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A microfluidic biosensor for rapid simultaneous detection of waterborne pathogens

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ABSTRACT

A microfluidic based biosensor was investigated for rapid and simultaneous detection of *Salmonella*, *Legionella*, and *Escherichia coli O157:H7* in tap water and wastewater. The biosensor consisted of two sets of focusing electrodes connected in parallel and three sets of interdigitated electrodes (IDE) arrays. The electrodes enabled the biosensor to concentrate and detect bacteria at both low and high concentrations. The focusing region was designed with vertical metal sidewall pairs and multiple tilted thin-film finger pairs to generate positive dielectrophoresis (p-DEP) to force the bacteria moving toward the microchannel centerline. As a result, the bacterial pathogens were highly concentrated when they reached the detection electrode arrays. The detection IDE arrays were coated with three different antibodies against the target bacterial pathogens and a cross-linker to enhance the binding of antibodies to the detection electrode. As the binding of bacterial pathogen to its specific antibodies took place, the impedance value changed. The results demonstrated that the biosensors were capable of detecting *Salmonella, Legionella,* and *E. coli 0157:H7* simultaneously with a detection limit of 3 bacterial cells/ml in 30 − 40 min.

1. Introduction

The identification and detection of pathogens in water and food supplies are crucial for ensuring food safety and public health, and minimizing economic losses. Many ("Water quality and health strategy 2013–2020," n.d.) diseases (such as guinea worm infection, diarrhea, skin problems, cholera, dysentery, and typhoid) are caused by pathogens found in water sources (World Health Organization (WHO) report). Annually, the waterborne pathogens are responsible for 7.15 million disease cases, 6,630 deaths, and significant economic losses costing the healthcare system over $3.3 billion (Collier et al., 2021). Therefore, developing a rapid, sensitive, and specific biosensing system for efficient monitoring waterborne pathogens is critically important to public health ("Drinking Water Requirements for States and Public Water Systems | US EPA," n.d.).

The U.S. Food and Drug administration (FDA) has established the official microbiological culture method for monitoring pathogens in clinical and food products (Lee et al., 2015; Pan et al., 2014). It is noted that bottled water is classified as food and regulated by the FDA (Simons, 2014), while public water supplies are regulated by the EPA (US Environmental Protection Agency (EPA), 2016). The microbial culture method is based on bacterial enrichment culture and subsequent colony counting (McEgan et al., 2013; Scaturro et al., 2020). Although the bacterial culture technique is generally reliable, it is time-consuming and requires 2–5 days to complete the detection. In addition, some bacterial species are fastidious and difficult to culture (Mobed, 2019). Thus, the culture method is not adequate for rapid detection of bacterial pathogens in public water supplies. Nucleic acid-based assays, such as PCR and qPCR (Park et al., 2011; Zhou et al., 2013), have been used to detect pathogens in water and food samples. Although these methods are known for the high sensitivity, selectivity, and specificity, multiple steps and involved, including pre-enrichment, nucleic acid extraction, and final detection. The total turn-around time is 24 h or more (Hockman et al., 2017; Zheng et al., 2016). Enzyme-linked immunosorbent assay (ELISA), is based on the specific binding of the antibody to the target bacterial analyte. It is rapid, but often used after pre-enrichment

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culture to achieve the required sensitivity JOHNSON, 1995; Wei et al., 2016, e.g., the commercially available Solus Scientific Testing Solutions can detect *Salmonella* in 36 h ("Salmonella ELISA Kits - Creative Diagnostics," n.d.). Although the abovementioned methods are useful in monitoring water sources and supplies, the turnaround time remains to be improved. In comparison, our impedance biosensor selectivity is excellent and is similar to those techniques that depend on antibody antigen binding process. However, the sensitivity is lower than the traditional biosensing techniques that depend on bacterial culture.

Alternative diagnostics techniques have been investigated. Electrochemical biosensors have been studied extensively (Wang et al., 2015; Zou et al., 2018) such as amperometric (Brosel-Oliu et al., 2019a), potentiometric (Güner et al., 2017; Joung et al., 2013), and impedimetric (MacKay et al., 2015; Xu et al., 2016). These biosensing technologies demonstrated high specificity, sensitivity, and rapid detection (Furst and Francis, 2018). Impedance biosensors, e.g., achieved Limit of Detection (LOD) as low as 10 cells/ml (Hoyos-Nogués et al., 2016). In another study, an impedance biosensor was immobilized with an antimicrobial peptide and detected *E. coli O157:H7* strain in a water sample from 10^2 to 10^6 cells/ml at a pH value between 7 and 9 (Jiang et al., 2015). Detailed reviews of various impedance biosensors showed a wide range of biosensor designs, with the recent devices having much higher sensitivity (Bratov et al., 2017; Brosel-Oliu et al., 2019b; Kim et al., 2021; Leva-Bueno et al., 2020). The impedance sensing technique has been combined with technologies, such as magnetic beads/magnetic nanoparticles for cell separation (Molinero-Fernández et al., n.d.; Wang et al., 2016) and screen printed IDE array achieving a LOD of 1.04×10^3 cells/ml for *Salmonella* (Xu et al., 2015). In comparison, the LOD of our impedance biosensor was lower than that of all other impedance biosensors found in literature.

Optical biosensors such as surface plasmon resonance (SPR) (Boulade et al., 2019), surface-enhanced Raman spectroscopy (SERS) (Wu, n.d.), chemiluminescence (Wang et al., 2017), and fluorescence (Ding et al., 2015) have achieved high sensitivity and rapid detection of pathogens. For example, noble metal (such as gold) nanoparticles (NPs) are functionalized with appropriate ligands for detection of the target analyte. The reaction results in a visible color that can be used for quantitative enumeration (Zheng et al., 2019). A SERS-based lateral flow assay (LFA) has been investigated for simultaneous detection of *Listeria monocytogenes* and *Salmonella* with a linear response between 10^2–10^7 cells/mL (Wu, n.d.). The paper-based techniques have reached a LOD of 30–300 cells/mL in food matrices with direct readout (ARY Suaifan et al., 2016). Although these sensing techniques are very promising, expensive equipment is involved and the sensitivity is slightly lower than our impedance biosensor. Other techniques, such as Mass-based biosensors (Zheng et al., 2016), (Ozlap, 2015) are reboust, but less sensitive.

