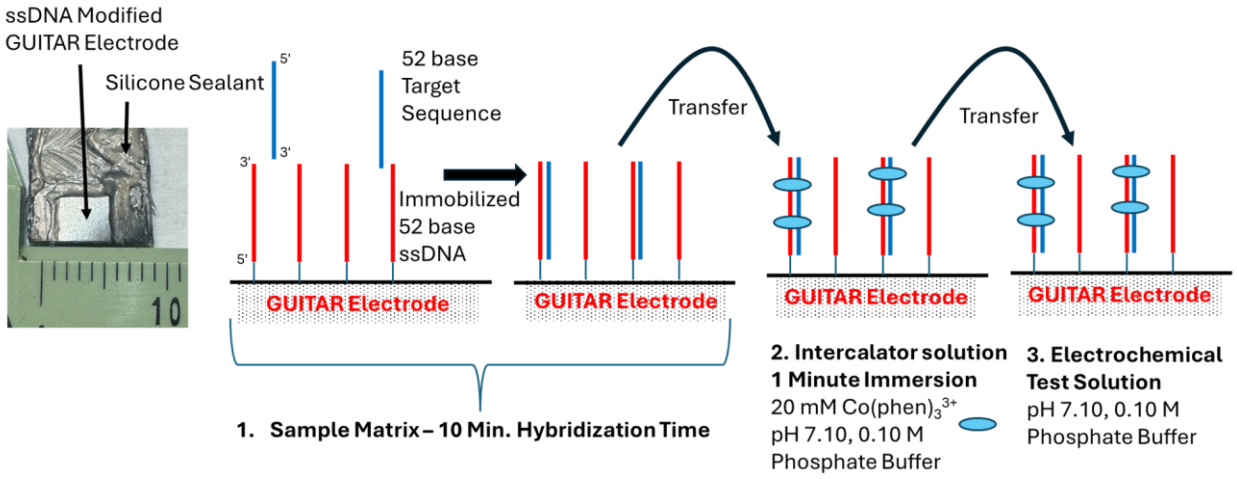


***Figure 1**. Schematic for the electrochemical detection of target ssDNA (Table S1) on a GUITAR electrode. A photograph of the electrode (far left)*

The surface-bound ssDNA on GUITAR and GC electrodes is allowed to hybridize to the target complementary DNA (Table S1), forming double-stranded DNA (dsDNA) in a 0.1 M phosphate buffer (PB), pH 7.10, at 55 °C for 10 minutes. The volume of the target solution was 1.0 mL. The electrode is then rinsed with 0.1 M PBS buffer, pH 7.10, at 45 °C to remove any non-hybridized DNA and is immersed in a 0.020 M $Co(Phen)_3^{3+}$ solution for 1 minute. Afterward, the electrode is rinsed with 0.1 M PBS buffer, pH 7.10 and the voltammetric response of the electrode surfaces is taken in that buffer system. Figure 2 displays the square wave voltammetric response (SWV) of the ssDNA modified GUITAR (Figure 2A) and GC electrodes (Figure 2B). In the presence of 100 pM target, the ssDNA-modified GC response is a much lower current response than the corresponding GUITAR electrodes. This is probably due to a lower surface density of tethered ssDNA on GC relative to GUITAR. The

balance of this investigation focused on GUITAR for this reason. In Figure 2A, the zero-concentration target signal gives background current due to a weak affinity of $Co(Phen)_3^{3+}$ bound to ssDNA by electrostatic interactions [16,17,34].

