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ABSTRACT

*Listeria monocytogenes* is one of the most common causes of food illness deaths worldwide, with multiple outbreaks in the United States alone. Current methods to detect foodborne pathogens are laborious and can take several hours to days to produce results. Thus, faster techniques are needed to detect bacteria within the same reliability level as traditional techniques. This study reports on a rapid, accurate, and sensitive aptamer biosensor device for *Listeria* spp. detection based on platinum interdigitated array microelectrodes (Pt-IDEs). Pt-IDEs with different geometric electrode gaps were fabricated by lithographic techniques and characterized by cyclic voltammetric (CV), electrochemical impedance spectroscopy (EIS), and potential amperometry (DCPA) measurements of reversible redox species. Based on these results, 50 \(\mu\)m Pt-IDE was chosen to further functionalize with a *Listeria monocytogenes* DNA aptamer selective to the cell surface protein internalin A, via metal-thiol self-assembly at the 5' end of the 47-mer's. EIS analysis was used to detect *Listeria* spp. without the need for label amplification and pre-concentration steps. The optimized aptamer concentration of 800 nM was selected to capture the bacteria through internalin A binding and the aptamer hairpin structure near the 3' end. The aptasensor was capable of detecting a wide range of bacteria concentration from 10 to 10\(^6\) CFU/mL at lower detection limit of 5.39 ± 0.21 CFU/mL with sensitivity of 268.1 ± 25.40 (Ohms/log [CFU/mL]) in 17 min. The aptamer based biosensor offers a portable, rapid and sensitive alternative for food safety applications with one of the lowest detection limits reported to date.

Keywords: Aptasensor, lab-on-a-chip, pathogen detection, food safety, impedimetric sensor

1. INTRODUCTION

Recent foodborne outbreaks have heightened public concern about food safety and created a greater impetus to improve methods for pathogen detection. In recent years, many studies on rapid-screening methods for food safety have been focused on development of biosensor platforms for reliable and faster results than conventional techniques such as aerobic plate counting (APC), enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) \(^1\). A number of devices have recently been developed for monitoring foodborne pathogens based on immobilization of capture agents, i.e., the bio-recognition element component of a biosensor, such as aptamers on sensor platform \(^2,3\). Aptamers are synthetic oligonucleotides, either DNA or RNA, that have an ability to bind specifically to target molecule or bacteria such as cell surface proteins, extracellular biomolecules, or viruses \(^4,5\). Due to aptamers inherent advantages of simple production, easy storage, good reproducibility, target versatility, easy modification, and convenient regeneration, they are considered to be ideal recognition elements for biosensor applications \(^6\). Recent efforts have expanded the library of aptamers for common foodborne pathogens; therefore, aptamers are being used to construct biosensors called aptasensors.

Aptasensor has been designed to detect various pathogens including an aptamer-based sensor, which was developed utilizing impedimetric detection of *Salmonella Typhimurium* via aptamers self-assembly onto a gold nanoparticle-modified screen-printed carbon electrode \(^6\). Another aptamer-based biosensor was developed using gold nanoparticles for detection of *Escherichia coli* O157:H7 based on colorimetric detection \(^8\). An antibody-aptamer functionalized fiber-optic biosensor for detection of *Listeria monocytogenes* in food was designed with detection limit of 10\(^3\) CFU/mL in...
pure solution and 10^2 CFU/25 g of food sample after 18 h-enrichment. A gold electrode aptamer-based sensor was designed for *E. coli* O111 detection based on a target-induced aptamers displacement strategy with detection limit of 1.1 x 10^2 CFU/mL in phosphate buffer saline and 3.1 x 10^2 CFU/mL in milk. However, to date, few, rapid, sensitive aptasensors for *Listeria* detection have been demonstrated in field conditions for monitoring food safety.

Electrochemical impedance spectroscopy (EIS) technique has been employed for the detection of foodborne pathogens over other categories such as optical based biosensors that require light (e.g., surface Plasmon resonance or fluorescence) and piezoelectric biosensors that use mechanical motion (e.g., quartz crystal microbalance or resonant cantilever) due to their low cost, low power, ease of miniaturization, portability, simplicity, faster, response, and label-free detection capabilities. EIS is used to monitor the impedance change at the electrode-solution interface when the target analyte interacts with the functionalized surface of the electrodes designed to capture it. Impedance changes occur due to change in electrical properties at the electrode surface solely due to the presence of the target analyte.