Flow-through chemiluminescence microarray readout system has been investigated for rapid and simultaneous detection of multiple waterborne pathogens (Wolter et al., 2007). When the technique was applied on tap water samples, it achieved a LOD of 3×10^6, 1×10^5, and 3×10^3 cells/mL for *Salmonella typhimurium*, and *Legionella pneumophila*, and *E. coli O157:H7*, respectively, and overall assay time of 13 min (Karsunke et al., n.d.; Wolter et al., 2007)

The advantages of impedance biosensors include high sensitivity, low limit of detection, specificity, selectivity, short detection time, and small sample volume required for testing. The addition of nanoparticles, a nanoporous membrane, nanobeads, a focusing region and microfluidic microchannels has significantly improved the performance. However, the impedance-based biosensors still have certain limitations. First, the sensitivity with some sample matrices, such as ready to eat poultry products for which the testing technique/device must meet the AOAC standard for certification (1 cell/325 gr of product). Therefore, a short enrichment step to increase the bacterial concentration to a detectable level is required. Second, the cost associated with antibodies, manufacturing the biosensor and single use of the devices due to difficulty in removing antigen-antibody cross-linker complex from the electrode surface after testing. However, point of care applications are generally considered expensive especially in poor regions of the world. Developing a technique to clean the electrode surface will allow multiple uses of the devices, thereby reducing the testing cost.

In this paper, we present a microfluidic based impedance biosensor for rapid and simultaneous detection of waterborne pathogens, including *Salmonella, Legionella*, and *E. coli 0157:H7*. The advantages of this biosensor include excellent sensitivity, specificity, selectivity, and rapid detection. The biosensor can detect as low as 3 bacterial cells/ml in 30 to 40 min without the need for a sample enrichment step. The biosensor consists of two regions, one for concentrating the bacterial cells and the other for capturing and detecting via specific antibodies coated on the surface of electrodes. When the antibodies capture the target bacterial cells, the impedance changes which is then recorded. Compared to all other reported biosensing techniques, our impedance biosensor has unique advantage in simultaneously detecting multiple pathogens. This feature enables lowering the cost to a value comparable to that of the commercial biosensors for detection of a single pathogen.

2. Materials and methods

2.1. Biosensor design

In this study, the biosensing device was designed and simulated to concentrate low quantities of waterborne pathogens in water samples, capture and detect the pathogens simultaneously, using three sets of interdigital electrodes. Each electrode set was coated with one type of antibody for specific detection of *Salmonella, Legionella,* and/or *E. coli O157:H7*. The device was tested and validated using tap water samples as well as wastewater samples that were spiked with the abovementioned bacteria. The measured bacterial cell concentration/count for each tested sample was confirmed by bacterial culture. The design of the device includes the following innovative features and shown in Fig. 1. (1) A region for concentrating the waterborne pathogens in the centerline of the microfluidic channel. This region consisted of 2 sets of focusing electrodes connected in a parallel fashion, in a single horizontal microfluidic microchannel with a length, start and end widths of 3 mm, 100 μm, and 300 μm, respectively. Each set consisted of gold electroplated vertical electrode pairs along with tilted gold thin film finger pairs with a ramp-down microchannel.

The design was aimed to generate positive dielectrophoresis (p-DEP) forces to concentrate the bacteria, e.g., *Salmonella*, into the center of the microchannel before the bacteria reaching the sensing microchannel. The device discarded a volume exceeding 90% of the original sample that does not contain bacterial cells via the outer microchannel towards the waste outlets. The ramp down microchannel design generated hydrodynamic forces that also aided the focusing process. The combination of the ramp-down vertical side wall and titled thin-film fingers generated a high E-field gradient that pushed the pathogens toward the centerline of the microchannel regardless of their location across the microchannel's width or height, resulting in a concentrated sample. (2) A region for pathogen (antigen) detection based on impedance change, consisted of a microfluidic microchannel specially designed with fluidic flow control and multiple IDE arrays for simultaneous detection of multiple pathogens including *Salmonella*, *E. coli O157:H7*, and *Legionella* with high sensitivity and no cross contamination. The width of each finger and the spacing between the two parallel fingers were designed and fabricated with 6 μm and 4 μm, respectively. The width and height of the detection region were 33 μm and 28 μm, respectively. (3) The combination of a focusing region and detection region for the detection of either a single or multiple pathogens simultaneously. The use of IDE arrays alone with/without microfluidic channel was studied by the biosensing and bacterial detection field and demonstrated its suitability for rapid detection. However, the devices did not achieve a sensitivity needed for the detection of low numbers of pathogens. Our biosensor

