Label-free, rapid *Listeria monocytogenes* biosensor based on a stimulus response nanobrush and nanometal hybrid electrode

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ABSTRACT

Recent outbreaks and food recalls associate with *Listeria monocytogenes* have heightened public concern about food safety and created a greater impetus to improve methods for foodborne pathogen detection. The goal of this study was to develop rapid, label-free *L. monocytogenes* biosensors based on composites of pH-responsive polymer nanobrushes and aptamers. Biosensors were characterized electrochemically by ESA and EIS analyses to evaluate chitosan-platinum nanobrush platforms. The detection range and response time were 10-10^7 CFU/mL and approximately 17 min, respectively; for both sensing platforms. The biosensors were also tested in the presence of equal concentrations of *L. monocytogenes* and *Staphylococcus aureus* and presented LOD as low as 31.1 ± 3.3 CFU/mL. The biosensors were tested in vegetable broth showing LOD as low as 36.4 ± 11.1 CFU/mL. The designed biosensor platforms show to have great potential for rapid *L. monocytogenes* detection.

Keywords: biosensor, food safety, pathogen detection, *Listeria monocytogenes*

1 INTRODUCTION

The most prevalent foodborne pathogens that persistently cause infections include *Salmonella* Typhimurium, *Listeria monocytogenes* and the "big six" diarrheagenic *Escherichia coli* pathotypes [1]. To meet the need of supplying fresh, high quality, and safe food to a growing world population, rapid and sensitive monitoring techniques are needed which can determine foodborne pathogen presence. Recent recalls related to *L. monocytogenes* contamination highlight the importance of rapid tools that could be used to monitor pathogens such as *Listeria* [2]. Conventional techniques such as aerobic plate counting (APC), enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) are laborious, time-consuming (hours to days to produce results) and require trained personnel [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]. 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 [4]. Impedance biosensors are a class of electrochemical biosensors that show promise for point-of-care analysis (i.e.; non-laboratory settings) due to ease of miniaturization, label-free operation, speed of analysis, and quantitative readouts that can be assessed by personnel with minimum training. Impedance changes occur due to change in electrochemical properties at the electrode surface solely due to the presence of the target analyte [5]. Biosensors functionalized with pH-responsive nanobrushes based on their swelling and shrinking behavior have the potential to improve bacteria capture and detection performance. The goal of this study was to develop rapid, label-free *L. monocytogenes* biosensors based on composites of pH-responsive polymer nanobrushes and aptamers. To achieve this goal two approaches were used, the first was to design the sensing platform by combining pH-responsive polymer nanobrushes and platinum nanoparticles structures, and the second was embedding the pH-responsive polymer nanobrush with platinum nanoparticles.

2 EXPERIMENTAL

2.1 Materias and Reagents

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 (Hydroxymetyl)aminomethane, potassium phosphate dibasic (K_2HPO_4), and sodium phosphate monobasic monohydrate (Na_2HPO_4) were obtained from J.T.Baker Chemical (Phillipsburg, NJ). Sodium phosphate monobasic monohydrate ($Na\ H_2PO_4.H_2O$), DL-Dithiothreitol, chitosan (Medium molecular weight, 75-85% deacetylated, 200-800 cP), and thioglycolic acid were purchased from Sigma Aldrich (St. Louis, MO). Potassium ferrocyanide trihydrate ($K_4Fe(CN)_6.3H_2O$) was purchased from Ward's Science (Rochester, NY). EDC (1-ethyl-3-[3-dimethylaminopropyl]- carbodiimide), sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1carboxylate) and sulfo- NHS (N-hydroxysuccinimide) were purchased from ThermoScientific (Waltham, MA)

Listeria monocytogenes thiol terminated aptamers that target protein Internalin A (A8, 5′-ATC CAT GGG GCG GAG ATG AGG GGG AGG AGG GCG GGT ACC CGG TTG AT-3′, 47 mers) [6] were purchased from GeneLink (Hawthrone, NY).

2.2 Bacterial cultures

Listeria monocytogenes (ATCC 15313) and *Staphylococcus aureus* (ATCC 25923) cultures were stored at -80°C. Before use, stock cultures of *L. monocytogenes* and *S. aureus* were resuscitated through 2 consecutive 24 h growth cycles in tryptic phosphate and tryptic soy buffers (TPB and TSB, Becton, Dickinson and Co., Franklin Lakes, NJ), respectively 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 and Tryptic Soy Agar media (MOX and TSA, Becton, Dickinson and Co.) for *Listeria* and *S. aureus* were used [7].

