



PlcA-based nanofabricated electrochemical DNA biosensor for the detection of *Listeria monocytogenes* in raw milk samples

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Received: 22 April 2020 / Accepted: 23 June 2020
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Abstract

The electrochemical DNA biosensor has been developed for the detection of *Listeria monocytogenes* in raw milk samples. The electrochemical studies of the developed biosensor was recorded by cyclic voltammetry (CV) and electrochemical impedance (EI) using methylene blue (MB) and potassium ferricyanide $K_3Fe(CN)_6$ as redox indicators. The selectivity of the developed biosensor was demonstrated using complementary and mismatch oligonucleotide sequences. The sensitivity (S) of the developed sensor was recorded as 3461 ($\mu A/cm^2$)/ng and limit of detection (LOD) was found to be 82 fg/6 μl with the regression coefficient (R^2) 0.941 using CV. The sensor was characterized by field emission scanning electron microscopy (FE-SEM). The electrode was found to be stable for six months, with only 10% loss in the initial CV current.

Keywords DNA biosensor · *plcA* gene · CNF · AuNP · Foodborne pathogens · *listeria monocytogenes* · Selectivity

Introduction

Nowadays, the consumption of ready to eat (RTE) food is a leading cause of majority of problems related to human health. *Listeria monocytogenes*, a gram-positive, facultative anaerobic bacterium that grows at temperatures between 0.4 and 45 °C is one of the most virulent pathogen found in both unprocessed and processed foods (Valimaa et al. 2015). *Listeria monocytogenes* is the common cause of Zoonosis (Vizzini et al. 2019). The genus *Listeria* includes seven species, out of which only *L. monocytogenes* and *L. ivanovii* are pathogenic, facultative intracellular bacteria which can cross intestinal barrier and can proliferate within macrophages, epithelial, and endothelial cells.

The bacterium contains different virulent factors that disrupt the vacuolar membrane and may cause listeriosis in humans. Phosphatidylinositol-specific phospholipase C gene (*PlcA*) of *L. monocytogenes* is a virulent gene and encodes a 33-kDa protein responsible for the lysis of primary

single-membraned vacuoles (Vazquez et al. 2001). *L. monocytogenes* causes listeria infection especially in pregnant women, newborn babies, and immunocompromised patients (Soni et al. 2014). This pathogen is responsible for mild fever to gastroenteritis infection, meningitis, and septicemia. The infection rate of this pathogen is high in neonates and in people above 65 years of age, and regarded as one of the factor for abortions and still birth in India (Jamshidi and Zeinali 2019). The reported rate of hospitalization with infection by listeriosis is found to be 94%, with a fatality rate of 12–17% (Zhao et al. 2017), and mortality rate of 20–30% (Soni et al. 2018).

In past decade, various techniques have been used for the detection of *L. monocytogenes* and include bacterial culturing methods, sugar fermentation, haemolytic properties, immunological methods such as enzyme-linked immunosorbent assay (ELISA), enzyme-linked fluorescent assay (ELFA), (Jaakohuhta et al. 2007) and molecular methods viz, polymerase chain reaction (PCR), and microarray study (Ingiani et al. 2001; Palumbo et al. 2003; Volokhov et al. 2002; Sergeev et al. 2004; Chemburu et al. 2005). The colony morphology, sugar fermentation, and haemolytic properties are considered as a gold standard methods for identification of *L. monocytogenes*. All the methods mentioned above have some limitations of being expensive and less sensitive, and some of these methods also lead to false-positive results (Valimaa et al. 2015). Culture-based

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methods, however, are simple, but time-consuming (require up to 5–7 days), labor-intensive, and may not be appropriate to test foods with short shelf life (Jadhav et al. 2012; Datta et al. 2013). To overcome all above problems, there is a need for the development of rapid and sensitive method of detection. In present study, an electrochemical DNA biosensor with high sensitivity and specificity was developed for the detection of *L. monocytogenes* with short time of assay. Also, this is the first *plcA*-based electrochemical DNA biosensor developed for the detection of *L. monocytogenes* in raw milk samples using CNF/AuNP-based electrode.

Materials and methods

Strains and chemicals

The bacterial strains of *L. monocytogenes* (MTCC 657), *Salmonella enterica* (MTCC 9844), *Klebsiella Pneumoniae* (MTCC 39), and *Escherichia coli* (MTCC 50) were procured from MTCC, Institute of Microbial Technology, Chandigarh, India. Chloroform, EDTA, isoamyl alcohol, phenol, sodium chloride, sodium di-hydrogen orthophosphate and di-sodium hydrogen orthophosphate, nitric acid (HNO_3), and brain heart infusion broth were purchased from Hi-media, India. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-Hydroxysuccinimide (NHS), methylene blue (MB), potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), *R*nase, and Proteinase K were purchased from Sigma-Aldrich, USA. All other chemicals used were of analytical grade. Screen-printed CNF/AuNP electrode was procured from DropSens, Spain, and modified in the lab.

Oligonucleotides

5'-NH₂ modified *plcA* gene-specific single-stranded DNA (ssDNA) probe and all other oligonucleotides related to *L. monocytogenes* and negative control were designed and procured from Eurofins, Bengaluru, India. The stock solution of 100 pmol μl^{-1} of probe and oligonucleotides were prepared with autoclaved distilled water and stored at -20°C . The specificity of the *plcA* gene probe for the DNA sensing was

verified by using BLAST. The sequence of oligonucleotides used in this study is shown in Table 1.