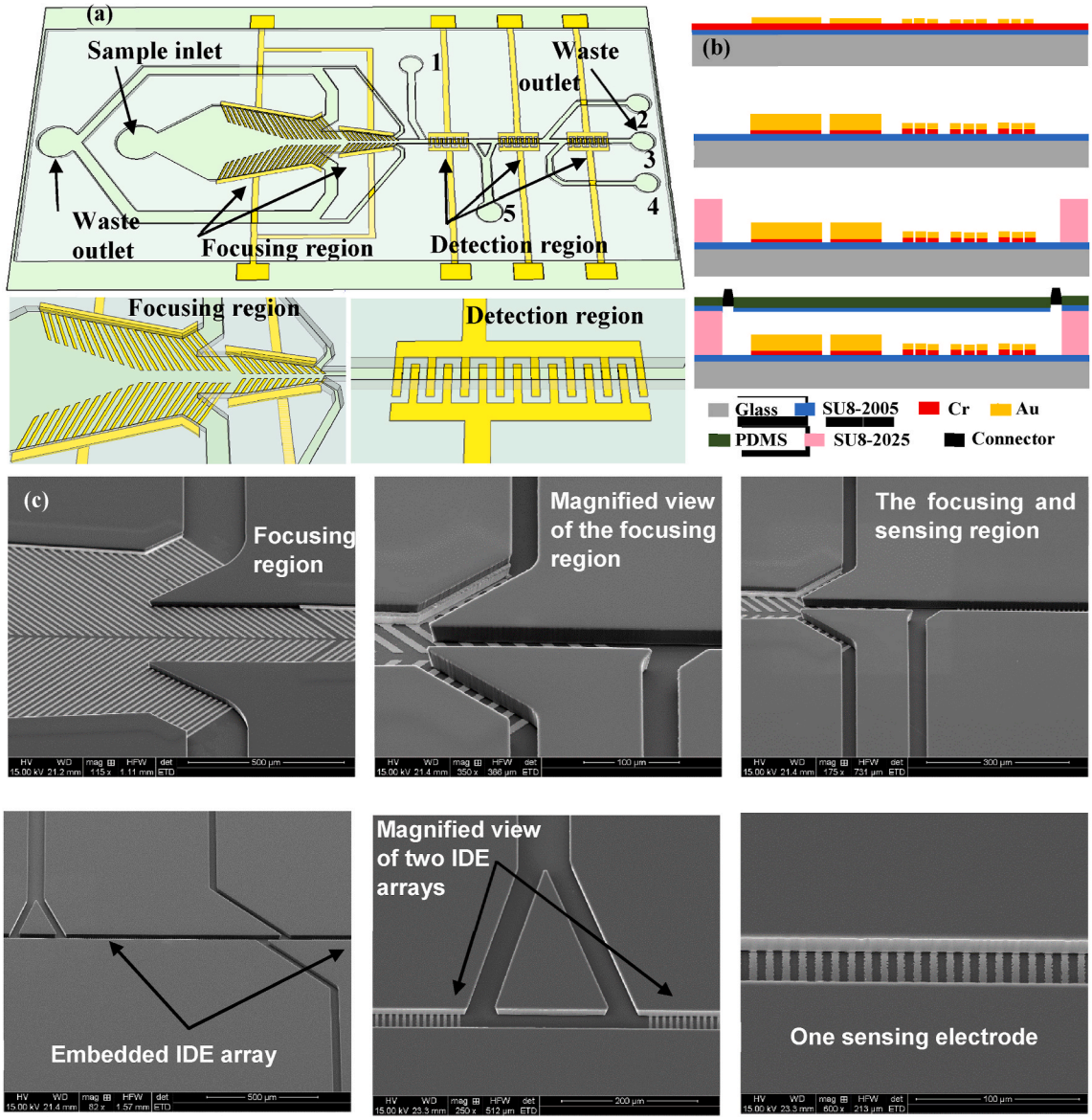

Fig. 1. (a) Three dimensional schematics of the biosensor showing the focusing and the detection regions. (b) Cross-sectional views of the biosensor fabrication processing steps, (c) SEM micrographs of the fabricated device showing the focusing electrodes, the detection electrodes.

with the addition of a focusing region has addressed this issue.

Initially, the electrode surfaces were functionalized with specific antibodies for *Salmonella*, *E. coli O157:H7,* and *Legionella* antigens via the antibody inlets while avoiding any cross contamination, and the antibody did not reach the focusing electrodes. The testing sample, e.g., *Legionella*, was injected from the sample inlet toward the focusing region, and once the detection channel was filled with the sample, the flow was stopped for 10 min so that the *Legionella* antigen can bind to its specific antibody.

2.2. Modelling and simulation

The electric field (E-field) intensity and gradient of the focusing electrode pairs were simulated, using COMSOL Multiphysics software, in order to determine the optimum electrode dimensions that provide the highest sensitivity. To do so, an optimal AC voltage of $V_{p\text{-}p}$ of 6 V at a frequency of 6 MHz was applied to the focusing electrode pairs. The results showed that the E-field gradient was high at the centerline of the microchannel and decreased elsewhere, as shown in Fig. 2(a). Therefore, the pathogens were forced to move toward the microchannel's center due to the generated p-DEP forces, regardless of the height of the microchannel. The concentrated pathogens continued to move toward the detection microchannel while the fluid without pathogens moved toward the outer microchannels and exited via the waste outlets. The ramp down shaped microchannel produced hydrodynamic forces that helped focusing the pathogens too. However, the ramp down feature alone would only achieve a low sensitivity unless the microchannel is very long, which is not practical for such a device. The detection electrode was also simulated using COMSOL. The results demonstrated that the micron scale dimensions of the IDE array significantly increased the detection sensitivity. The spacing between electrode fingers had a greater effect on the E-field gradient strength than the width of the fingers. In this paper, the width of the fingers and spacing between them were chosen as 6 μm and 4 μm, respectively Fig. 2(b).

2.3. Microfabrication

The impedance-based biosensor was fabricated using surface micromachining processes on a glass substrate, as shown in Fig. 1(b). The substrate was first cleaned using the piranha solution, which