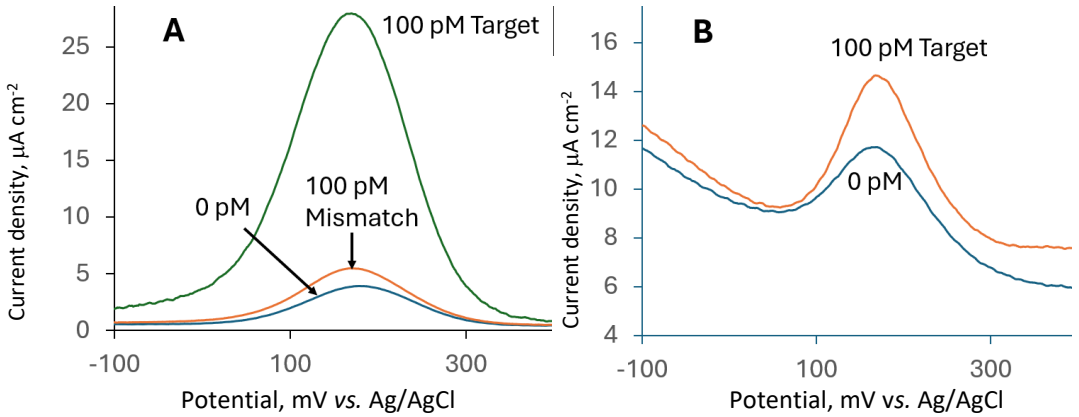


Figure 2. *(A) SWV response of $Co(Phen)_3^{3+}$ after intercalation into surface-bound ssDNA modified GUITAR electrode, with 100 pM of target (Table S1) and a 10-base mismatch (Table S1) in 0.1 M PB, pH 7.10. (B) The responses with a modified glassy carbon electrode*

The SWV response of the interaction between the probe ssDNA-GUITAR electrode and the target ssDNA with the 10-base mismatch in Table S1 is shown in Figure 2A. The latter produced a SWV response close to the background.

Determination of limit of detection of DNA target by hybridization

The LOD of the modified ssDNA electrode was estimated through the variation of the concentration of the target complementary DNA. The SWV responses are shown in Figure 3A and the calibration curve is presented in Figure 3B. The proportionality of voltammetric peak currents to the log of the concentration of $Co(Phen)_3^{3+}$ intercalated to surface dsDNA has been observed several times in literature [35-37]. The response was used to create a calibration curve to quantify the target complementary ssDNA. The LOD is calculated using Equations (2) and (3).

$$j = m \log c_{DNA} + b \quad (2)$$

$$j_{LOD} = j_{Blank} + 3\sigma_{Blank} \quad (3)$$

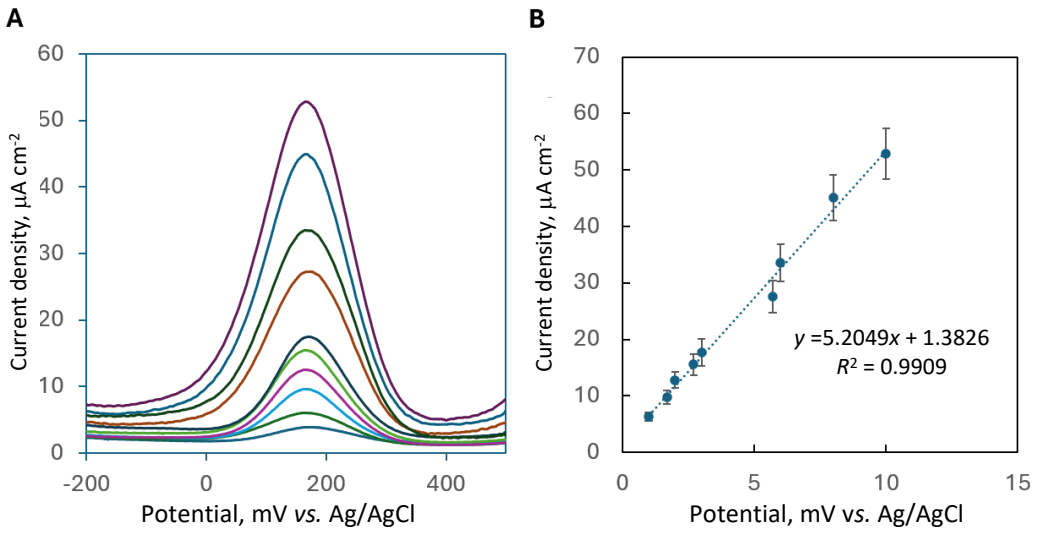


Figure 3. *A) The SWV i_p response of surface-bound intercalated dsDNA $Co(Phen)_3^{3+}$ with target DNA (Table S1) varying from 10 aM to 10 nM. The standard deviation bars are shown for n = 3 trials; (B) linear relationship between current response* vs. *log of DNA concentration after intercalation with $Co(Phen)_3^{3+}$*

The LOD is estimated to be 2.0 aM, where σ is the standard deviation (0.55 µA) of the blank solution with n = 7 trials, b is intercept, j is the current density and m is the slope (5.20 µA (log aM)$^{-1}$) of the gradient of the line shown in Figure 3B.