2.3 Electrode fabrication

Pt/Ir electrodes were cleaned prior to use following the procedures in Burrs et al (2015). For the first approach, a base nanoplatinum (n-Pt) was electrodeposited onto Pt/Ir using pulsed sonoelectrodeposition using 1.44% (w/w) chloroplatinic acid and 0.002% (w/w) lead acetate at a frequency of 500 mHz for 60 s at 10 V versus a bare platinum wire based on [8, 9]. Next, for nanobrush deposition, nPt decorated electrodes were immersed in 10 mL of the chitosan solution and connected to the cathode of a DC power supply with a 1 mm diameter platinum wire as the anode. Electrodeposition time (2.5 to 10 min) and voltage (1.75 to 3 V) was optimized. For the second approach, chitosan was initially modified to have thiol termination using TGA via EDC-NHS bioconjugation chemistry [10]. Next, simultaneous deposition of chitosan-TGA and n-Pt onto Pt/Ir electrode was carried out via pulsed sonoelectrodeposition using 1.44% (w/w) chloroplatinic acid and 0.002% (w/w) lead acetate containing chitosan-TGA (0.5 to 1% w/v).

Thiol labeled aptamers for *L. monocytogenes* consisted of 47-mer and it targets the internalin A protein [11]; the 5' end was modified with a terminal thiol group. Disulfide modified *Listeria* thiol aptamers were reduced using the dithiothreitol (DTT) reduction protocol [10]. For the first approach, aptamers were attached to the chitosan nanobrushes using sulfo-SMCC bioconjugation chemistry [10]. For the second approach, aptamers were attached to the chitosan-TGA nanobrushes using the sulfur atoms of the thiol group forming a self-assembled monolayers (SAM) via covalent adsorption between thiol-platinum [12]. A stock solution of 100 µM *L. monocytogenes* aptamers was further diluted to various concentrations in 10 mM Tris, 1 mM EDTA, pH 7.5 buffer. Then, electrodes were submerged in 250 µL of aptamer solution with or without Sulfo-SMCC (first and second approach, respectively), and agitated for 2 h at room temperature. 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 Electrochemical analysis

All electroactive surface area (ESA) and electrochemical impedance spectroscopy (EIS) tests were performed with a 3 electrode cell set up with a Ag/AgCl reference electrode and a platinum auxiliary electrode using a CH Instruments potentiostat (CHI6044E; Austin, TX). The tests were all conducted in a 20 mL electrochemical cell. Cyclic voltammetry (CV) was conducted in 4 mM potassium ferricyanide trihydrate and 1 M potassium chloride to determine ESA via the Randles-Sevcik theorem as previously described [13, 14]. CV was performed using a sweep range of 650 mV with a switching potential of 650 mV and a 30 second quite time. EIS was performed in 4 mM potassium ferricyanide trihydrate and 1 M potassium chloride. An initial DC voltage of 0.25 V was applied with a frequency range of 1 Hz to 100 kHz and an AC amplitude of 100 mV. For chitosan actuation tests the pH of ferrocyanide solution was adjusted to pH 5 or pH 7 using a 2.5 M HCl or NaOH solution; solution pH was monitored during tests to ensure reported pH did not change by more than 0.5 pH units

EIS was used to determine the limit of detection (LOD), range and sensitivity of each biosensor. **Fig 1** shows the conceptual approach used to create an impedimetric sensors for both approaches using EIS analysis method for *L. monocytogenes* detection. Nyquist (complex plante diagrams) and Bode (impedance versus frequency) plots were generated to analyze the total impedance response of each biosensor with increasing bacteria concentration Bode plots were used to determine the impedance at a fixed cutoff frequency (Z"). Change in normalized impedance ($\Delta Z_N''$) was determined from Bode plots based on equation 1, where Z_o'' is the measured imaginary impedance in the absence of bacteria (i.e., baseline impedance). The sensitivity was taken as the linear slope of the calibration curve prepared by plotting ($\Delta Z_N''$) versus bacteria concentration in CFU/mL. LOD was estimated using the 3 sigma method (99.7% confidence interval) according to [15, 16]. Selectivity was measured by calculating the net percent change in sensitivity and LOD in the presence of *Listeria* and another gram positive bacteria (*S. aureus*) for concentrations from 10 to 10^7 CFU/mL in buffer or vegetable broth as noted.

$$\Delta Z_N'' = \frac{Z'' - Z_o''}{Z_o''} \quad (1)$$

2.5 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.

