Simultaneous detection of *E. coli* K12 and *S. aureus* Using a Continuous Flow Multijunction Biosensor

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Abstract: Rapid detection and identification of potentially harmful bacteria is ideal for food manufacturers to prevent foodborne illness outbreaks. Continuous monitoring method of foodborne pathogens levels and trends in food gives real-time results. Therefore, the objectives of this study were to fabricate and characterize the continuous flow multijunction biosensor for simultaneous detection of *Escherichia coli* K12 and *Staphylococcus aureus*. Junction biosensors were fabricated using gold plated tungsten wires coated with polyethylenimine and single walled carbon nanotubes. Each junction was functionalized with streptavidin and biotinylated antibodies specific to *E. coli* K12 and *S. aureus*. Then, single or 2 biosensors for each targeted analyte were connected to tubing, perpendicular to the flow direction. Pure serial diluted samples of *E. coli* K12 and *S. aureus* and microbial cocktail samples were continuously pumped at a 0.0167 mL/s into the detection zone. Changes in the electric current by biorecognition reactions between antibody and antigens were calculated. The developed junction sensor coupled with the fluidic channel showed the enhancement of the electric signal responses for detection of *E. coli* K12, compared to the stationary sensor. A linear regression was observed for both the *E. coli* and *S. aureus* functionalized array sensors in the detection range of 10^2 to 10^5 CFU/mL. Multiplexed detection of bacteria at the sensing levels as low as 10^2 CFU/mL for *E. coli* K12 and *S. aureus* was achieved within 2 min. Therefore, the continuous flow multijunction biosensor shows potential for rapid and continuous multiplexed detection of foodborne pathogens.

Keywords: rapid detection, carbon nanotubes, multiplexing, pathogens

Introduction

Rapid identification and detection of bacterial pathogens in food is urgently needed to ensure food safety. Centers of Disease Control and Prevention estimated annual number of domestically acquired, 47.8 million foodborne illnesses, 128000 hospitalizations, and 3000 deaths due to 31 pathogens and unspecified agents transmitted through food. Most of these diseases are caused by known foodborne pathogens such as *Escherichia coli* O157:H7, *Salmonella* (nontyphoidal), *Listeria monocytogenes*, *Clostridium perfringens*, *Campylobacter spp.*, *Staphylococcus aureus*, and *norovirus* (CDC 2011). Rapid identification of pathogenic bacteria contaminated in food products before distribution to grocery stores, restaurants and manufacturing facilities is a solution to reduce the number of foodborne illnesses (Shriver-Lake and others 2007). A range of the number of pathogenic bacteria ingested that can cause infection is from 1 to 10^7 cells depending on the species, their serotype, age and health of host and the type of contaminated food consumed (Kothary and Babu 2001). The infection dose of pathogenic *E. coli* groups is ranging from 10^7 to 10^{10} cells; however, *E. coli* O157:H7, an enterohemorrhagic *E. coli* serotype, causes the illness with very low number of cells, in the range of 10 to 100 cells (Croxen and others 2013). *S. aureus* population exceed 10^5 organisms/g in food can create 1 μg of toxin causing food poisoning (Evenson and others 1988). Therefore, a limit of detection for pathogens in food should be less than the infective dose. The powerful analytic methods with sensitive and reliable for detecting pathogens at pre-infectious levels in foods are required to prevent the widespread outbreaks of disease.

Conventional methods based on colony counting, immunoassay, and nucleic acid amplification, remain highly reliable and have been successful for detecting bacterial pathogens (Leonard and others 2003; Velusamy and others 2010). However, current detection techniques rely on specific microbiological and biochemical identification. In addition, these methods require long assay time, labors and initial enrichment steps to detect pathogens with low concentration in food (Leonard and others 2003).

Biosensors have emerged as useful tools for pathogen detection, especially, electrochemical biosensors have the advantages of high sensitivity, rapidity, low cost and amenability of microfabrication (Sadik and others 2009). The electrochemical biosensor has been studied with the immune reaction between the analyte and the corresponding recognitions molecules. The antigen–antibody complexes are formed on the biosensor's transducer to create measurable electrical signals. The detection process based on label-free electrochemical impedance spectroscopy was demonstrated to reliably detect pre-infectious levels of *Salmonella typhimurium* at 500 CFU/mL with a detection time of 6 min, including 5 min for data acquisition and 1 min for analysis (Nandakumar and others 2008).