Zeptomolar limit of detection: electrode-based polymerase chain reaction for detection of Salmonella DNA

Solid phase PCR (sp-PCR) has been demonstrated in the literature. A previous study examined the immobilization of a primer on an indium tin oxide electrode followed by differential pulse voltammetry of a ferrocene tag [38,39]. The investigators conducted sp-PCR of an oligonucleotide, but not in a complex matrix. Another study used a commercial sp-PCR kit on a blood matrix, followed by detection with a fluorescent probe with a sub-aM LOD [40]. However, this process required 3 hours. The oE-PCR system is relatively inexpensive and ensures rapid 17-minute detection of target DNA in a complex matrix after just 5 PCR cycles with a lower LOD of 35 zM. Typical PCR procedures require 25-50 cycles with isolation procedures from complex sample matrices and master mix solutions. These procedures may take up to 3 hours [41,42]. Furthermore, nonspecific amplification can occur with an excessive number of PCR cycles [43-45]. With these problems in mind, it is always beneficial to keep the number of cycles to a minimum while still maintaining LOD below the fM required for early pathogen detection [14,15]. In this portion of the study, PCR was conducted directly on the surface of electrodes modified with immobilized forward primer sequences (Table S2 in the Supplementary material). The GUITAR electrode configuration was in the form of coated carbon fibres [31]. The feature of these fibres is that they are immersible in standard PCR vials. Figure 4 contains the steps for the oE PCR process.