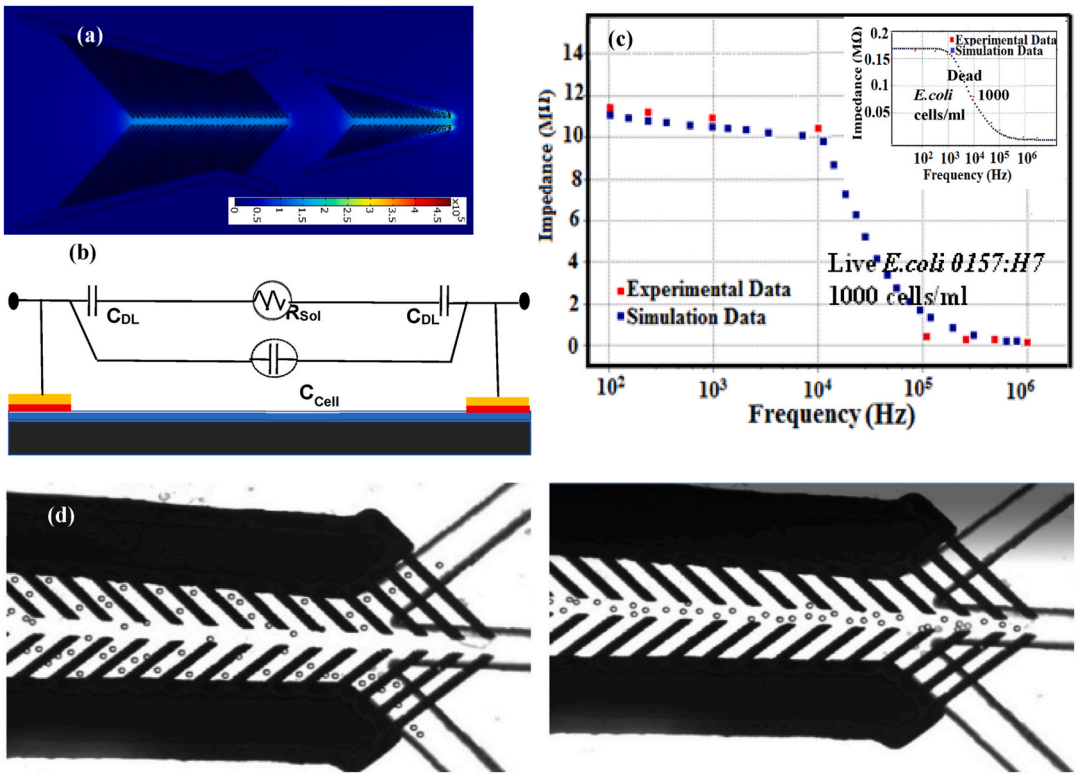

Fig. 2. (a) E-Field simulation of the focusing and detection electrodes, (b) an equivalent circuit of the biosensor, (c) experimental and simulation results for live and dead cells, (d) optical image of the focusing region with (left) no focusing effect, (right) focused microbeads in the center of the channel.

consisted of H_2O_2 and H_2SO_4 at 1:3 ratio, for 5 min to remove all the contaminants and dirt, washed with DI water, and then dried with N_2. The fabrication steps were as follows. (1) SU-8 2005 negative photoresist was coated on the glass substrate using a spinner, prebaked at 65 ° C for 1 min and 95 °C for 2 min on hotplates, UV flood exposed for 10s, followed by post baking at 65 °C for 30 s and 95 °C for 1.5 min to achieve a layer with a thickness of 5 μm. The substrate was then baked at 150 °C for 30 min to harden the photoresist layer. The SU-8 2005 was used to improve the adhesion property between the glass slide surface and the subsequent SU-8 2025 microchannel. (2) Cr and Au thin films were deposited by e-beam evaporator with thicknesses of 50 nm and 150 nm, respectively. The Au layer was patterned using a photoresist (Shipley 1813) and wet etched using a mixture of KI, I_2, and DI water to form the focusing and detection electrodes, the traces and bonding pads, and electroplating seed layer for the vertical electrode sidewall. (3) A thick mold was formed using a photoresist (AZ 4620) layer for the purpose of electroplating gold to form the sidewalls of the focusing electrodes with a thickness of 12 μm. (4) gold was then plated using ready gold electroplating solution (Technic gold 25 ES). The solution was heated and stirred at 54 °C and 70 RPM, respectively, and a current of 60 μA was applied. The photoresist mold was then washed away using acetone and isopropanol. (5) The Cr thin layer was etched using ready to use chrome etchant solution for 20 − 30 s. The substrate was then washed with DI water. (6) To form the microchannel, a negative photoresist (SU8 2025) was coated on the substrate using a spinner followed by prebake at 65 ° C and 95 ° C for 2 min and 5 min, respectively, exposed with UV light for 8 s, post bake at 65 °C, and 95 ° C for 1.5 min, and 4.5 min, respectively, developed, and hard baked at 150 °C for ½ hour to form the microchannel with a height of 28 μm. (7) The microchannel was sealed using two layers of polydimethylsiloxane (PDMS) along with microfluidic connectors for the inlets and outlets. The first PDMS layer was exposed to an oxygen plasma to change the surface property to a hydrophilic, coated with a thin layer of negative photoresist (SU-8 2005) with a thickness of 5 μm, and baked at 95 °C for 10 min to improve the stiction between the PDMS layer and the substrate. The PDMS layer was aligned manually and bonded to the glass substrate with the help of the markers of the inlets and outlets mask and baked at 50 °C for 10 min on a hotplate. During baking, a weight of 4 kg was placed on the substrate to enhance the bonding strength. The second PDMS layer contained microfluidic connectors. It was prepared similar to the first layer and bonded to the first PDMS layer. (8) Finally, the device was placed on a PCB board that was patterned with traces and bonding pads. The device and the PCB were connected together via the bonding pads, a wire, and silver paste. The impedance analyzer (Agilent 4294A) wire was connected to the wire that was fixed on the PCP board, as shown in Fig. 3(a). Scanning electron micrographs (SEMs) are shown in Fig. 1(c).

2.4. Bacterial sample preparation

Salmonella Typhimurium (a lab stock), *E. coli* O157:H7 (ATCC700728), and *Legionella pneumophila* (ATCC 33152) were used in this study. Overnight cultures of the bacteria were mixed with sterile 50% glycerol at 1: 1 ratio and stored at −80 °C as the spiking stocks. The concentrations of the stocks were determined by plating serially dilutions of *Salmonella* and *E. coli* stocks on LB agar plates and *Legionella* on BCYE agar plates and incubating the plates at 37 °C for 24 to 48 h followed by an enumeration of colony forming units (CFUs). Before each test, the bacterial stocks were thawed on ice and used to spike distilled water, tap water, or wastewater. The spiked water samples were serially diluted with the same type of unspiked water to the desired concentrations, such as 100 CFU/ml. One set of the diluted spiked water samples served as the antigens for testing by the biosensor. The other set of diluted spiked samples were plated on agar plates as described above, to confirm bacterial concentrations.