Incorporation of nanomaterial to the sensor platform, in particular single walled carbon nanotubes (SWCNTs)-based sensor is a promising detection alternative due to SWCNT's bio and size compatibility, structural flexibility, and electrical conductivity for enhancing the sensor's performance (Katz and Willner 2004;

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Kang and others 2006; Allen and others 2007). The SWCNTs incorporated the biosensor have been used as field-effect transistors (FETs), which changes the electrical conductance by binding of analyte molecules to recognition molecule (Trojanowicz 2006). García-Aljaro developed the carbon nanotubes-based immunosensors for detection of *E. coli* O157:H7 and the bacteriophage T7. The detection limit and response time were 10^3 CFU/mL with 60 min for *E. coli* O157:H7 detection and 10^3 PFU/mL within 5 min for bacteriophage. The biosensor exhibited the selectivity against *E. coli* K12 and MS bacteriophage (García-Aljaro and others 2010). Bio-nano combinational junction sensor with functionalized SWCNTs has shown the potential for high-performance biosensing to detect foodborne pathogens (Yamada and others 2014, 2015). The junction sensor works to convert bioaffinity binding between target bacteria cell and antibodies into measurable electrical current signals (Figure 1A). The researchers achieved rapid (within 2 min), sensitive and selective detection of *E. coli* K12 and *S. aureus* (limit of detection: 10^2 CFU/mL) in pure and cocktail bacterial samples. The combination of CNTs and specific antibodies provided excellent sensitivity and selectivity in electrochemical biosensors for detection of pathogens.

Although most biosensors exhibited high sensitivity with a fast detection time, they tended to work for small sample volumes. The volume of bacterial solution ranged from a droplet about 5 to 100 μL (Nandakumar and others 2008; Lu and others 2013; Yamada and others 2014). The sample volume for detection of bacterial cells might could limit adequate quantitative microbial

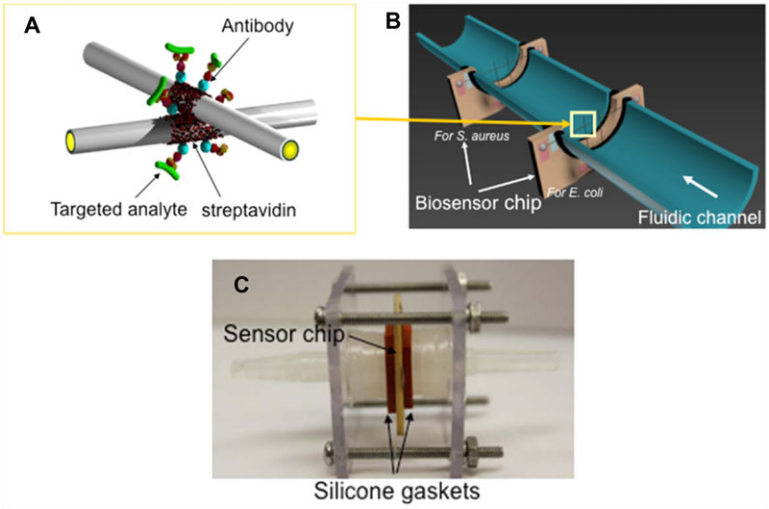

Figure 1–A continuous flow multijunction sensor device. (A) Illustration of bio-nano functionalized junction (Yamada and others 2014). (B) Conceptual design of multijunction biosensor for multiplexed detection in a continuous flow mode (cross sectional view). (C) Individual sensor chip ready to use.