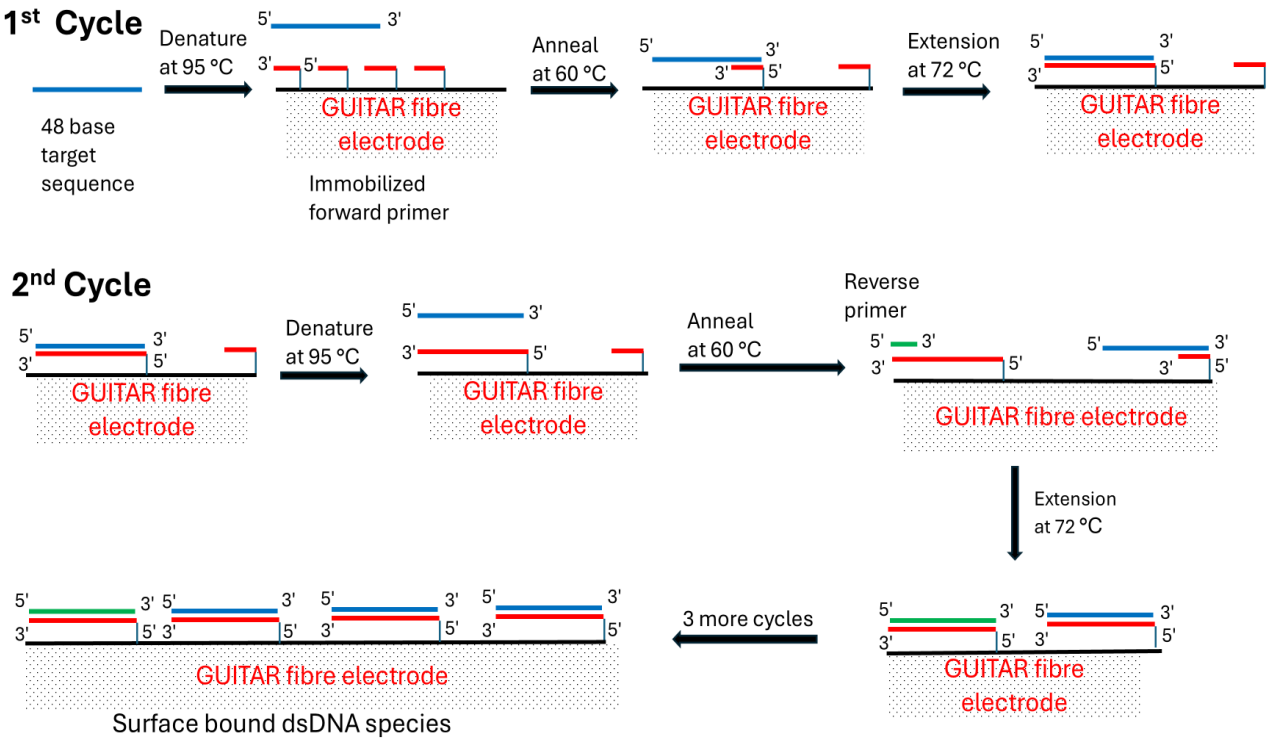


***Figure 4**. Schematic for the oE-PCR technique*

In the first step, the 48-base target in the GUITAR fibre electrode with immobilized 20-base forward primer, 21-base reverse primer and the PCR master mix solution (see Tables S2, S3 and S4) are combined in a PCR vial. The fibres are then subjected to PCR thermocycling. The initial

denaturation is at 95 °C for 1.5 min, followed by annealing at 60°C for 0.5 mins for 5 cycles. After completion, the extension is at 72 °C for 4 min [13].

Supplementary material includes the optimization study for the number of PCR cycles (Figure S1). 5 cycles yielded the same results as 25 cycles, indicating saturation of the electrode surface after 5 cycles. Fewer than 5 PCR cycles offer little time advantage. After PCR, the fibres are rinsed three times with 0.1 M PB buffer pH 7.10 and intercalated with 0.020 M $Co(Phen)_3^{3+}$ at pH 7.10, 0.10 M PB for 1 min. The fibre is then removed from this solution and placed in a 3-electrode electrochemical cell test solution consisting of pH 7.10, 0.10 M phosphate buffer solution. The total time for the process is 17 minutes. The signal for DNA-intercalated $Co(Phen)_3^{3+}$ was determined by SWV. The study of the SWV signal with the concentration of target DNA is presented in Figure 5. The *p*-values (n = 4) are relative to the blank which contains only the immobilized forward primer, master mix, but zero concentration of target and reverse primer. The no template control (NTC) contains the immobilized forward primer, reverse primer, master mix and zero concentration of target. The sequences for target, primers, and the random strand are in Table S2. The response between 2.0 pM and 35 zM was nearly constant. This indicates that the immobilized primers on the surface of the GUITAR electrodes are fully saturated with dsDNA after extension in this range of target concentrations. Based on the *p*-values (0.00004), the LOD for the formation of the amplified target *Salmonella* sequence is estimated to be 35 zM. The *p*-value for the target concentration of 5.0 zM indicates that it is not detectable after the 5 PCR cycle procedure (Figure 5, $p = 0.34$). The presence of 10 nM random sequence did not interfere with the detection of 35 zM target. This is based on the two studies in the forward in Figure 5.