Impedance based biosensors do not require special reagents and are preferred for label-free detection of target bacteria. Miniaturized impedance biosensors based on lab-on-a-chip systems utilize microfabrication techniques to fabricate microelectrode arrays. Microelectrodes have been used for various sensing devices due to small ohmic potential drop, fast establishment of steady-state signals, faster response time, and increased signal-to-noise ratio. Specifically, interdigitated array microelectrode (IDEs) can be fabricated using lithography techniques allowing controlled dimensions of the microelectrodes to study electrochemical behavior.

In this study, aptamers, as the biorecognition element, were used to functionalize platinum interdigitated array microelectrodes (Pt-IDEs) for selective binding of Internalin A in the cell membrane of the target bacteria, *Listeria* spp. Internalin A (In1A), a surface protein, is one of the major invasion proteins involved in pathogenesis that represent a complex family of leucine-rich-repeat-containing protein that interacts with E-cadherin leading to bacteria growth in the host cells. In1A is found in all *L. monocytogenes* strains and serves as a molecular marker for the detection of the pathogenic bacteria. Aptamer specific for In1A was used in this study to detect *Listeria innocua*. *Listeria innocua* is a non-pathogenic microorganism, which has been shown to be an excellent surrogate for *Listeria monocytogenes*. Herein, the optimization of various aptamer concentrations loading on the surface of Pt-IDEs was determined based on the saturation point derived from the electrochemical impedance analysis. Optimized aptamer concentration was used at saturation point to cover the maximum surface area of Pt-IDEs to maximize the capture of the bacteria cells and consequently biosensor performance. Furthermore, optimized aptamer concentration was used to evaluate the response of miniaturized aptasensor to detect *Listeria innocua* in phosphate buffered solution. Aptasensor performance parameters were measured and compared to current published biosensors.

### 2. EXPERIMENTAL

#### 2.1 Materials and Reagents

*Listeria monocytogenes* thiol SS-C6 aptamers that target protein Internalin A (A8, 5′-ATC CAT GGG GCG GAG ATG AGG GGG AGG ACC CGG TTG AT-3′, 47 mers) were purchased from GeneLink (Hawthrone, NY). Ethylenediaminetetraacetic acid (EDTA), disodium salt, dihydrate was purchased from EMD Performance Materials (Sommerville, NJ). Potassium chloride (KCl) and sodium chloride were purchased from EM Science (Hatfield, PA). TRIS (Hydroxymethyl)ammonium, potassium phosphate dibasic (K_HPO_4), and sodium phosphate monobasic monohydrate (Na_H2PO_4) were obtained from J.T.Baker Chemical (Phillipsburg, NJ). Sodium phosphate monobasic monohydrate (Na_H2PO_4.H2O) and DL-Dithiothreitol were purchased from Sigma Aldrich (St. Louis, MO). Potassium ferrocyanide trihydrate (K₄Fe(CN)₆.3H₂O) was purchased from Ward’s Science (Rochester, NY). Sulfuric acid and hydrogen peroxide were purchased from Avantor Performance Materials (Center Valley, PA). Sodium acetate (NaC₂H₃O₂.3H₂O) was purchased from MCB Reagents (Cincinnati, OH).

#### 2.2 Pt-IDEs fabrication

The fabrication process consisted of several steps of photolithography processing including dual layer lift-off and electrodeposition requiring one layer mask step on a flat silicon oxide wafer. Mylar mask was used to delineate interdigitated array microelectrodes and bonding pads. Ti was deposited as a sacrificial layer to achieve adhesion of platinum layer with the thickness of 15 nm of Ti and 100 nm of Pt. Each interdigitated array microelectrode was 25 by 15 μm with electrode gaps of 50 μm, a total active area of 0.81 cm² and bonding pads 200 x 200 μm. Electrical wires that connected the microchips to the potentiostat were attached with the conductive silver epoxy to the bonding pads followed by curing at 65 °C for one hour in the oven.
2.3 Aptamer functionalization onto Pt-IDEs