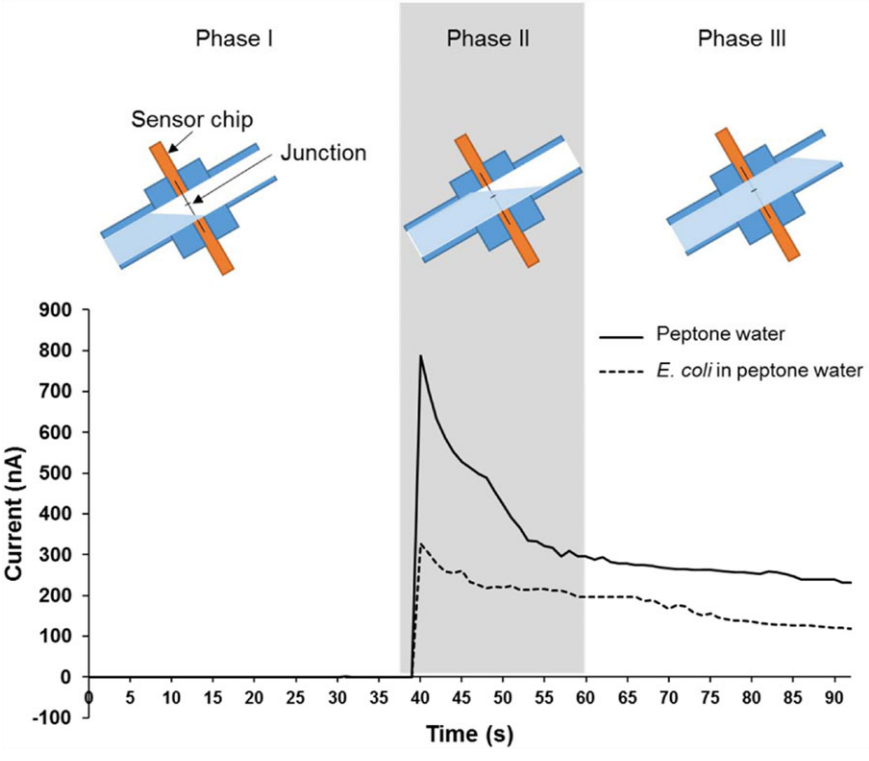

Figure 2–A current change profile of the continuous flow junction sensor for pure peptone water (solid) and *E. coli* K12 suspended in peptone water (dot) in Phase I (when the junction was open), Phase II (when the junction met the flow head) and Phase III (when the antigen–antibody reaction fully developed). The concentration of *E. coli* K12 suspended was 10^4 CFU/mL.

analysis because it does not represent the whole population. It should entail a pre-enrichment step to concentrate low numbers of pathogens, resulting in an increase in the detection time. Continuous system is able to detect the pathogens with large volume of samples as well as it can be monitored food safety in real-time (Kim and Park 2003; Hartwell and Grudpan 2010) for food processing applications that involve continuous flow food products. In addition, flow-based detection techniques such as flow-injection, sequential injection and microfluidic system increase the potential for assay automation (Fintschenko and Wilson 1998; Gubitz and others 2001) and the ratio of immobilized surface area to sample volume, offering increased antibody–antigen encounter (Abdel-Hamid and others 1999). However, most studies for flow-based biosensor have been done in microfluidic channel. Also, rapid and multiplexed detection method for commercial use in flow condition was hardly studied. Therefore, the objectives of this study were to fabricate the continuous flow multijunction biosensor for continuous detection of 2 different bacteria and to evaluate the sensing performances in a macrofluidic channel.

Materials and Methods

Junction sensor fabrication

The methods for individual sensor fabrication consisted of microwires coating, junction assembly, and antibody immobilization and were followed Yamada et al.'s procedure (Yamada and others 2014). 7% gold plated tungsten wire (50 μm in diameter, ESPI Metals, Ashland, Oreg., U.S.A.) was washed by sonicating in distilled water, followed by 70% alcohol for 5 min each and dried in a furnace at 175 °C for 10 min. The sanitized microwires coated with 1% polyethylenimine (PEI) and then single walled carbon nanotubes (SWCNTs; SWNT PD1.5L, NanoLab, Inc.,