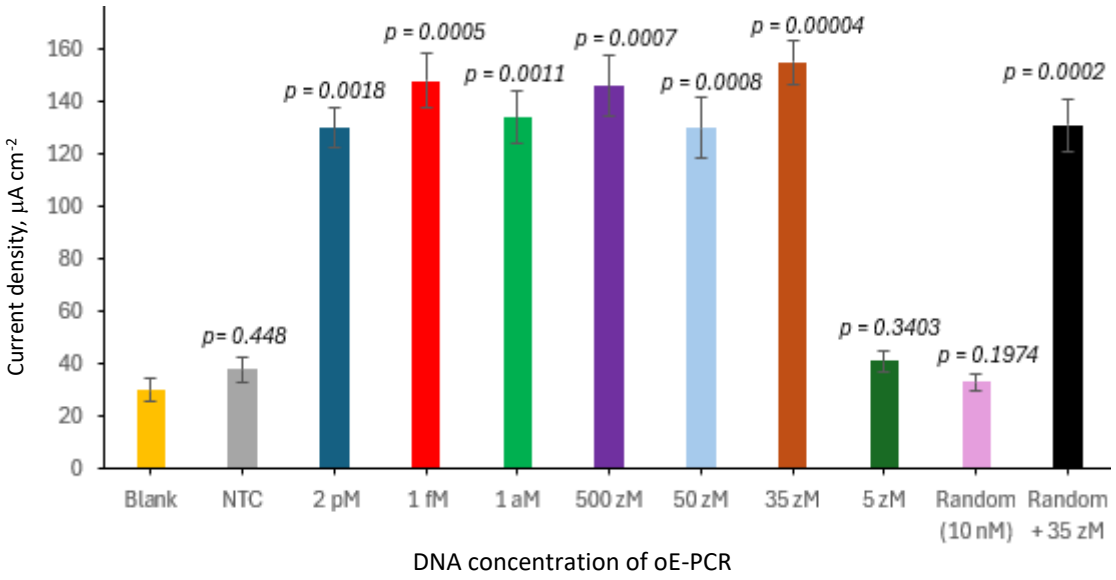


Figure 5. *SWV peak current signals for GUITAR fibre electrodes modified with forward primer (Table S2) after the 15-minute 5 PCR cycles protocol. All electrodes were exposed to 20 mM* $Co(Phen)_3^{3+}$ *prior to voltammetric measurements. The* p*-values are relative to the blank. The LOD was estimated to be 35 zM of target ssDNA*

Detection of a single DNA molecule

Single-molecule detection of target DNA has been demonstrated in the literature [46,47]. In general, these techniques require expensive or elaborate instrumentation and isolation of target DNA from a complex matrix before detection. Srisa-Art *et al.* [46] also did not perform detection in a complex matrix. Hui *et al.* [47] used a genomic DNA and a restriction enzyme to break it apart, which takes 1 h 30 min and detection by atomic force microscopy (AFM) was done in a buffered matrix. On the other hand, the oE-PCR system is based on relatively inexpensive and unmodified commercial instruments and reagents. The conditions for the 35 zM LOD study indicate that the

oE-PCR process detects 1 molecule of 48-base target *Salmonella* ssDNA (Table S2) within 17 minutes. This is based on 10.0 µL spikes of 173.3 ± 8.8 zM stock solution. A study of the delivery of 0.5 molecules of ssDNA to the PCR vial (17 zM) was conducted as replicate measurements would indicate a 40/60 positive/negative rate based on Poisson statistics [48]. The probability (*P*) of delivery is given by Equation (4):

$$P = (\lambda^k e^{-\lambda}) / k! \quad (4)$$

where λ is the expected number of molecules in the aliquot, in this case 0.5, and k is the actual number of molecules received in that aliquot. For zero molecule delivery (k = 0) this is, $P = e^{-0.5}$ = = 0.606 (or 60.6 %). The spikes consisted of 1.7 µL of 500.0±22.5 zM and 5.0 µL spike of 173.3±8.8 zM stock solutions. Results of 11 oE-PCR experiments are shown in Figure 6. The p-values are relative to the blank of Figure 5. In these studies, 7 of 11 (63.6 %) OE-PCR detections were negative. This is close to the expected value predicted by Equation (4). This indicates that the oE-PCR protocol is able to capture, amplify and detect a single molecule of DNA within 17 minutes.