Apparatus

Cyclic voltammetry (CV) and electrochemical impedance (EI) studies were carried out using the potentiostat, (Palm-sens 4 Netherland) and data analysis system PStace 5 software. The CNF/AuNP electrode consists of a three-electrode system having working electrode (WE) and counter-electrode (CE), made of carbon, and the reference electrode (RE), made of silver. The DNA was quantified using nanodrop spectrophotometer (QIAXpert System, Netherland). Electrode surface characterization studies were carried out using field emission scanning electron microscopy (FE-SEM), Hitachi-SU8010 microscope.

Isolation of genomic DNA from culture

The target bacterium *L. monocytogenes* was grown in brain heart infusion broth culture for 24 h at 37°C and the genomic DNA (G-DNA) was isolated using phenol–chloroform method (Kaushal et al. 2012). The purity of isolated G-DNA (A_{260}/A_{280}) and quantity (A_{260}) was measured using nanodrop spectrophotometer.

Construction of the sensor

The screen-printed CNF/AuNP electrode was first cleaned with Milli Q water for 30 s and air-dried at room temperature. The working area of the electrode was further washed with 1 mM of HNO_3 solution (6 μl) for 10 min, followed by washing with PBS (pH 7.4) to remove the unbound reagents. Finally, the screen-printed electrode was dried at room temperature. The working area of the electrode was further treated with a mixture of 10 mM each of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide EDC:*N*-Hydroxysuccinimide NHS (1:1, v/v in PBS, pH 7.4) for 1.5 h to activate the carboxyl groups on the surface (Rahman et al. 2009). The electrode was washed with PBS (pH 7.4) and dried at room temperature. The 5'-NH₂-labeled probe (6 μl) was added onto the working surface of the electrode followed by overnight incubation. This allowed the formation

Table 1 Oligonucleotide sequences used in the present work

Oligonucleotides	Sequences
Probe (20 bases)	5'- NH ₂ -TTACTTGGTTAGGTGCGCCG-3'
Negative control (20 bases)	5'- NH ₂ -TTACTTGGTTAGGTGCGCCG-3'
Complementary sequence (20 bases)	5'- CGGCGCACCTAACCAAGTAA-3'
Complementary sequence (35 bases)	5'-TAGCCGACGGCGCACCTAACCAAGTAAGCATCGAA-3'
Single mismatch (20 bases)	5'- CGGCGCACCTTACCAAGTAA-3'

of an amide bond between the NH_2 group of the probe and COOH group of the C-nanofibres through EDC-NHS chemistry (Rahman et al. 2009). The electrode was then washed with TE buffer (10 ml of 1 M Tris-HCl buffer and 2 ml of 0.5 M EDTA and distilled water added to make final volume upto 1000 ml and pH adjusted at 8) to remove the unbound probe and air-dried at room temperature before further electrochemical analysis. The schematic illustration of the fabrication of the sensor is shown in Scheme 1.

Hybridization with ssDNA

Different concentrations of isolated genomic DNA of *L. monocytogenes* were prepared and denatured at 95 °C for 5 min. The single-stranded DNA probe (ssDNA) was hybridized on working electrode surface using different concentrations of single-stranded genomic DNA (ssG-DNA). After hybridization, the electrode surface was washed with TE buffer (pH 8). Further electrochemical analysis of electrode with CV and EI was done by potentiostat using 1 mM MB and 2.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ that works as an artificial redox indicator (Zhu et al. 2008; Nasef et al. 2010). Electrode

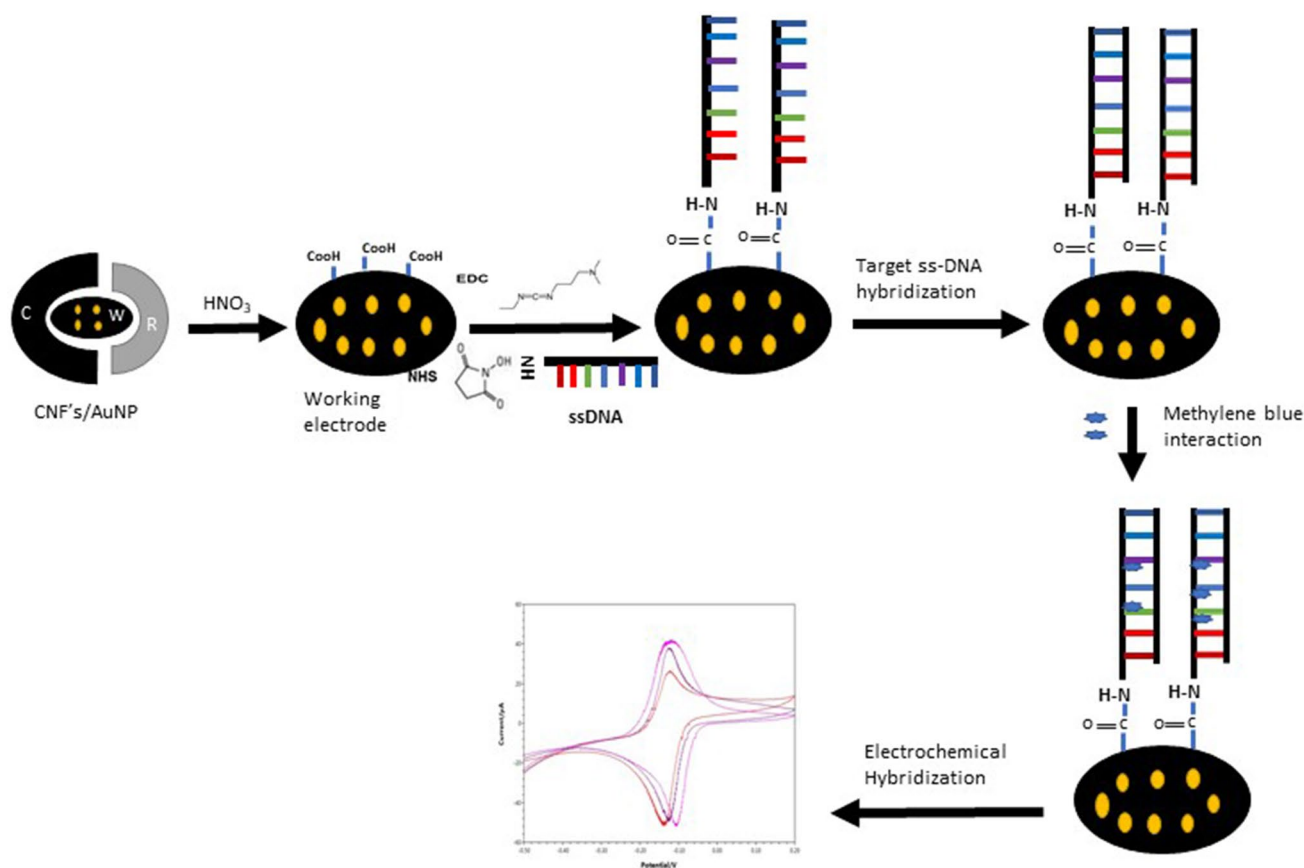
surface was regenerated after every measurement using PBS buffer (pH 7.4) that removes the hybridized DNA strands from the electrode surface except immobilized probe and TE (pH 8) was used to provide favorable electrochemical conditions.