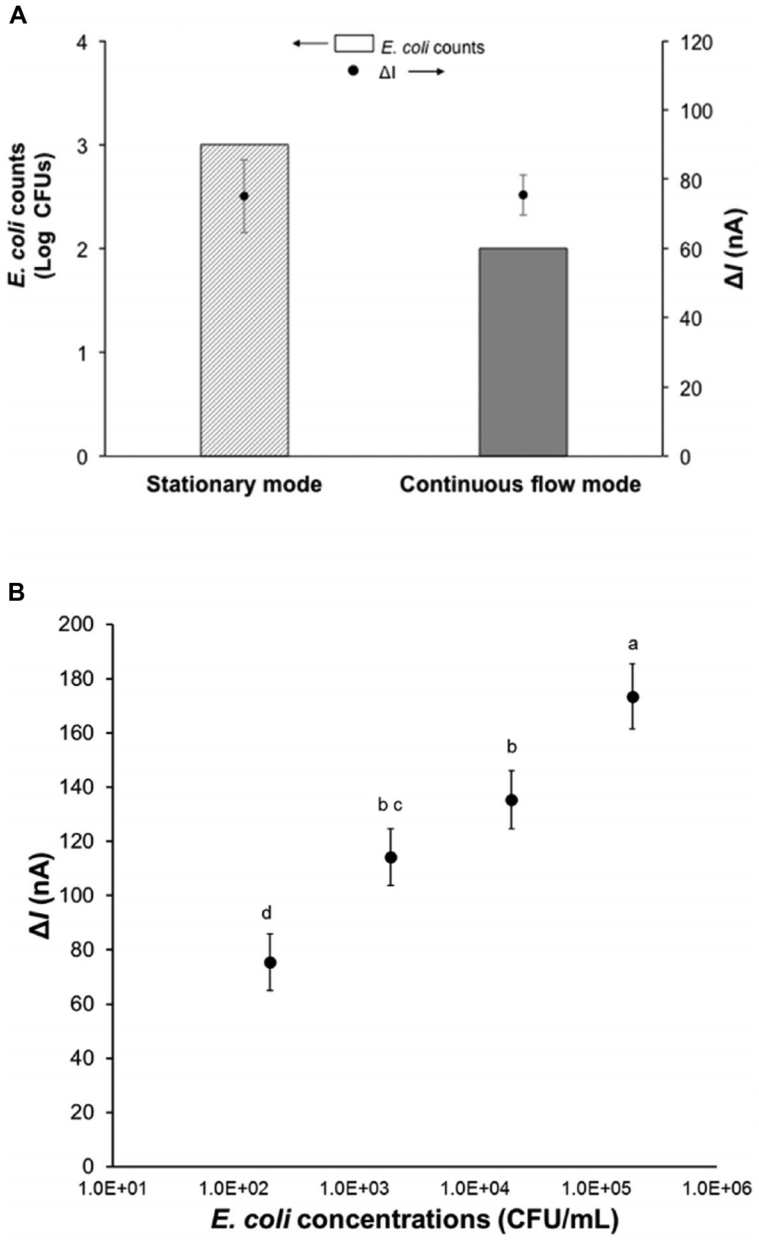

Figure 3–Sensing signal enhancement of a continuous flow junction sensor vs. a stationary sensor on the basis of the equivalent current reading (75 nA): (A) Increase of the sensing sensitivity by a factor of 10 and (B) Current changes of the continuous flow sensor with *E. coli* K12 in the range of 10^2 to 10^5 CFU/mL.

Waltham, Mass., U.S.A.) dispersed in N,N-dimethylformamide (DMF; Sigma Aldrich, St. Louis, Mo., U.S.A.) by dipping and withdrawing method using XYZ stage and stepping motor (Franklin Mechanical & Control Inc., Gilroy, Calif., U.S.A.) at a withdrawal velocity of 100 μm/s. For sensor chip fabrication, copper clad printed circuit board (1.5 mm thick, McMaster-Carr, Santa Fe Springs, Calif., U.S.A.) were cut into small pieces (26 × 26 mm^2) and drilled a hole the center with 10 mm in diameter for fluidic channel. Then, they were etched to form electrode connector pads at the 4 sides of the circuit board. The hole was filled with polydimethylsiloxane (PDMS; Sylgard 184 silicone elastomer curing agent and base, Dow Corning, Midland, Mich., U.S.A.). PDMS layer was used as a support during antibodies immobilization at the junction and detection of bacteria in a stationary mode, but was removed after functionalized process for continuous flow testing. PEI-SWCNT coated wires were orthogonally soldered to the connector pads on the circuit board to form a single crossbar array with 2 wires or multijunction array with 4 wires. Mica sheets (McMaster-Carr) were placed to create the gap between wires at the junction. Two 100 g weights were used to adjust the tension of the wire during soldering to withstand flow pressure. Each junction was functionalized with 5 μL of streptavidin (from *Streptomyces avidinii*, Sigma Aldrich) for 5 min and then the droplet of streptavidin was removed. Subsequently, 5 μL of biotinylated polyclonal *E. coli* (from rabbit, #PA1-73031, Thermo Fisher Scientific, Waltham, Mass., U.S.A.) and *S. aureus* (from rabbit, #PA1-73174, Thermo Fisher Scientific) antibodies was applied to junctions for another 5 min. Each biosensor chip was prepared for each targeted analyte. Functionalized bio-nano junction sensor was dried and stored at refrigerator before use for cell detection.