2.5. Antibody preparation

Salmonella antiserum group B (BD Biosciences, NJ), *E. coli* O157:H7

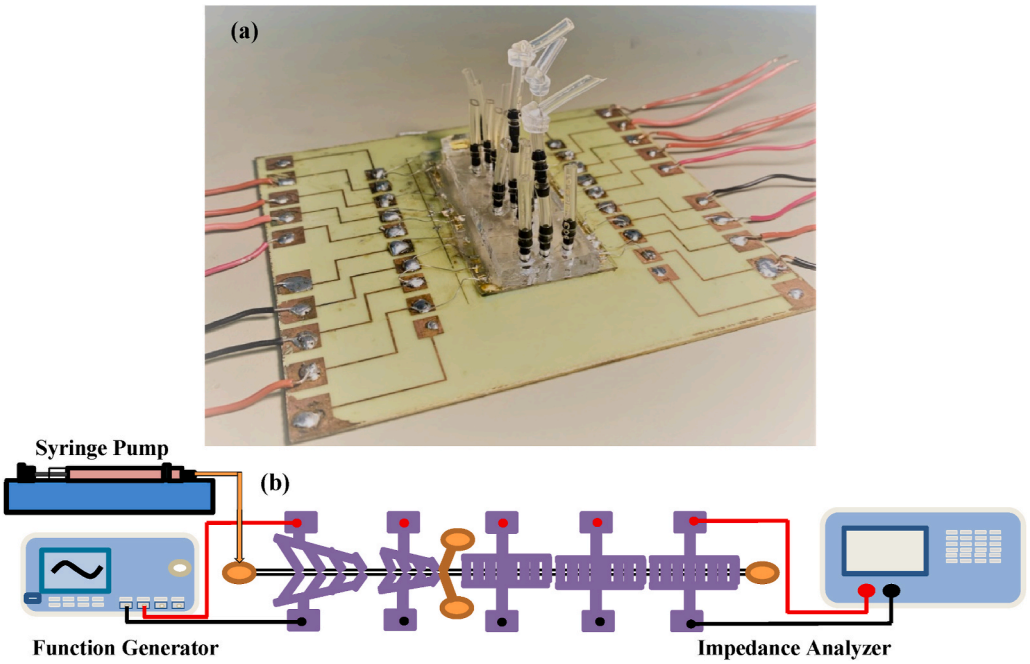

Fig. 3. (a) A photo of the packaged biosensor on a PCB board. (b) A schematic of the pathogens testing setup showing the equipment needed for device testing.

antibody (Invitrogen, MA), and *Legionella* antibody (Invitrogen, MA) were used in the study. Before each test, the antibodies were treated with the cross-linker, sulfosuccinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate (sulfo-LC-SPDP), to enhance their binding to the detection electrode arrays. Briefly, a 180 μL of antibody solution was incubated with the same volume of 20 mM sulfo-SPDP water solution at room temperature for 1 h. Thirty minutes before delivering the cross-linker treated antibody into the biosensor, a 120 μL of DTT solution (0.1 M sodium acetate buffer, 0.1 M NaCl, pH 4.5) was added to reduce disulfide bonds. Preliminary tests had determined that the optimal concentrations were 20 mg/ml for antibodies against *E. coli* O157:H7 and *Legionella* and 1/x dilution for *Salmonella* antiserum.

2.6. Impedance measurement

Antibodies against *E. coli O157:H7*, *Legionella*, and *Salmonella,* were mixed with the cross-linker separately as described in the Antibody preparation section. The solution was then placed at the antibody inlets and directed toward the antibody outlet via applying suction to the antibody outlet. Each electrode was coated with one type of antibody without causing any cross contamination. Initially, the first two IDE arrays were coated with antibodies. This was achieved by placing the antibodies at inlets #1 and #2 while applying suction to inlet #5. After filling the detection channel with the antibody cross-linker mixture, and the flow rate was reduced to zero for a period of 60 min to allow uniform immobilization of the antibody onto the detection electrodes surfaces. The microchannel was then washed using distilled water, and the impedance was measured. The same process was repeated to coat the third electrode via inlet #3 and outlet #4. The impedance was measured for the three electrode arrays, from 100 Hz to 10 MHz. Tap water as well as wastewater samples were spiked with waterborne pathogens, e.g., *Legionella*, and injected from the inlet specified for delivery of the bacterial sample, which was connected to the focusing region. The bacterial antigen solution subsequently entered the detection microchannel. After filling the detection microchannel with the bacterial sample, the flow rate was reduced to zero for a period of 10 min to expedite the contact and binding of antigens to antibodies, e.g., *Salmonella* to anti-*Salmonella* antibody selectively. The microchannel was then washed with distilled water, leaving only the antibody-antigen complex on the IDE arrays. The impedance of each electrode was measured and recorded again. The impedance of each waterborne pathogen, e.g., *Legionella*, was established by subtracting the impedance value of the antibody from the impedance that was measured after antibody-antigens binding.

2.7. Testing setup

The impedance-based biosensor was tested for the detection of waterborne pathogens using the experimental testing setup shown in Fig. 3(b). Syringe pumps (a Harvard Apparatus PHD 2000) were used for injection/suction of the antigens and antibodies through the biosensor sample and antibodies inlets and outlets. A function generator was used to apply an AC voltage at a particular frequency to the focusing electrode pairs. In addition, an impedance analyzer (Agilent 4294A) was used to measure the impedance of the IDE array, between 100 Hz and 10 MHz, before and after coating the antibody and after delivering the bacterial sample. An inverted microscope was used to observe the fluid flow through the microchannel during the experiment.

3. Results and discussions

3.1. Equivalent electrical circuit

We have constructed an equivalent electrical circuit to represent the impedance response of the biosensor. The electrical circuit consisted of the resistance of the solution (R_{Sol}) and the double-layer capacitance (C_{DL}) between the IDE array surface and the solution. Both R_{Sol} and C_{DL} were connected in series. The impedance response of the IDE array was simulated when the bacterial antigen solution reached the detection area. The equivalent resistance of solution R_{Sol} was generated when an AC voltage was applied to the IDE array, where a current was directed through the circuit. The dielectric capacitance ($C_{Parallel}$) was another generated component which was directly connected in parallel with both C_{DL} and R_{Sol}. The value of this capacitance depended on the dielectric constant of the solution and the structural geometry of the IDE array. In addition, electrical wiring could affect the electrical circuit. However, it was ignored since its value was very small in comparison with the measured resistance of the solution, and thus was considered to be zero. Therefore, the total value of the impedance of the equivalent

circuit included the R_{Sol} and the two capacitors C_{DL}. Fig. 2(b) presented the primary equivalent circuit where S is the area for electrode and D the space between the consecutive fingers of the IDE array. The resistance value was expressed by the following equation.

$$R = \rho \frac{D}{S}$$

Where the solution resistivity represents the main factor that had a significant effect on the resistance. Again, the wiring resistance was neglected due to its a small value. Hence, the equivalent impedance of the circuit could be obtained by summing the branches of resistance R and the capacitor C. The total impedance was expressed by:

$$Z_{Dl} = 2Z_{Dl} + R_{sol}$$

$$Z_{Dl} = {}^{1}/_{i\omega c}$$

$$C = \frac{\varepsilon_r \varepsilon_0 s}{D}$$

Where ε_0 and ε_r are the vacuum permittivity, and the relative permittivity of the solution, respectively. ω is the angular frequency in radians per second. The impedance was totally independent of the capacitance C and depended on the solution resistance because the frequency bandwidth was low. In contrast, the impedance of this capacitor had a substantial impact on the equivalent impedance value at the high frequency. An EIS spectrum analyzer was utilized in this section to analyze the equivalent circuit response where a bacterial testing solution filled the microchannel. The value of C and R_{Sol} for the *E. coli O157:H7* antigen with a concentration of 1000 cells/ml were obtained to be 20 nF and 3.5 MΩ, respectively. R_{Sol} had large values because of the significant large number *of E. coli O157:H7* cells that were captured on the electrodes' surfaces. Fig. (2) clearly showed the two main parts of the impedance response for the equivalent circuit in terms of frequency. For example, Fig. 2(c) shows that the impedance value depends only on the dielectric capacitance at higher frequencies. Consequently, the bacterial cells did not have a substantial impact on the impedance value.