Surface characterization of DNA biosensor

Surface morphology characterization of CNF/AuNP electrodes at different stages of fabrication viz. CNF/AuNP-based electrode, CNF/AuNP immobilized with NH_2 -labeled probe ssDNA, and CNF/AuNP/dsDNA after hybridization with 50 ng/6 μl ssG-DNA of *L. monocytogenes* were carried out using field emission scanning electron microscopy (FE-SEM).

Specificity, selectivity, and stability of DNA biosensor

The specificity of the DNA biosensor was studied by hybridization of ssG-DNA of *L. monocytogenes* and other selected foodborne pathogens (*E. coli*, *K. pneumoniae*, and



Scheme 1 Fabrication of electrochemical sensor using immobilization of 5'- NH_2 -labeled ssDNA probe of *plcA* gene on CNF/AuNP and hybridization with ssG-DNA of *L. monocytogenes*. EDC:NHS chemistry was used in fabrication of biosensor

S. enterica) with ssDNA probe specific to *plcA* gene of *L. monocytogenes*. The relative peak current (I_p) in each case was calculated with respect to the immobilized probe.

The selectivity of the sensor was evaluated using different oligonucleotides mentioned in Table 1. The hybridization efficiency of the negative control, complementary, and mismatch oligonucleotides with respect to the immobilized probe DNA was investigated using CV analysis. The stability of the developed sensor on storage at 4 °C was studied using CV analysis at regular interval of 30 days each for upto 6 months.

Validation of DNA biosensor

The validation study of developed biosensor was done using 20 raw milk samples and 3 milk samples artificially spiked with *L. monocytogenes*. The peak current (I_p) values of CV after hybridization with ssG-DNA of raw milk samples were compared with the probe, standard positive (samples artificially spiked with *L. monocytogenes*), and negative control (milk sample without *L. monocytogenes*), respectively.

Results and discussion

In this study, an electrochemical biosensor based on CNF/AuNP electrode was developed to detect *L. monocytogenes* in milk samples with high specificity and sensitivity. A 5'-NH₂-labeled single-stranded probe DNA (ssDNA) specific to *plcA* gene was immobilized on the working surface of nanofabricated electrode. The complementary ssG-DNA of *L. monocytogenes* was hybridized with immobilized probe and was detected using CV and EIS. The sensitivity and limit of detection (LOD) of the sensors was improved by using nanomaterials, i.e., carbon nanofibers fabricated with gold nanoparticles on screen-printed electrode. These CNF/AuNP screen-printed electrode have a high surface area-to-volume ratio and excellent dimensional stability with high thermal and high electrical conductivity (Zhu et al. 2013, 2015). The results of this study are discussed as follows:

Cyclic voltammetry (CV) studies of CNF/AuNP-based biosensor

Cyclic voltammetry studies were performed to analyze the immobilization of 5'-NH₂-labeled ssDNA probe and hybridization of different concentrations of the ssG-DNA on CNF/AuNP electrode as shown in Fig. 1. The CV peak current of ssDNA probe was found higher, i.e., 114 μ A (curve a) as compared to bare electrode (not shown), and on further increase in the concentration of DNA, an increase in CV peak current was observed. The peak current for CV after hybridization with different DNA concentration was

found as 177 μ A (curve b), 209 μ A (curve c), 248 μ A (curve d), 297 μ A (curve e), 330 μ A (curve f), 362 μ A (curve g), 401 μ A (curve h), 427 μ A (curve i), and 445 μ A (curve j), respectively. This increase in electrode peak current was attributed to the presence of more unhybridized nitrogenous base pairs that lead to oxidation of methylene blue molecules at electrode surface. The plot between the relative I_p of probe and the ssG-DNA was found to be hyperbolic (Fig. 1 inset [A]) with a regression coefficient (R^2) of 0.941 (Fig. 1 inset [B]). The sensitivity (S) of DNA sensor was calculated using the formula $S = m/A$ and was found to be 3461 (μ A/ $\text{cm}^2/\text{ng}^{-1}$) where m is the slope of the linear equation and A is the area of the working electrode (0.126 cm^2). The LOD of the DNA sensor was calculated using the formula $\text{LOD} = 3 (\sigma/S)$, where σ is the standard deviation and S is the sensitivity, and was found to be 82 fg/6 μ l (Fig. 1 inset [B]).