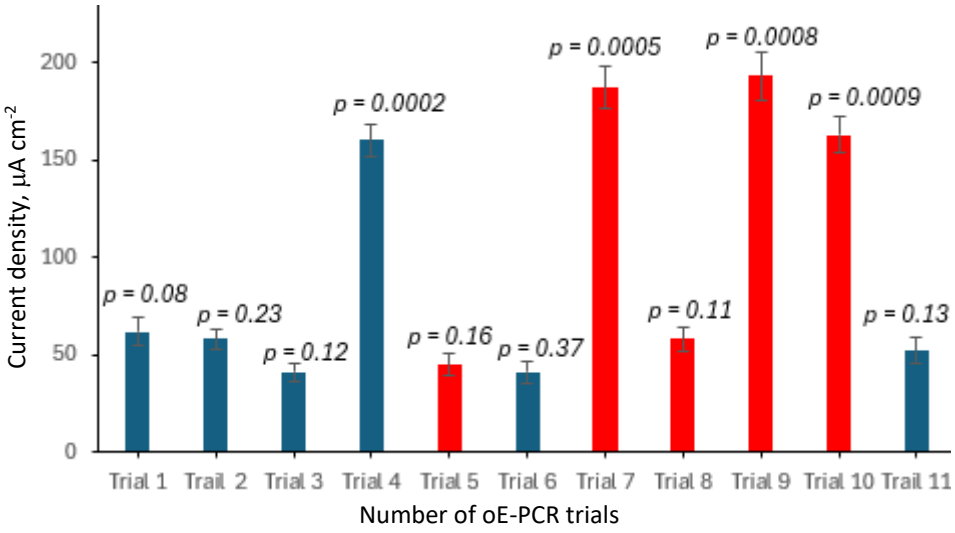


Figure 6. *Results of the oE-PCR technique with 17 zM target DNA (0.5 molecule of ssDNA) after 5 surface-based PCR cycles. Red bars are results from a spike volume of 5.0 µL of 173.3± 8.8 zM stock and blue from 1.7 µL of 500 ± 22.5 zM stock of target salmonella DNA. The* p*-values are in comparison with the blank of Figure 5*

Specificity of PCR and rapid isolation of amplified DNA in a complex matrix

The effects of a complex sample matrix on the oE-PCR process were examined. It is noteworthy that standard PCR in chicken juice was found to be inhibited in a previous study [13]. This agrees with the literature [49-51]. The source of this inhibition can be one or both: deactivation of Taq polymerase or detection of PCR products with fluorescent tags in standard procedures. In addressing the latter, oE-PCR has a distinct advantage: it is a surface-based phenomenon. Fluorescence signals require transmission through the bulk; interference is apparent when particulate suspensions scatter light from this process. The oE-PCR procedure was examined in the chicken juice matrix (Table S4 in the Supplementary material) spiked with 35 zM (single ssDNA molecule) of 48 base targets (Table S2). This is from the Salmonella CP014358.1 GenBank [52]. The forward primer (Table S2) was immobilized onto the GUITAR fibre electrode. The oE-PCR proceeded as described above. Figure 7 shows the SWV signal of the 35 zM target in the presence of chicken juice. The p-values are relative to the blank study in Figure 5. The values indicate the same response regardless of whether chicken juice is present. The chicken juice blank was not statistically significant from the blank in Figure 5 ($p > 0.05$). On the other hand, chicken juice did not interfere with the detection of 35 zM of the target ($p = 0.0003$).

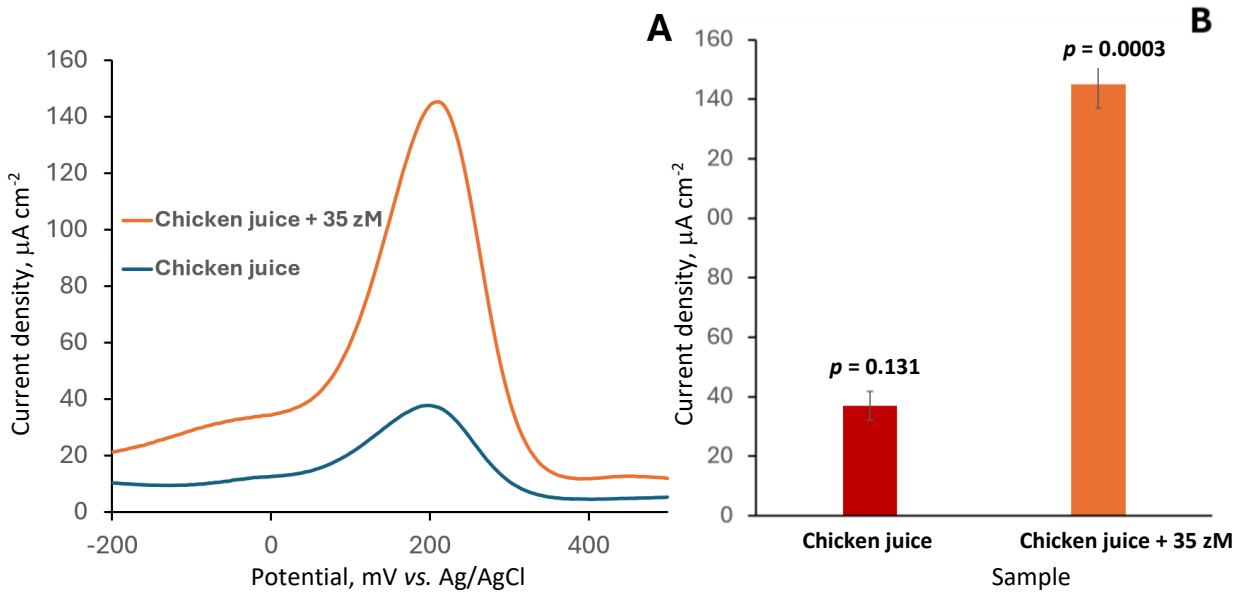


Figure 7. *A) SWV after intercalation with $Co(Phen)_3^{3+}$ response of GUITAR modified GUITAR after 5 cycles of PCR with chicken juice and spiked 35 zM target sample and chicken juice. (B) SWV signal response with p-values in comparison with the blank in Figure 5*