The result showed that the cell resistance has a larger impact on the impedance value while dielectric capacitance had a smaller impact on the impedance value at the low-frequency range. In other words, the number of bacterial cells in the solution was considered to be the main cause behind the change in the impedance response. For that reason, the range of frequency was performed between 100 Hz and 1 MHz to achieve the acceptable Bode plot.

3.2. Focusing effect

To demonstrate concentrating the bacterial cells, dielectric beads with a diameter of 4 μm were injected from the sample inlet toward the focusing region. At the same time, an optimum AC voltage at a specific frequency was applied to the focusing electrode pairs to generate p-DEP forces that forces the beads to move toward the centerline of the microchannel. The optimum voltage and frequency were determined to be Vp-p of 6 V and 6 MHz, respectively. The focused and concentrated microbeads continued to flow into the detection region. Fig. 2(d) shows the microbeads inside the microchannel before and after generating the p-DEP forces. The figure shows a large number of microbeads focused into the centerline of the microchannel. Polystyrene microbeads were chosen because of their similar permittivity to that of bacterial pathogens and the ease of visualization under a microscope. The relative permittivity of the microbeads and bacteria, e.g., *Salmonella*, to free space are 3 ("The Engineering ToolBox," n.d.), and 4 (Chiara Biagi et al., 2015), respectively. The magnitude of the impedance change of the IDE arrays indicates the status of the sample as either positive or negative.

3.3. Antibody coating time

Antibodies against each target pathogens (*Salmonella*, *E. coli O157: H7,* and *Legionella*) were mixed with a cross-linker separately. The cross-linkers were used to improve the adhesion of antibodies to gold detection electrodes surfaces. The antibodies cross-linker mixtures were immobilized on the gold surface of the three IDE arrays via the inlets designed for injection of antibodies without causing any cross contamination. After filling the detection channel with the antibody cross-linker mixture, and the flow rate was reduced to zero for a period of 60 min to allow uniform immobilization of the antibody onto the detection electrodes. The microchannel was then washed using distilled water, and the impedance was measured. Tap water samples and wastewater samples were spiked with waterborne pathogens and injected from the sample inlet toward the focusing region. The bacterial antigen solution subsequently flowed into the detection microchannel, where each electrode was functionalized with one specific antibody to allow selective and simultaneous antigen detection. After filling the detection microchannel with the bacterial sample, the flow rate was reduced to zero for a period of 10 min to help increasing the contact and binding of antigens to antibodies, e.g., *Salmonella* to anti-*Salmonella* antibody selectively. The microchannel was then washed with distilled water again, leaving only the antibody-antigen complex on the three detection electrodes. The impedance of each electrode was measured and recorded again. The impedance of waterborne pathogens, e.g., *Legionella* alone, was established by subtracting the impedance value of the antibody from the impedance that is measured after antibody-antigens binding.

To determine the antibody coating time, the antibody crosslinker mixture for *Legionella* were injected from the antibody inlets toward the detection electrodes without causing any cross contamination. Once the detection channel was filled, the flow was stopped for a specific period of time to allow *legionella* antibody to be adsorbed non-specifically to the gold surface of the IDE arrays. Four different coating time periods, ranging from 0.5 to 3 h, were chosen. The microchannel was washed with DI water for 15 min to remove the unbounded antibodies and particles. The impedance of each IDE array was measured as a baseline impedance. Next, bacterial suspensions containing a known number of targeting *Legionella* (e.g., 100 cells/ml) were injected over the immobilized antibodies via the sample inlet. The target *Legionella* bound to the antibody due to the specificity of the capture antibody for the *Legionella* strain. Any unbound bacteria were washed away using distilled water, leaving the securely bonded antigen/antibody complex on the sensor array. After incubation, the impedance was measured again. The impedance of *Legionella* antigen alone was determined by subtracting the antibody impedance from the total impedance after antibody-antigens binding. The results demonstrated that the 1 h antibody binding provided optimal antibody coating, as shown in Fig. 5(d). Longer binding (more than 1 h) did not significantly increase the impedance. Therefore, 1 h of binding was used throughout this study.

3.4. Testing of waterborne bacterial pathogens

The presence or absence of waterborne pathogens was tested using tap water samples. The samples were spiked with *Legionella, E. coli O157: H7, and/or Salmonella* at various concentration concentrations between 3–1000 cells/ml. First, each detection electrode was coated with antibodies for *E. coli O157:H7, Legionella, and/or Salmonella* using the steps and procedure described earlier, then the bacterial samples were flown into the biosensor. The antibody impedance was measured after cleaning the channel with distilled water.

The first set of experiments was performed using samples spiked with a single type of pathogens e.g., *Legionella.* After filling the detection channel with the bacterial samples, the flow rate was reduced to zero for 10 min. Then distilled water was injected into the biosensor for the purpose of cleaning the microchannel, leaving only the antibody-antigen complex. The impedance was measured again, and the

antibody impedance was subtracted. The difference (impedance change) confirms the presence/absence of targeted pathogenic cells. The value of the impedance change indicated if the sample had a low, medium, or high concentration of bacterial cells. The results demonstrated that antibody/antigen binding resulted in a strong impedance change. The limit of detection (LOD) was 3 cells/ml. The device was also measured without applying the p-DEP forces to the focusing electrode Fig. 4(a–c). The results with and without p-DEP effect were compared and plotted as a function of concentration in Fig. 4(d–f). It is clear that the addition of the focusing electrodes has significantly increased the detection sensitivity by a factor between 5 and 6.2.