The electrochemical impedance (EIS) of CNF/AuNP-based biosensor

The Nyquist plot for electrochemical impedance studies of the immobilized probe and the hybridization of different concentration (6.25, 12.5, 25, 50, and 100 ng/6 μ l) of ssG-DNA using 1 mM K₃Fe(CN)₆[−] is shown in Fig. 2. In the Nyquist plots, the diameter of the semicircle reflected the charge transfer resistance (R_{ct}) value (Fan et al. 2019). The R_{ct} value of immobilized probe was found higher ($0.9 \times 10^3 \Omega$, curve a) than the bare electrode (not shown in the figure). This may be attributed to the negatively charged phosphate backbone of ssDNA, which prevented the K₃Fe(CN)₆[−] ions from reaching the electrode surface. The R_{ct} values for different concentrations of ssG-DNA after hybridization with the immobilized probe were found to be $1.1 \times 10^3 \Omega$ (curve b), $1.3 \times 10^3 \Omega$ (curve c), $1.6 \times 10^3 \Omega$ (curve d), $1.8 \times 10^3 \Omega$ (curve e), and $2.0 \times 10^3 \Omega$ (curve f), respectively. This may be due to the increase in the number of negatively charged phosphate groups after hybridization on the electrode surface that causes an increase in surface thickness which in turn resulted in an increase in the R_{ct} value (Park and Park 2009). Total bacterial detection time of developed sensor was found to be 30 min that includes DNA processing, immobilization, and hybridization with immobilized probe.

FE-SEM analysis of CNF/AuNP-based biosensor

In this study, morphological changes occurring on the surface of developed sensor at different stages of fabrication were recorded. Figure 3a shows CNF/AuNP bare electrode with a smooth and uniform fibrous structure that confirms the carbon nanofibers on the electrode surface. In Fig. 3b, CNF/AuNP immobilized with NH₂-labeled probe showed a change in the surface morphology. In Fig. 3c, CNF/AuNP

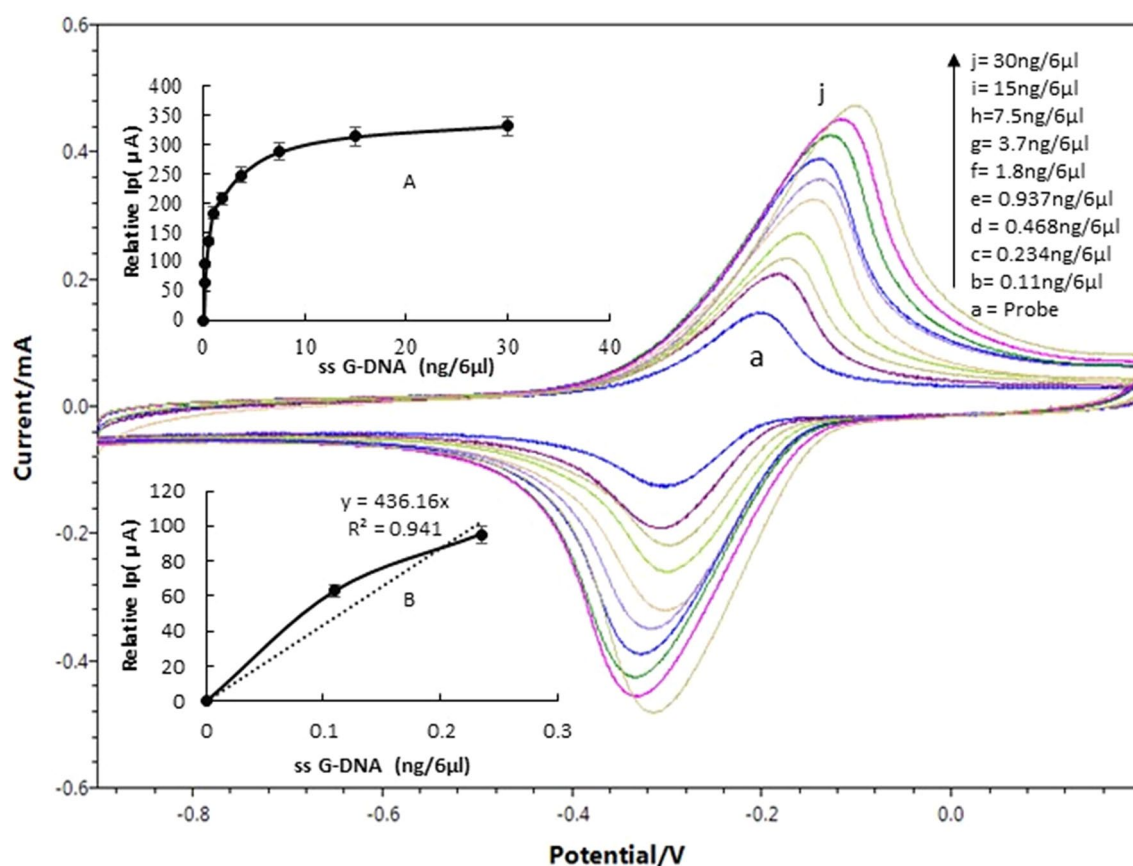


Fig. 1 CV study of developed CNF/AuNp electrochemical biosensor at different stages of fabrication (a) ssDNA-NH₂/CNF/AuNp electrode and (b–j) hybridization with 0.11–30 ng/6 μl of *L. monocytogenes* ssG-DNA at 50 mVs⁻¹ using 1 mM MB in 50 mM PBS,