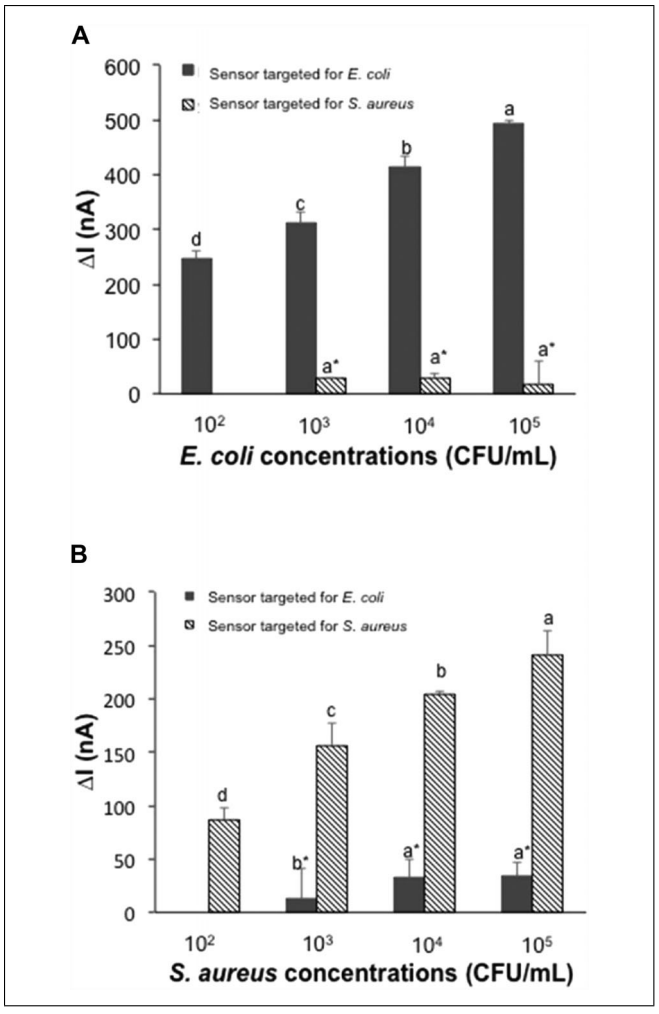

Figure 4–Specificity testing for the developed sensors functionalized with anti-*E. coli* (A) and *S. aureus* (B): Microbial concentrations ranged from 10^2 to 10^5 CFU/mL.

Microbial preparation

Frozen stock cultures of *E. coli* K12 and *S. aureus* were obtained from the Food Microbiology Lab, University of Hawaii. 100 μL of each stock was inoculated separately in 10 mL of tryptic soy broth (BBL^{TM} Trypticase^{TM} soy broth, BD diagnostic systems, Franklin Lakes, N.J., U.S.A.) and incubated for 24 h at 35 °C. Three milliliters aliquots of cultured bacteria was put into conical tube and centrifuged at 8000 rpm for 10 min. After the supernatant was removed, the pellets were washed with phosphate buffer saline at twice and with distilled water by centrifuging at 6000 rpm for 5 min per each washing step. The resulting pellets were suspended in 30 mL of sterilized 0.1% peptone water (Bacto^{TM} Peptone, BD diagnostic systems) by autoclave. Then, bacteria suspension was serially diluted in the peptone water to obtain varying concentrations of each organism for target sensing experiments. For microbial cocktail sample, individual resulting pellets from both bacterial cultures using same procedure above were added to the peptone water at once and serially diluted to prepare 10-fold sample cocktail dilutions. Standard plate counting method on plate count agar (Difco^{TM} Plate count agar, BD diagnostic systems) was used to determine the initial concentrations of *E. coli* and *S. aureus* stock cultures. The initial concentrations of *E. coli* and *S. aureus* in culture were 2.0 × 10^9 and 2.1 × 10^9 CFU/mL, respectively. All experiments were conducted in a certified Biosafety Level II laboratory.