Thiol labeled aptamers for *L. monocytogenes* consisted of 47-mer and it targets the internalin A protein; the 5' end was modified with a terminal thiol group. Disulfide modified *Listeria* thiol aptamers were reduced using the dithiothreitol (DTT) reduction protocol provided by the manufacturer. The aptamers were attached to the Pt-IDEs surface using the sulfur atoms of the thiol group forming a self-assembled monolayers (SAM) via covalent adsorption between thiol-platinum. The Pt-IDEs were first cleaned with piranha solution with the ratio of 3:1 concentrated sulfuric acid to hydrogen peroxide for one minute, thoroughly rinsed with DI water, and air dried. A stock solution of 100 μM *Listeria monocytogenes* aptamers was further diluted to various concentrations of 100, 150, 200, 300, 400, and 800 nM in 10 mM Tris, 1 mM EDTA, pH 7.5 buffer. Then, 65 μL of each concentration was used to functionalize the biosensor by drop coating and air drying for two hours inside a biosafety cabinet. Next, the unbound aptamers were washed off in 10 mL phosphate buffered saline (PBS) solution (pH 7.4) followed by DI water rinsing and then subjected to electrochemical analysis.

2.4 Bacterial cultures

*Listeria innocua* (NRCC B33076) cultures were stored at -80°C. Before use, stock cultures of *L. innocua* were resuscitated through 2 consecutive 24 h growth cycles in tryptic phosphate buffer (TPB, Becton, Dickinson and Co., Franklin Lakes, NJ) at 35°C to obtain working cultures containing approximately 10^8 CFU/mL. For bacterial enumeration via spread plating after serial dilution in buffered peptone water (BPW, Becton, Dickinson and Co.), modified Oxford’s medium (MOX, Becton, Dickinson and Co.) for *Listeria* were used.

2.5 Electrochemical analysis

Electrochemical impedance spectroscopy (EIS) was carried out in a solution of PBS (pH 7.4) using a two-electrode setup on CHI 600E potentiostat/impedance analyzer. One of the two Pt-IDEs was connected to the working electrode, and the other Pt-IDEs was connected to the reference and counter electrodes of the CHI 600E potentiostat/impedance analyzer. A sine-modulated AC potential of 100 mV was applied across the Pt-IDEs and impedance was measured for a frequency range of 1 Hz to 100 kHz at 30 points per decade. Bode plot (log frequency versus impedance) and Nyquist plot (imaginary versus real impedance) were generated to analyze the aptamer functionalization at various concentrations. The saturation point was determined by comparing the impedance value at each concentration relative to the bare Pt-IDE.

EIS analysis was used to characterize the aptamer based biosensor response with increasing concentration of *Listeria innocua* (NRCC B33076), from 10 to 10^8 CFU/mL. Figure 1 shows the conceptual approach used to create an impedimetric aptasensors using EIS analysis method for *Listeria* spp. detection. Aptamer functionalized Pt-IDEs impedance without bacteria addition was measured and established as a baseline to detect the impedance difference after introducing the bacteria cells. The applied potential was 100 mV (AC) with a frequency range of 1 Hz to 100 kHz at 30 points per decade. PBS (pH 7.4) was used to run the analysis with functionalized Pt-IDEs immersed in 17 mL PBS solution. The bacteria cells at each concentration were immobilized on the aptamer coated Pt-IDEs for 15 minutes to allow successful binding to the aptamers. Nyquist and Bode plots were generated to analyze the total impedance response of the biosensor with increasing bacteria concentration from 10 to 10^8 CFU/mL. The total impedance change was correlated to the bacteria concentration by measuring the baseline of the aptamer coated Pt-IDEs without bacteria. The difference in impedance was calculated as the change in impedance at the bacteria concentration from the baseline and plotted as a calibration curve to determine the sensitivity, range, and lower detection limits of the aptamer based biosensor. The total impedance was measured at 1 Hz based on the spectrum of EIS scan from 1 Hz to 100 kHz.

2.6 Statistical Analysis

JMP v. 11 Software (SAS Institute, Cary, NC) was used for all statistical analyses. Means, error bars and standard deviations were calculated based on triplicate tests. Differences between variables was tested for significance using one-way analysis of variance (ANOVA) and significantly different means (p < 0.05) was separated using Tukey’s Honestly Significant Differences (HSD) test.