pH 7.4. The inset-A shows hyperbolic curve from 0 to 30 ng/6 μl with linear peak current (Ip) up to 0.234 ng/6 μl of ssG-DNA of *L. monocytogenes*. Inset-B shows the linear plot from 0–0.234 ng/6 μl ssG-DNA for the calculation of sensitivity and LOD

hybridized with 50 ng/6 μl ssG-DNA of *L. monocytogenes* recorded a further dense surface morphology, and globular surface which confirms the binding of DNA onto the probe.

Specificity, selectivity, and the stability of the developed sensor

The specificity of the developed DNA biosensor was observed using CV. After CV analysis, the peak current of all the foodborne pathogens was found almost similar to the immobilized probe except with *L. monocytogenes* (Fig. 4) where it was found higher even at the lower concentration of genomic DNA (10 ng/6 μl). Based on these observations, the sensor is found to be highly specific for the *plcA* gene of *L. monocytogenes*.

The selectivity studies of the developed sensor were confirmed by CV analysis. The oxidation peak current of MB with ssDNA (probe) on the CNF/AuNP electrode (curve a) was found lowest as shown in Fig. 5. After hybridization with negative control, the peak current was almost equal to probe peak current (curve b). On subsequent hybridization

with single base-pair mismatched ssDNA sequence (curve c), 35 mer complementary ssDNA sequence (curve d), and 20 mer complementary ssDNA sequence (curve e), a gradual increase in the peak current was recorded. This indicated the hybridization of ssDNA with complementary base-pair confirming the selectivity of the electrode surface (Sun et al. 2010). The statistical analysis was performed at 5% ($p=0.05$) level of significance.

In the present study, the developed sensor was found stable on storage at 4 °C for up to six months with about 10% loss in CV current (Fig. 6).

Validation of developed sensor with raw milk samples

The sensor was validated using 20 raw milk samples and 3 milk samples artificially spiked with *L. monocytogenes* and the results were recorded using CV analysis (Fig. 7). The change in relative peak current with respect to probe showed by negative control (milk sample without *L. monocytogenes*) and positive control (milk sample with *L. monocytogenes*)

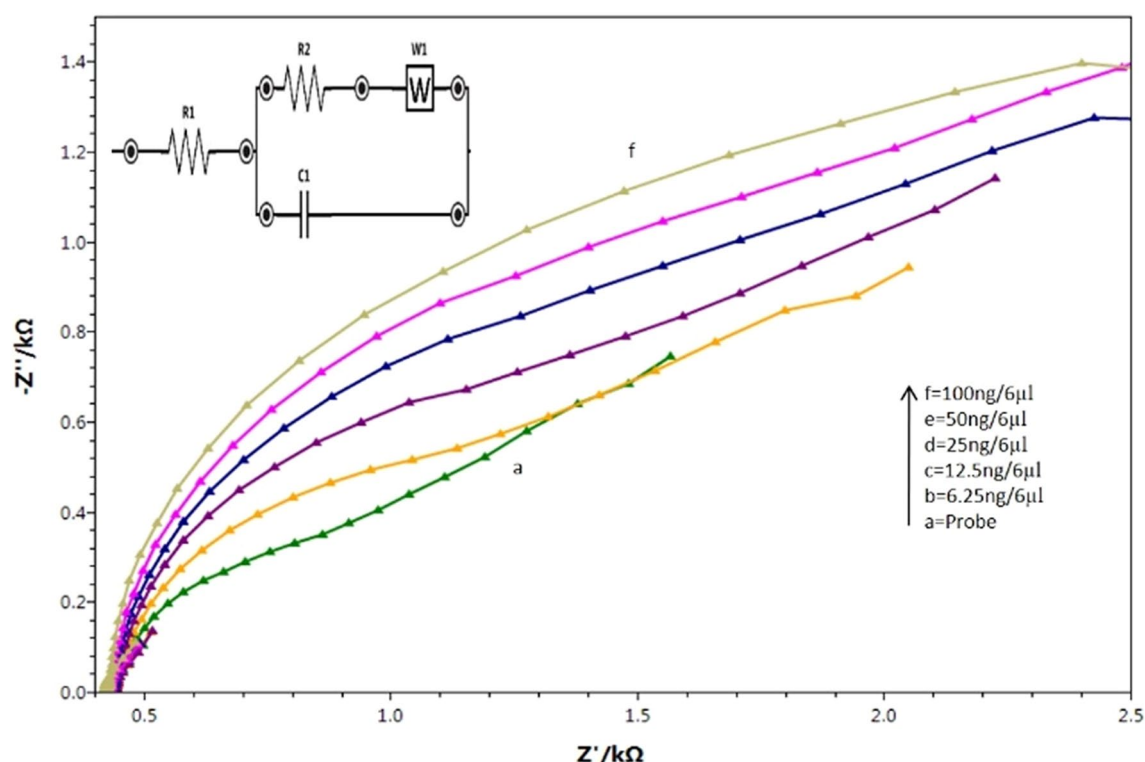


Fig. 2 Impedance-based study of developed CNF/AuNp electrochemical biosensor at different stages of fabrication (a) ssDNA-NH₂/CNF/AuNp and (b–f) hybridization with 6.25, 12.5, 25, 50, and