Continuous flow detection

The fabricated biosensor chips were placed in a fluidic channel perpendicular to the flow direction (Figure 1B) and fixed by 2 acrylic boards with fasteners. Stepping tubing connectors (4.7 to 9.5 mm in diameter, Bel-art product, Pequannock, N.J., U.S.A.) were embedded onto the center of acrylic boards (Figure 1C). Two silicone gaskets (1.6 mm thick, McMaster-Carr) were used to prevent liquid from leaking. Pure serial diluted samples or microbial cocktail sample was continuously pumped into the detection zone using a syringe pump (KDS 100, KD Scientific Inc., Holliston, Mass., U.S.A.) at a flow rate of 0.0167 mL/s. The bacterial suspension was filling the upward tube (4 mm in diameter) with a 30° angle. The biosensors were connected to the multiplexing circuit, which was composed of a power source to generate 1 $V_{DC,}$ a switch and a picoammeter (6485, Keithley, Cleveland, Ohio, U.S.A.) to measure the current at the 4 junctions in real-time. The electric current readings were collected for 1 min when 1 mL of the injected sample solution fully reacted with the sensing junction. Then the valid data set was selected when the readings reached the steady state. To compare the sensor characteristics between the continuous flow and stationary junction sensors, 10 μL of 1 of serial diluted *E. coli* solutions (10^4 CFU/mL) was applied to the junction for 1 min to permit full antibody–antigen reactions (Yamada and others 2014). Then, the junction was rinsed with distilled water for elimination of nonspecific binding *E. coli*, and

the current value was measured in the existence of 1 droplet of 10 μL of distilled water.

Sensitivity and selectivity test

The sensitivity of the multijunction sensor was evaluated using pure serially diluted *E. coli* and *S. aureus* cultures with the concentrations of 10^2 to 10^5 CFU/mL. Single anti-*E. coli* functionalized multijunction sensor chip was installed in the fluidic channel and tested for specific with 10^2 to 10^5 CFU/mL of *S. aureus* solutions. The selectivity test for anti-*S. aureus* functionalized sensor was conducted in the presence of pure *E. coli* K12 solutions. Two biosensors for each targeted analyte were placed inside the channel to evaluate the multiplexed sensing to simultaneously detect *E. coli* and *S. aureus*. Mixed cultures of *E. coli* and *S. aureus* were applied to the sensing area and current values were measured. Sterile 0.1% peptone water was used as negative controls.

Data analysis

The current values measured at each of 4 junctions were averaged to obtain a representative current (I) measurement for 1 sensor chip. The ΔI signal response was determined by calculating the difference between the electrical current output of the negative control (I_{antibody}), and that of the sample ($I_{\text{antibody-bacteria}}$), as given by

$$\Delta I = | I_{(\text{antibody}-\text{bacteria})} - I_{(\text{antibody})} | \quad (1)$$

Statistical analysis was conducted based on the Duncan's multiple range tests using a single factor analysis of variance (ANOVA)