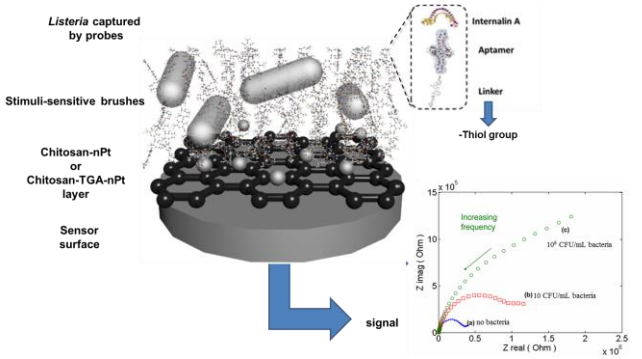

Figure 1: Conceptual approach used to create impedimetric sensors for both approaches using EIS analysis method for *L. monocytogenes* detection.

3 RESULTS AND DISCUSSION

Optimization of both sensing platforms by measuring ESA values while varying electrodeposition time (2.5 to 10 min) and voltage (1.75 to 3 V); and sonoelectrodeposition cycles (pulsed from 40 to 360 s) for the first and second approach, respectively. For the first approach, chitosan nanobrushes electrodeposition was optimized to 0.5% (w/v) low molecular weight chitosan at 2 V for 5 min, increasing the ESA to 0.101 ± 0.004 cm^2. While for the second approach, the ESA values for electrode submitted to the one-step grafting for 240 s was equivalent ($p < 0.05$) to the electrodes obtained with a 3-step deposition (n-Pt, chitosan, n-Pt), 0.063 ± 0.004 and 0.065 ± 0.008 cm^2, respectively using 0.5% (w/v) low molecular weight chitosan.

Using the optimal sensing platform for each approach, charge transfer resistance (R_{ct}) percetange change values (relative to unloaded, i.e., no aptamers) obtained from EIS analysis were used to determine the optimum aptamer loading concentration. **Fig 2** shows the results for the first approach (chitosan-nPt platform). The best concentrations based on the highest R_{ct} percentage change values obtained were 1000 nM and 400 nM for chitosan-nPt and chitosan-TGA-nPt platforms, respectively.

In order to verify sensing platform pH-responsiveness, actuation was measured initially without cell present and later in the presence of *L. monocytogenes* in buffer (PBS), **Fig 3** shows representative CV curves obtained from pH-actuation of chitosan-nPt platform. Actuation tests of chitosan nanobrushes revealed pH 4 and pH 8 were the ideal pHs for capturing and sensing bacteria, respectively. While for chitosan-TGA-nPt platform, actuation tests revealed pHs 8 and 4 were the ideal pHs for capturing and sensing bacteria, respectively.

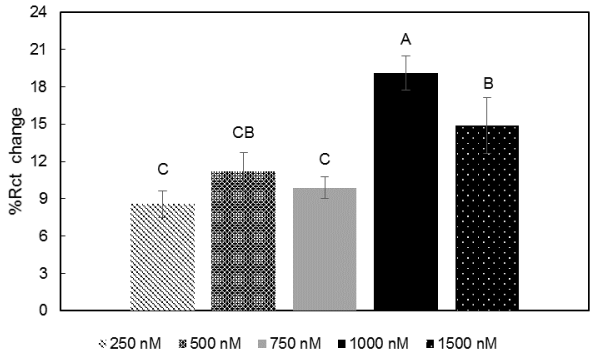

Figure 2: Charge transfer resistance (R_{ct}) percentage change values from EIS analysis obtained at different aptamer loading onto chitosan-nPt platform. Error bars were based on the standard deviations (n = 3). Different letters indicate significance at $P < 0.05$.