The device was subsequently tested on tap water samples spiked with two types of waterborne pathogens (*Legionella* and *Salmonella, Salmonella and E. coli O157: H7,* Fig. 5(a and b) and all three pathogens, *Salmonella*, *Legionella*, and *E. coli O157:H7* as shown in Fig. 5(c), at a concentration of 5 bacterial cells/ml and 100 bacterial cells/ml.

To maximally mimic real samples, non-autoclaved wastewater (pH 7) was tested. Briefly, wastewater samples collected in April 2020 were tested and confirmed negative for *Legionella pneumophila* via bacterial culture. The wastewater samples without further treatment (such as autoclaving) were spiked with *Legionella pneumophila* at concentrations of 100, 10, and 3 bacterial cells/ml. One set of spiked samples were plated on BCYE agar plates to verify bacterial concentrations. The plates were incubated at 37 °C for 48 h, and then the colonies were counted. When the spiking concentrations >100 CFU/mL, 100 μL of each sample was plated, and two plates were prepared. The actual concentration of *Legionella pneumophila* in the sample was calculated as the average number of colonies on the two plates, and was multiplied by ten. For the concentration <100 CFU/mL samples, 1 mL sample was plated on ten plates, and the sum of colonies on the plates was the actual concentration of *Legionella pneumophila* in the samples. The second set of the spiked samples were tested by the impedance biosensor. Fig. 6(a) shows

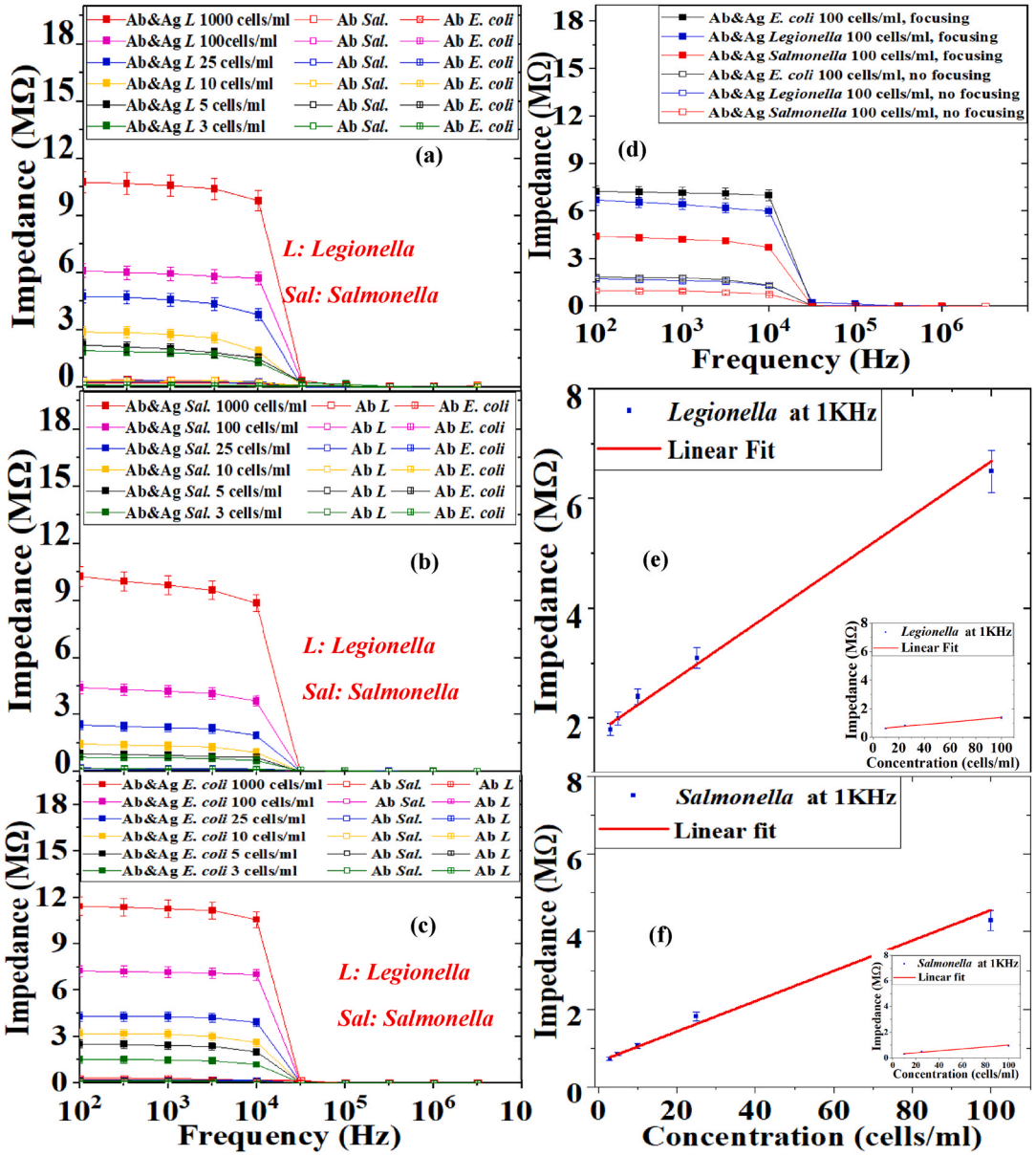

Fig. 4. (a) Detection of (a) *Legionella,* (b) *Salmonella, and* (c) *E. coli 0157: H7 with a concentration of* 1000, 100, 25, 10, 5 and 3 cells/ml. The three electrodes are coated with antibodies for *Legionella, Salmonella, and E. coli 0157: H7* without causing any cross contamination. The impedance change was plotted, at 1 kHz, as a function of concentration for (d) a comparison between impedance changes with the focusing electrode on and off condition. The results demonstrate that the addition of focusing region have improved the detection sensitivity by a factor ranged between 5 – 6.2. (f) *Legionella,* and (e) *Salmonella.* The results show the impedance change increases linearly with concentration. The inset shows the impedance change without applying focusing effects.