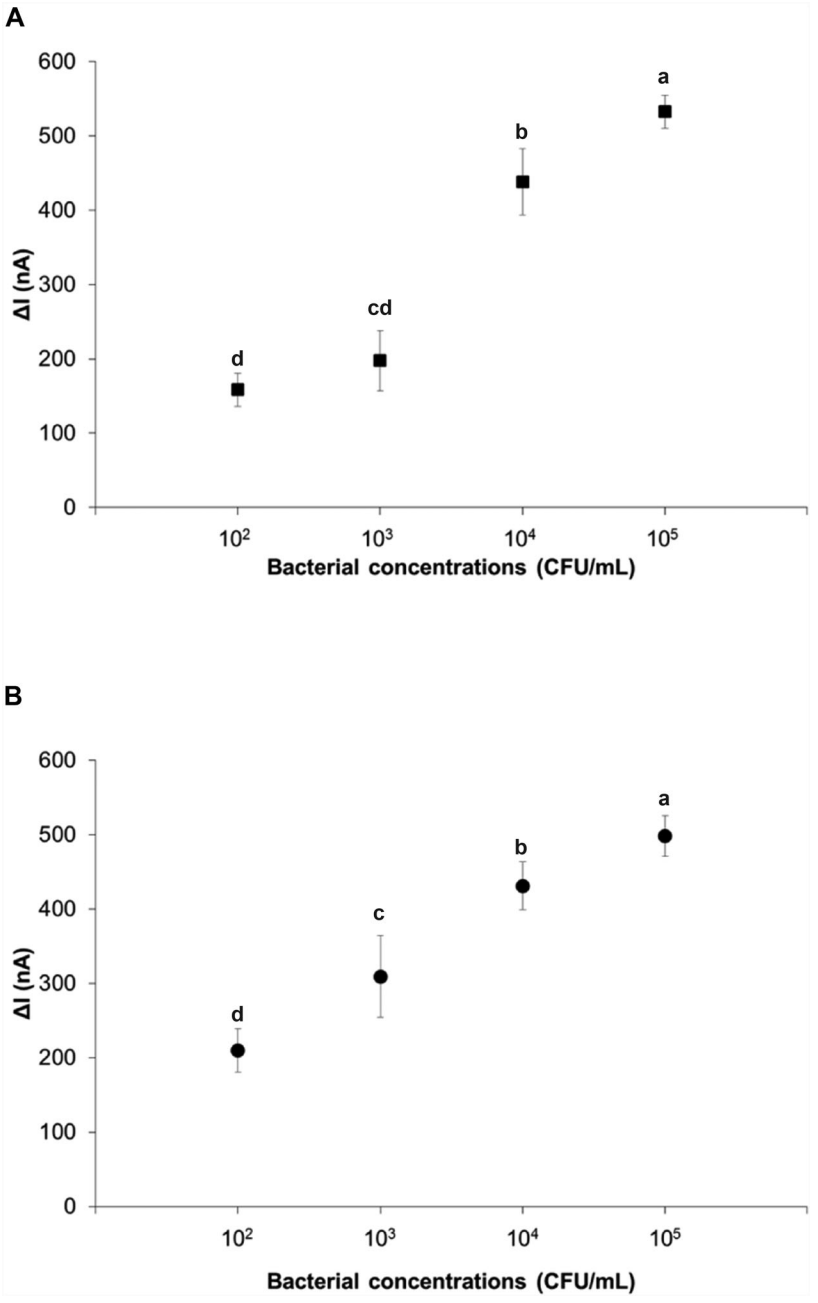

Figure 5–Electrical signal responses of multijunction sensors specific for *E. coli* K12 (A) and *S. aureus* (B) when mixture samples of *E. coli* K12 and *S. aureus* were used.

offered by Statistical Analysis Software (SAS version 9.4, SAS Institute Inc., Cary, N.C., U.S.A.). The bacterial concentration dependence of the biosensor responses was statistically different at 95% confidence level ($P \leq 0.05$).

Results and Discussion

Detection of *E. coli* K12 with a single junction sensor in a continuous flow mode

A single junction biosensor was evaluated for continuous flow detection of *E. coli* K12 in a macrofluidic channel. Before the flow head of the microbial solution reached the junction, there was no electric signal change because the gap between the microwires was open. An electrical signal response was obtained when the solution filled the junction zone. The signal amplitude decreased as the solution passed through the channel; eventually, the observed current values were at equilibrium after 1 min measurement (Figure 2). The reaction time required for signal stabilization was consistent with our former study done at the batch-sensing mode (Yamada and others 2014).

The performance of the continuous flow junction biosensor was compared with the stationary junction biosensor equipped with 1 sample-holding platform (Figure 3A). Since different volumes of bacterial solutions were used, bacterial cell counts applied at the junctions in both detection modes were estimated using Lu and others's (2013) equation. The change in the current was 75.1 nA when *E. coli* suspension with 3 log CFU cell counts was applied to the stationary junction sensor. However, the continuous flow junction sensor in the fluidic channel required only 2 log CFU cell counts to obtain the equivalent magnitude (75.5 nA). It is noted that bacterial loads needed for equivalent current outcomes were reduced by a factor of 10 when the continuous flow sensor was used. The continuous flow sensing mode appears to reduce the likely steric hindrance between analytes (*E. coli* cells) and transducer (functionalized junction) by allowing bacterial cells to continuously flow over the surface of microwire immobilized with antibodies. A linear trend for the current changes was observed as *E. coli* concentration increased from 10^2 to 10^5 CFU/mL ($R^2 = 0.988$) and was consistent with the trend observed in the stationary junction sensor (Yamada and others 2014). Therefore, the signal enhancement and the limit of detection (LOD) as 10^2 CFU/mL for *E. coli* cells were achieved using the developed junction biosensor in a continuous flow mode.