100 ng/6 μl of *L. monocytogenes* ssG-DNA using redox indicator 1 mM K₃Fe(CN)₆ in 50 mM PBS, pH 7.4

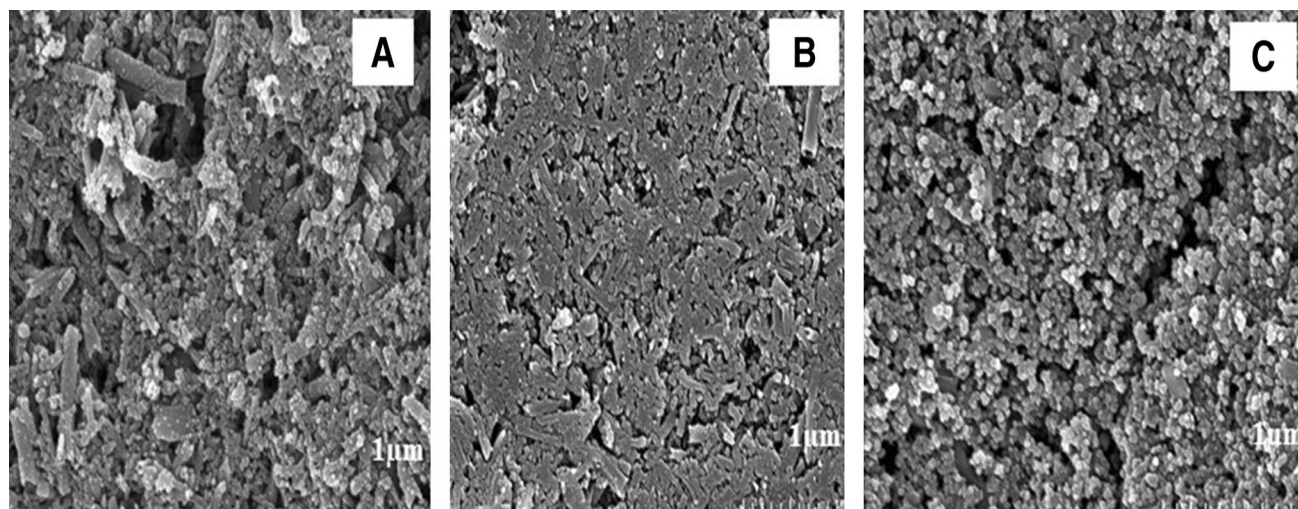


Fig. 3 FE-SEM micrograph of developed CNF/AuNp electrode biosensor. (a) bare electrode, (b) CNF/AuNp electrode/ssDNA probe, and (c) CNF/AuNp electrode/dsDNA after hybridization with 50 ng/6 μl ssG-DNA of *L. monocytogenes*

served as a threshold for milk samples. In Fig. 7, the y-axis denotes the relative peak current of CV with respect to the probe (as control). The negative control (without ssG-DNA in the sample to hybridize with probe) showed no increase in peak current, whereas the positive control (ssG-DNA

of *L. monocytogenes* was hybridized with probe) showed an increase in the peak current with respect to the control (unhybridized probe). The ssG-DNA of the milk sample containing *L. monocytogenes* was hybridized with probe, and the relative peak current was monitored using CV. The