This process is simple, rapid (15 minutes) and does not require the use of expensive instruments to reach a low detection limit. In principle, this technique will be able to rapidly detect the presence of various pathogens such as viruses, food-borne diseases and antibiotic-resistant genes, especially during the early outbreak of diseases and environmental surveillance [53-55]. The LOD, linear range and total processing time compare well with recent efforts [16,17,56-63] (Table 1). The 2.0 aM LOD for the non-PCR method is lower than that of most electrochemical-based detection systems. This is with a large linear range of 9 orders of magnitude. With the addition of the rapid (15 min) surface-based 5 PCR cycles protocol t,he LOD is extended down to 35 zM (n = 7). This may be the lowest LOD for a rapid form of DNA analysis. In terms of total time for sample processing and analysis, this technique requires only 12 to 17 min. It is difficult to make comparisons with literature, as typically this is not discussed in detail. Table 1 offers estimates, but it is clear that the GUITAR electrode systems is very rapid.

Table 1. *Comparison of GUITAR oE-PCR technique with literature*

Material	Method and signal indicator	Total time, min	Linear range	LOD	Ref.
GUITAR Non-PCR	SWV $Co(phen)_3^{3+}$	12	10 aM to 10 nM	2.0 aM	This work
GUITAR-Fiber 5 x PCR		17	2 pM to 5 zM	35 zM	
LAMP	N/A	30	692 fM to 0.07 zM	7 aM	[56]
RPA	N/A	75	17 fM to 1.6 aM	1.6 aM	[57]
One-pot RPA-CRISPR/Cas12a with electrochemical detection	CA	90	0.6 fM to 6 zM	6 aM	[58]
Gold nano-flower NiFe layered double hydroxide glassy carbon electrode	SWV Methylene blue	>10	25 fM to 50 nM	25 fM	[59]
Nanoporous gold electrode	CC $[Ru(NH_3)_6]^{3+}$	>120	80 aM to 1.6 pM	28 aM	[60]
Nanogold electrode	DPV $Co(phen)_3^{2+}$	> 60	50 nM to 150 nM	75 nM	[16]
Hollow gold nanospheres	DPV $Co(phen)_3^{3+}$	> 20	1 pM to 10 nM	1 pM	[17]
Multi-wall carbon nanotube polypyrrole nanowires on gold nanoparticles	DPV $Co(phen)_3^{3+}$	> 60	5 pM to 1 nM	0.43 pM	[61]
Gold electrode	SWV $[Ru(NH_3)_6]^3$	> 60	10 pM to 10 µM	2.9 pM	[62]
Carbon dots	Fluorescence	> 20	36 aM to 532 fM	2 aM	[63]

CC- Chronocoulometry, CA- Chronoamperometry, DPV-Differential pulse voltammetry, SWV- Square wave voltammetry, Fluorescence - inhibited by particles, expensive, more difficult to make portable

Conclusion

We demonstrated the OE-PCR technique to detect *Salmonella* DNA. Our approach is highly sensitive, specific, rapid, and will help speed up the overall PCR process. A low detection limit of 35 zM or one molecule of target *Salmonella* ssDNA was estimated for the oE-PCR technique. This approach offers portability and is cost-effective when compared to DNA assays like real-time quantitative PCR, digital PCR and other DNA amplification techniques that require the use of fluorescence probes and can be applied for rapid DNA diagnostics in a complex sample matrix.

Supplementary material

Additional data are available at https://pub.iapchem.org/ojs/index.php/JESE/article/view/3403, or from the corresponding author on request.

Acknowledgment: *The work presented in this article was conducted independently with partial support from REU-NSF 2348001 for Glen S. Soderquist.*

Funding: *National Science Foundation, Grant number NSF 2348001*

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