Sensitivity and selectivity of multijunction sensors

Electrical signal responses in multijunction sensors targeted for each microorganism were shown in Figure 4A and B. When *E. coli* suspension was flowed in channel, the sensor functionalized with anti-*E. coli* was responded with high linearity ($R^2 = 0.994$) in current change values as *E. coli* concentrations increased in range of 10^2 to 10^5 CFU/mL. The ΔI values were calculated from 246.2 to 493.2 nA. However, current changes in anti-*S. aureus* functionalized sensor against *E. coli* ranged between 17.8 and 29.7 nA, which might be attributed to background noise and nonspecific binding of *E. coli* on the junctions. The sensitivity and selectivity tests of the sensor functionalized with anti-*S. aureus* were conducted using the *S. aureus* suspension flow. The anti-*S. aureus* multijunction sensor demonstrated a linear relationship ($R^2 = 0.978$) between the ΔI value and concentrations of *S. aureus* suspension in the range of 10^2 to 10^5 CFU/mL. The ΔI value obtained from the anti-*S. aureus* junction sensor ranged from 86.1 to 240.8 nA, whereas The ΔI value obtained from the anti-*E. coli* sensor in the presence of interfering *S. aureus* was as low as 12.5 to 34.1 nA. Therefore, the detection limits of both anti-*E. coli* anti-*S. aureus* junction sensors were as low as 10^2 CFU/mL and the sensing selectivity were fully validated. The positive false results from the selectivity tests could imply that interfering nontarget bacteria might latch onto the nonspecific region of the sensing junction. However, the signals were as low as 20 to 30 nA and there were no significant sensing differences for various concentrations of interfering bacteria. The magnitudes of ΔI values observed in the multijunction sensor were greater than those from the single junction. The ΔI values in the single junction for detection of *E. coli* were between 75.5 and 173.5 nA, one-third of the values obtained from the multijunction sensor. The reason might be due to enhanced sensing areas and accordingly more frequent antigen–antibody binding reactions permitted in the multijunction sensor.

Simultaneous detection of *E. coli* and *S. aureus*

The cocktail samples in the presence of *E. coli* and *S. aureus* at the equal concentrations were used to investigate the response of the multijunction sensor for simultaneous bacteria detection. Figure 5A and B shows the ΔI values obtained from the sensors targeted for *E. coli* and *S. aureus* in the cocktail solutions. Both bacteria were equally (1:1 mixing ratio) mixed at the concentrations ranging from 10^2 to 10^5. The ΔI values increased as the concentrations of microorganisms increased due to more analytes landed on the bio-nano modified surface. Both sensors showed similar measurement trends with a linearity between ΔI and bacterial concentration (R^2 values of 0.932 and 0.988 for *E. coli* and *S. aureus*, respectively). However, the magnitudes of current responses obtained from mixed bacterial samples were higher than those from pure samples. The anti-*E.coli* functionalized sensor responded the change in current with 413.5 nA to pure solution but calculated 438.1 nA current difference for mixed solution at cell concentrations of 10^4 CFU/mL. This phenomenon observed for anti-*S. aureus* functionalized sensor that the electrical signal was 204.3 nA in pure *S. aureus* suspension, but the signal was changed to 431.1 nA in presence of *E. coli* at the concentrations of 10^4 CFU/mL for both analytes. These signal shifts might be due to the attachment of nonspecific binding bacteria at the sensor surface. The developed continuous flow biosensors showed a great potential for single step multiplexed detection of bacteria.

Conclusions

The limits of detection for pure and mixed microbial samples were achieved in range of 10^2 to 10^5 CFU/mL with a detection time of 2 min. The developed sensing device provided sensitive and selective detection of *E. coli* K12, and *S. aureus* with a flow rate at 1 mL/min. The continuous flow multijunction platform showed a potential for rapid and multiplexed detection of different pathogens with large volume (minimum 1 mL) in comparison to the stationary junction sensor (10 μL). This sensing method can be applied for food safety and quality testing with advantageous rapidity and portability. The sensor was tested for liquid samples in this study but it could be applied for bacterial detection in solid samples including semi-solid or colloid if followed by pretreatment steps, that is dilution and filtration of large particles. Future study will include the establishment of minimum preparation methods for real food samples. The sensor performance will be enhanced by application of electric field; for example, dielectrophoresis (DEP) force to enable viable cells to concentrate toward the junctions, and development of durable and reusable multijunction chips in the future.

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