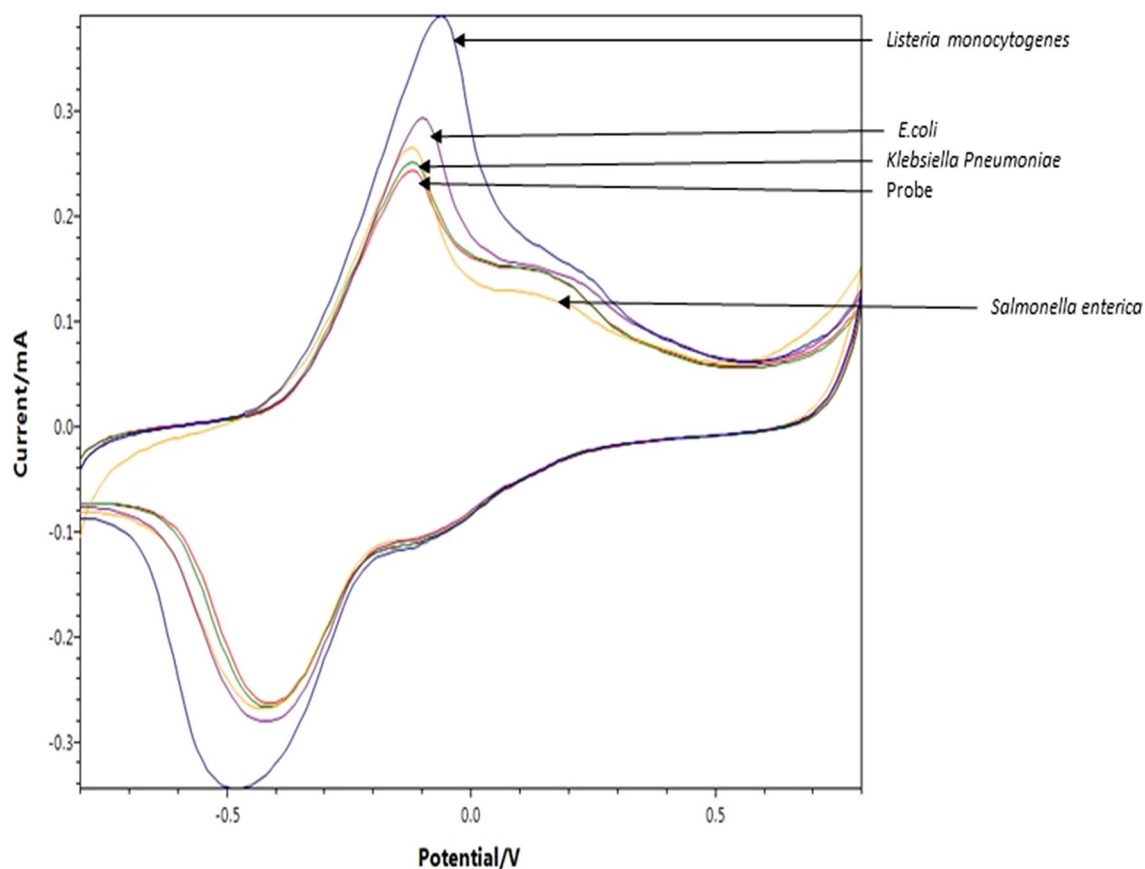
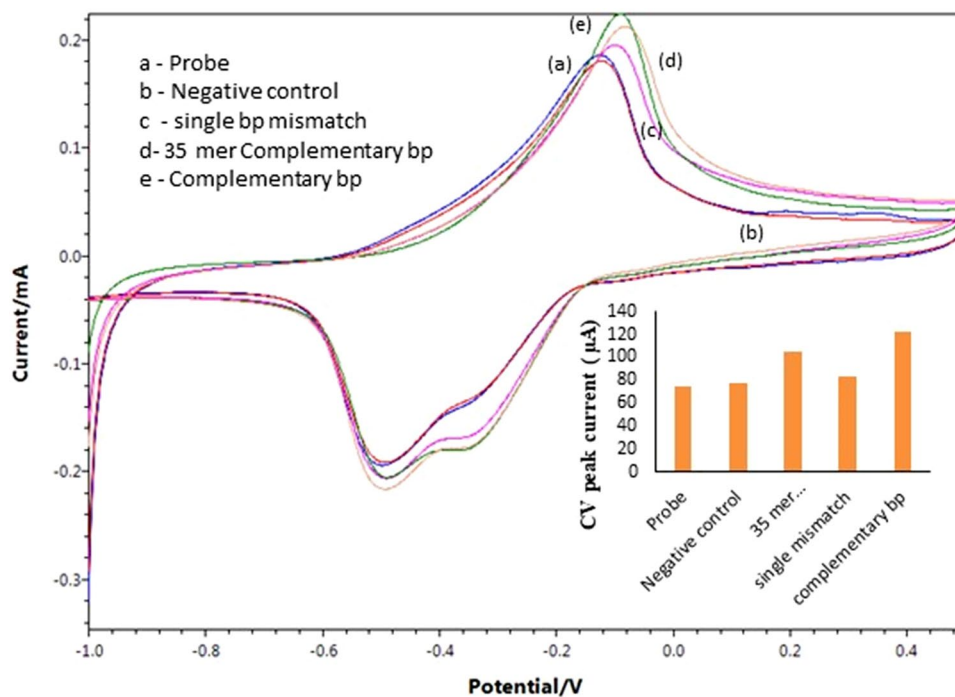


Fig. 4 Specificity study of DNA biosensor based on CNF/AuNp electrode for *L. monocytogenes* and other foodborne pathogens. The relative I_p value of CV with respect to immobilized probe as zero after hybridization with 50 ng/6 μ l ssG-DNA of other pathogens and *L. monocytogenes*

Fig. 5 Selectivity study of developed CNF/AuNp-based electrochemical biosensor using (a) ssDNA probe, (b) negative control, (c) 20 mer single bp mismatch, (d) 35 mer complementary bp, (e) 20 mer complementary bp. The concentration used for the hybridized oligonucleotides was 10 μ M



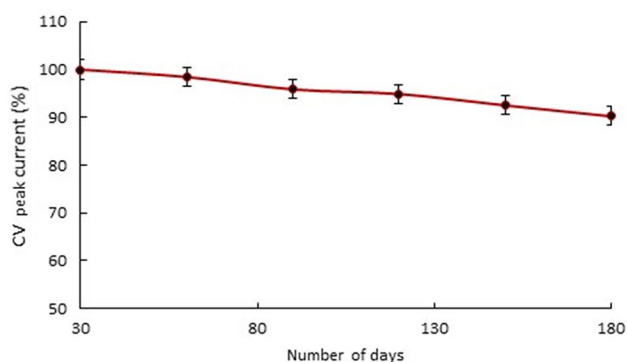


Fig. 6 Stability of the developed CNF/AuNP sensor measured as % relative peak current with respect to probe as zero using CV for regular interval of 30 days up to 6 months of storage at 4 °C

positive and negative sample peak current were plotted against above-mentioned controls. Negative controls without *L. monocytogenes* (without complementary ssG-DNA) and with other pathogens (non-complementary DNA or even no DNA) showed no significant increase in the peak current, whereas in positive samples (under similar ssG-DNA concentration), the peak current showed 10% variation in the average peak current.