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3. RESULTS AND DISCUSSION

3.1 Characterization of aptamer loading onto the Pt-IDEs

The adsorption of the thiolated aptamers onto the Pt-IDEs were expected to obstruct the current flow in the presence of buffer solution, therefore increasing the resistance on the surface of electrodes. This phenomenon was measured using the impedance value, which can describe the contributions of aptamer binding to the Pt-IDEs. Bode and Nyquist plots in Figures 2a and 2b demonstrate the behavior of the aptamer biosensor at different aptamer loading over the entire frequency range from 1 Hz to 100 kHz. The insets show the impedance values for different aptamer loading at 1 Hz frequency. Nyquist plots were used to show real and imaginary impedance values which reflect the resistance and capacitance of the electrochemical cell; respectively, over the entire range of frequency spectrum (1 Hz to 100 kHz).

Figure 2. EIS analysis A) Bode plot and B) Nyquist plot of aptamer loading at various concentrations onto Pt-IDEs in PBS over the frequency spectrum ranging from 1 Hz to 100 kHz. In B) $Z'$ is real impedance $Z''$ and is imaginary impedance. The inset shows the impedance values for various aptamer loading concentrations at 1 Hz.
Figures 2a and 2b established that at frequencies below 10 Hz the impedance response to the aptamer loading had high and distinct values due to the addition of the aptamer deposition onto the electrodes, with the aptamers being responsible for the resistance increase on the electrode surface. Based on these results, the frequency of 1 Hz was selected to determine the saturation limit of the aptamer loading as it showed the most distinguishable impedance changes among the treatments. The results indicated that the total impedance of the system increased, which is an expected trend due to increase in resistance as thiolated aptamers were adsorbed onto the electrode surface. Formation of SAMs of the thiolated aptamer onto the IDEs surface substantially increased the impedance values at 100, 150, 200, 300, 400, and 800 nM as compared to the bare IDEs indicating the formation of dense layers. The impedance value was significantly higher (p < 0.05) than the bare Pt-IDEs at any given aptamer concentration loading (Figures 2a and 2b).

In contrast, at higher frequencies the relationship between the total impedance value and the aptamer loading were not strongly correlated in distinct patterns. Similar results of overlapping curves at higher frequencies for Bode and Nyquist plots were obtained based on the studies by Dastider et al. 22 and Radke and Alocilja 23. These studies explained that the high frequency above 1 kHz might correspond to the ohmic resistance of the solution, which is indicated by the convergence of the impedance curves shown in Figures 2a.

Figure 3 illustrated the impedance magnitude at 1 Hz used to determine the saturation point of aptamer loading. The impedance values versus the aptamer concentrations were plotted at each concentration. The impedance values started to saturate at 300 nM and the IDEs biosensor reached steady state between 400 and 800 nM where the difference was between 15 to 20% relative to the bare. The 800 nM aptamer concentration is a safer design parameter, which guarantees that the electrodes are fully coated with aptamers. Hence, 800 nM aptamer loading was chosen to coat the biosensor for Listeria spp. detection.

3.2 Detection of Listeria spp. using Pt-IDEs aptasensor

Listeria innocua is commonly used as a non-pathogenic surrogate organism for Listeria monocytogenes due to their genetic and metabolic similarities and importance to the food industry 16, 24. Bode and Nyquist plots (Figure 4a and 4b) were obtained for increasing concentrations of bacteria. The inset show that the aptamer-bacteria binding resulted in increased impedance as the bacteria concentration was increased at the frequency of 1 Hz. The overlapping of impedance values at higher frequencies above 1 kHz in Figures 4a and 4b showed no distinct pattern whereas at lower frequencies below 10 Hz the impedance values were increased (p < 0.05) as the bacteria concentration was increased. Hence, the impedance values at 1 Hz were used to determine biosensor performance’s parameters, i.e., range, sensitivity, detection time, and limit of detection calculations.
A calibration curve for detection of *Listeria innocua* was obtained by plotting impedance difference (ΔZ) versus logarithm of bacteria concentration (Figure 5). Impedance difference was calculated using Equation (1):

\[
\Delta Z = \frac{Z_{\text{bacteria}} - Z_{\text{aptamer}}}{Z_{\text{aptamer}}}
\]