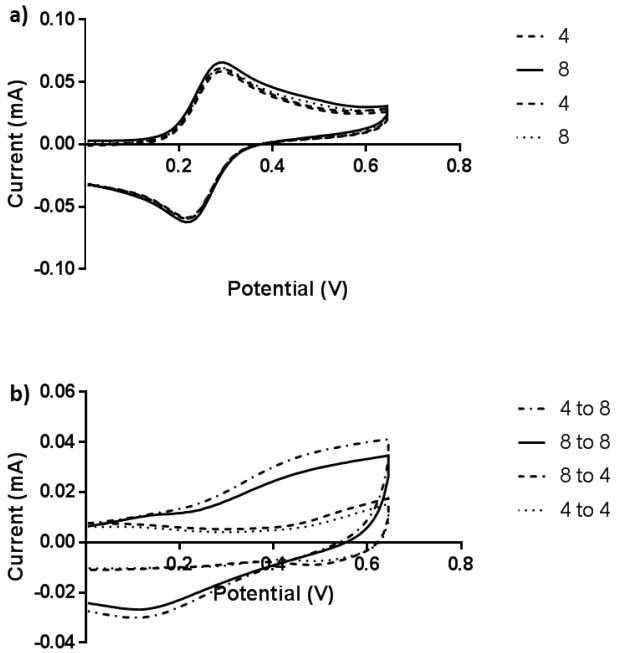

Figure 3: Representative CV curves showing change in peak current for different extension states (extended and collapsed) of chitosan nanobrushes (chitosan-nPt) at various pH levels. **a)** no bacteria using potassium ferricyanide redox probe solution; **b)** cell capture (10^3 CFU/mL of *L. monocytogenes*) in PBS.

Representative Nyquist plot (**Fig 4**) were obtained for increasing *L. monocytogenes* concentrations The inset show that the aptamer-bacteria binding resulted in increased impedance as the bacteria concentration was increased at 1 Hz frequency. The impedance values at higher frequencies above 1 kHz in **Fig 4** showed no distinct pattern whereas at lower frequencies below 10 Hz the impedance values increased ($p<0.05$) with increasing bacteria concentration. Hence, the impedance values at 1 Hz were used to

determine both biosensor platforms performance's parameters.

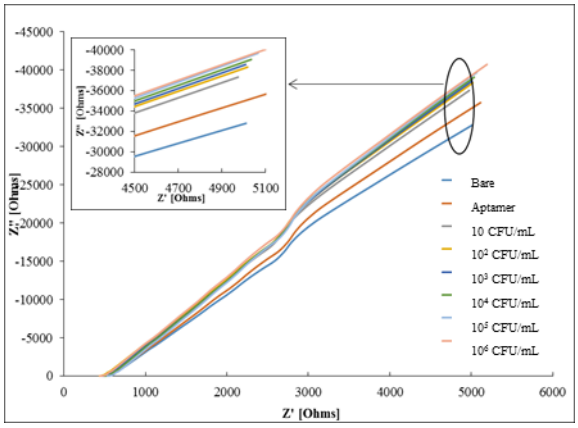

Figure 4. Representative Nyquist plot of *L. monocytogenes* detection in PBS solution over the frequency spectrum ranging from 1 Hz to 100 kHz. Bacteria concentration ranged from 10 to 10^6 CFU/mL. The inset in a) shows the impedance values below 10 Hz. *Z''* is imaginary impedance and *Z'* is real impedance.

The detection range and response time were 10 – 10^8 CFU/mL and approximately 17 min, respectively; for both sensing platforms. The LOD was as low as 4.3 ± 0.4 CFU/mL in PBS. In order to verify selectivity toward *L. monocytogenes*, the biosensors were also tested in the presence of equal concentrations of *Listeria monocytogenes* and *Staphylococcus aureus* and presented LOD as low as 31.1 ± 3.3 CFU/mL. Furthermore, the biosensors were tested in vegetable broth showing LOD as low as 36.4 ± 11.1 CFU/mL. The designed biosensor platforms show to have great potential to replace current highly technical and time consuming standard methods used by the food industry for rapid *Listeria monocytogenes* detection.

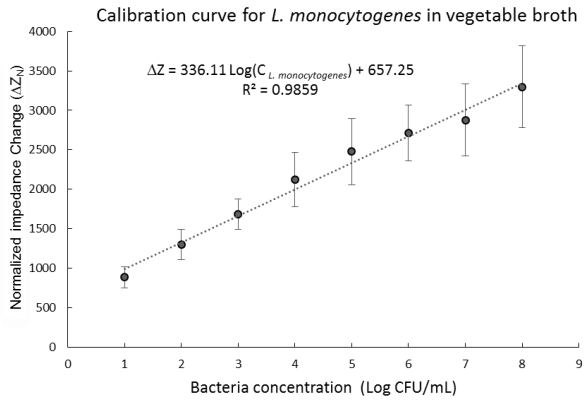

Figure 5. Detection of *Listeria monocytogenes* in vegetable broth using aptamer chitosan-nPt biosensor platform through impedance changes measured at 1 Hz. *Listeria monocytogenes* concentration ranged from 10 to 10^8 CFU/mL. Representative calibration curve. Error bars were based on the standard deviations of means (n = 3).

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