Comparative study of developed sensor with other reported sensors for detection of *L. monocytogenes*

The comparison of developed biosensor for the detection of *L. monocytogenes* causing food poisoning with other reported sensors is shown in Table 2. A similar work was carried out by Nagraik et al. (2019) in which an amperometric DNA-based biosensor was developed for the early detection of *Leptospira interrogans* by using MWCNT

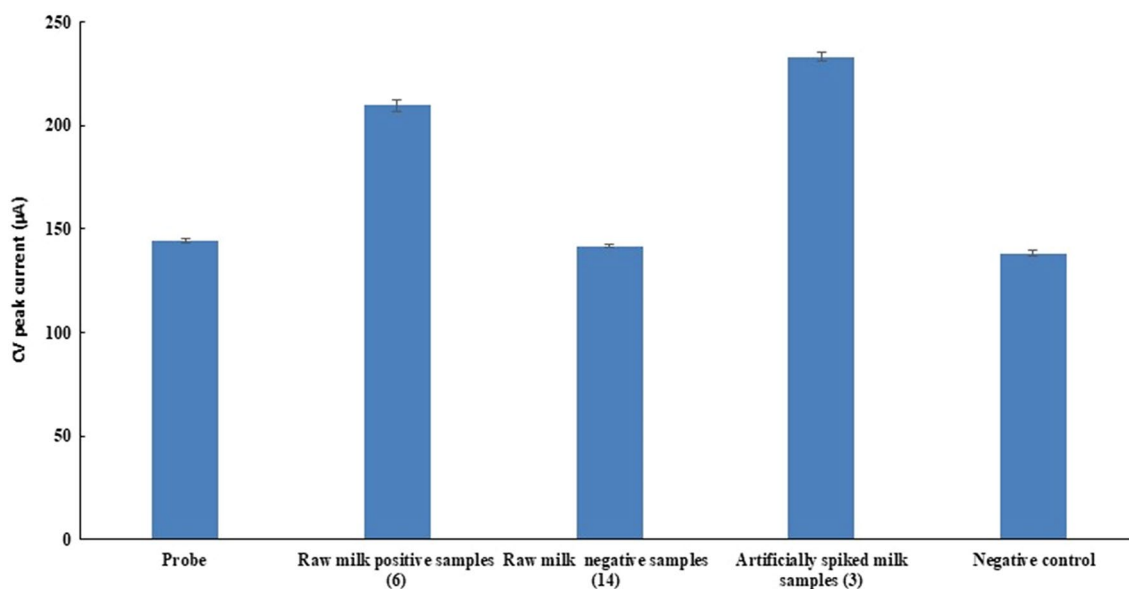


Fig. 7 Validation studies of developed CNF/AuNP biosensor for the detection of *L. monocytogenes* by measuring the relative I_p value of CV after hybridization with ssG-DNA from raw milk samples. Twenty raw milk samples tested with developed DNA sensor using

negative control (milk sample without *L. monocytogenes*) and positive control (milk sample with ssG-DNA of *L. monocytogenes*) with respect to probe (control) for validation of the sensor (significance level = 5%)

Table 2 Comparison of developed CNF/AuNP-based DNA sensor with some reported biosensors for the detection of *L. monocytogenes* in food samples

Detection method	Transducer	Analyte used as marker	Target gene	Indicator used	Limit of detection (LOD)	References
Electrochemical Biosensor	CILE	DNA	<i>hly</i> gene	MB	3.17×10^{-14} mol/L	Niu et al. 2017
Electrochemical Biosensor	Au/3DGR/CILE	DNA	<i>hly</i> gene	MB	3.3×10^{-15} mol L ⁻¹	Yan et al. 2017
Electrochemical Biosensor	CNF/AuNP	DNA	<i>plcA</i> gene	MB	82 fg/6 µL	Present study

SPGE screen-printed gold electrode, Au gold, 3DGR 3-dimensional graphene, CILE carbon ionic liquid electrode, MB methylene blue, ND not determined

electrode. In this study the electrochemical studies before and after hybridization were done using CV and DPV in the presence of methylene blue as a redox indicator. The biosensor showed sensitivity of $264.5 (\mu\text{A}/\text{cm}^{-2}/\text{ng}^{-1})$ and LOD of $0.015 \text{ ng}/6 \mu\text{l}$ using CV. Due to higher sensitivity, selectivity, and lower LOD, the nanofiber-based sensor is found better than earlier-reported methods. The sensitivity of the reported sensor was found to be $3164 (\mu\text{A}/\text{cm}^{-2}/\text{ng}^{-1})$ and the lower LOD was $82 \text{ fg}/6 \mu\text{l}$ at 0.126 cm^2 surface area of the working electrode.

Conclusions

The results demonstrated that the DNA biosensor could be effectively detect *plcA* gene of *L. monocytogenes*. The biosensor is highly specific, rapid, has low cost, and could efficiently detect *L. monocytogenes* specifically in raw milk. The sensitivity of biosensor was found to be $3164 (\mu\text{A}/\text{cm}^{-2}/\text{ng}^{-1})$ and the lower LOD of $82 \text{ fg}/6 \mu\text{l}$ at 0.126 cm^2 surface area of the working electrode. This is the first-reported electrochemical biosensor based on CNF/AuNP used for the detection of *plcA* gene of *L. monocytogenes* within 30 min. The present biosensor offers lower LOD as compared to the reported biosensors for the detection of *L. monocytogenes* (Table 2).

Acknowledgements The authors are thankful to Shoolini University, Solan, Himachal Pradesh, for providing the facility to carry out the present research work.

Compliances with ethical standards

Conflict of interest There is no conflict of interest for authorship or related to any other context between authors.

Ethical standards The authors have complied and worked within standard ethical norms.

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