where \(Z_{\text{bacteria}}\) is the total impedance (Ohms) measured for a given bacteria concentration, and \(Z_{\text{aptamer}}\) is the total impedance (Ohms) measured without bacteria and it was considered the baseline of the aptamer coated Pt-IDEs biosensor. Figure 5 demonstrated a linear relationship found between \(\Delta Z\) (Ohms) and the bacteria concentration, \(C_{\text{L. innocua}}\) in log (CFU/mL), to be depicted by the following equation \(\Delta Z = 268.1 \log(C_{\text{L. innocua}}) + 1009, R^2 = 0.9653\). The sensitivity was determined to be 268.1 ± 25.40 (Ohms/log [CFU/mL]). The lower detection limit (LDL) was calculated based on the signal/noise of 3 where noise is defined as measured impedance, \(Z_{\text{aptamer}}\) (control experiment) 20, 21. The lower detection limit of the aptasensor for *L. innocua* were found to be 5.39 ± 0.21 CFU/mL. The total detection time from introducing the bacteria samples to impedance measurements was 17 minutes. The protein binding of bacterial cells to the aptamer surface was allowed for 15 minutes while stirring the solution at 450 rpm at room temperature, followed by the EIS analysis which in average would take 2 min.

The developed impedance aptasensor to capture *Listeria* spp. had lower detection limit, higher sensitivity, and shorter detection time as compared to the biosensors reported in the literature shown in Table 1. The studies performed in the

Figure 5. Detection of *Listeria innocua* in PBS solution using aptamers functionalized Pt-IDEs through impedance changes measured at 1 Hz. *Listeria innocua* concentration ranged from 10 to 10^6 CFU/mL. Error bars were based on the standard deviations of means in triplicate tests. Lower detection limit (LDL) line was determined based on signal/noise ratio multiplied by 3.
past used antibodies or growth medium in order to detect *Listeria* as compared to the aptamers which offer the advantage of chemical synthesis with high purity avoiding batch-to-batch variations and longer shelf-life \(^1\). The study performed by Tolba et al. \(^{20}\) reported an impedimetric biosensor using the cell wall binding domain of bacteriophage-encoded peptidoglycan hydrolases (endolysin) immobilized on a gold screen printed electrode (SPE). However, the linear range and detection limits reported were significantly for high bacteria concentrations from \(10^4\) to \(10^8\) CFU/mL that do not meet the required detection levels in the food industry.

Table 1. Comparison of lab-on-a-chip impedimetric biosensors for detection of *Listeria* spp.

<table>
<thead>
<tr>
<th>Biosensor type</th>
<th>Impedance detection technique</th>
<th>Detection time</th>
<th>Limit of detection [CFU/mL]</th>
<th>Bacteria range [CFU/mL]</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Flow cell with embedded Pt-IDEs | Metabolites produced by bacterial cells as a result of growth in Tris-Gly-Dext (no biorecognition element) | 2 hours | \(1.9 \times 10^7\) – *L. innocua*  
3.80 \(10^7\) – *L. monocytogenes* | \(10^7\) to \(10^9\) | \(^{25}\) |
| TiO\(_2\) nanowire bundle microelectrodes | Biorecognition element – antibodies (Abs) | 50 minutes | \(4.7 \times 10^2\) | \(10^2\) to \(10^7\) | \(^{26}\) |
| Gold screen printed electrodes (SPE) | Immobilization of biorecognition element - Endolysin on SPE | 30 minutes | \(1.1 \times 10^3\) – pure bacteria  
1.1 \(10^5\) – 2% milk | \(10^4\) to \(10^8\) | \(^{20}\) |
| Pt-IDEs | Biorecognition element – *Listeria monocytogenes* aptamers | 17 minutes | 5.39 | \(10^1\) to \(10^6\) | This study |

4. CONCLUSIONS

An impedimetric aptasensor was developed using miniaturized Pt-IDEs with the biorecognition element, *Listeria monocytogenes* aptamers, for rapid, sensitive and lower detection limit of *Listeria* spp. The aptamer based biosensor was saturated at \(800\) nM, which was used to functionalize the Pt-IDEs surface to detect the targeted bacteria. EIS analysis was able to detect the surface modification due to aptamers attachment and protein binding of *Listeria innocua*; providing a direct technique for *Listeria* spp. detection without the need for label amplification or sample pre-concentration steps. The aptasensor was capable to detect *Listeria* throughout a wide range of bacteria concentration from \(10\) to \(10^6\) CFU/mL with a sensitivity value of \(268.1 \pm 25.40\) (Ohms/log [CFU/mL]) and lower detection limit of \(5.39 \pm 0.21\) CFU/mL in 17 min, which are lower than the values reported previously in the literature. The aptamer based biosensor offers a portable, rapid and sensitive alternative for food safety applications with one of the lowest detection limits reported to date.

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