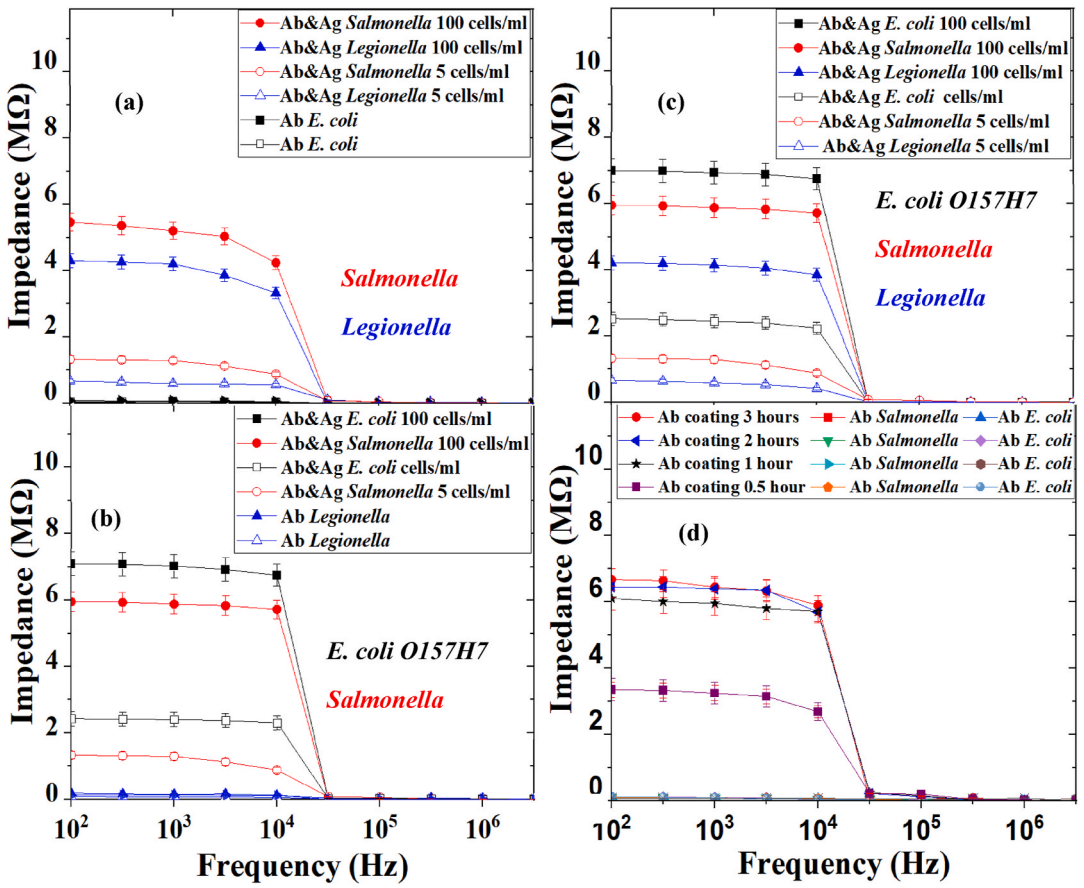

Fig. 5. Simultaneous detection of (a) *Salmonella* and *Legionella*, (b) *Salmonella* and *E. coli O157:H7, and* (c) *Salmonella, Legionella* and *E. coli O157:H7.* Tap Water Samples were spiked with pathogens equally with concentration of 100 cells/ml and 5 cells/ml. The three electrodes were coated with antibody-cross linker mixture for *Legionella, Salmonella, and E. coli 0157: H7* without causing any cross contamination. (d) Immobilization study of antibody mixed with cross linker, four different antibody coating time (0.5, 1, 2 and 3 h) were tested.

the impedance change of the wastewater samples that were spiked with 3 cells/ml, 10 cells/ml and 100 cells/ml. The results demonstrated that the device was able to detect pathogens in a complex sample at low concentrations. We have also tested the wastewater samples before spiking them with bacterial cells, and confirmed negative status, in agreement with bacterial culture as shown in Fig. 6(b). The impedance changes of the negative samples were slightly higher than tap water samples. However, this value was much lower than the impedance changes for the 3 cells/ml samples. Fig. 6(c) shows successful specificity testing result. The wastewater samples were spiked with *E. coli O157 and Salmonella* with concentration of 100 cells/ml with *Legionella* antibody.

3.5. Testing of bacterial dead cells

In this study, the ability of the device to differentiate dead and live bacterial pathogens were also investigated. The bacterial cells were killed by incubating at 90 °C for 3 min and then injected into the sample inlet. The impedance change was very small in comparison to that of live bacterial cells at the same concentration. The low impedance value could be due to damages to bacterial surface that decreased the binding probability. Fig. 6(d and e) shows the impedance values for high concentrations of live and dead *E. coli O157:H7*, *Legionella*, *Salmonella*. The biosensor successfully differentiated live and dead *Salmonella.* Fig. 6(f) shows impedance changes for the lowest concentration of live *E. coli O157: H7* (3 cells/ml), a high concentration of dead *E. coli O157: H7* (1000 cells/ml), and non-specific bindings of *Salmonella* and *Legionella* (1000 cells/ml) to antibody against *E. coli O157: H7*. It is clear that the device could differentiate the lowest concentration of *E. coli* from a high concentration of dead *E. coli O157:H7*, and non-specific binding of *Legionella* and *Salmonella*. In addition, the figure clearly showed that the device was able to detect *E. coli* while avoiding false positive results.

The minimum time needed to detect the pathogens was 30 to 40 min which included 10 min for antibody-antigens binding and 20 to 30 min for washing the microchannel after the sample was injected and left 10 min on top of the detection electrode. The antibody coating time was not included in the minimum time estimation because the device was treated as a disposable one, which means that the biosensors would be coated with antibodies before the sale.

In summary, the testing results demonstrated that this biosensor is important for the following reasons. (1) It is capable of simultaneously detecting three types of pathogens using impedance measurements. This feature allows to lower the biosensor manufacturing cost to a value competitive with that of the currently available commercial biosensors. (2) It has very low LOD, which is a crucial for reliable detection of pathogens at low concentrations and meeting the United States Environmental Protection Agency (EPA) requirements. Although the LOD was determined as 3 cells/ml, the device could detect lower than 3 bacterial cells/ml (See Fig. 4 a, b and c).

Nonetheless, the LOD of this device represents a significant improvement in sensitivity, compared to the impedance biosensors without a focusing region. The impedance change was >0.7 MΩ while the impedance of non-specific binding is < 0.3 MΩ. (3) The short turnaround time of is 30 to 40 min is beneficial to the end-users (such as a water plant or an onsite lab) in terms of operational efficiency as well as public health decision makers. For example, when a water source's pipe is broken, the city officials request the affected community to heat

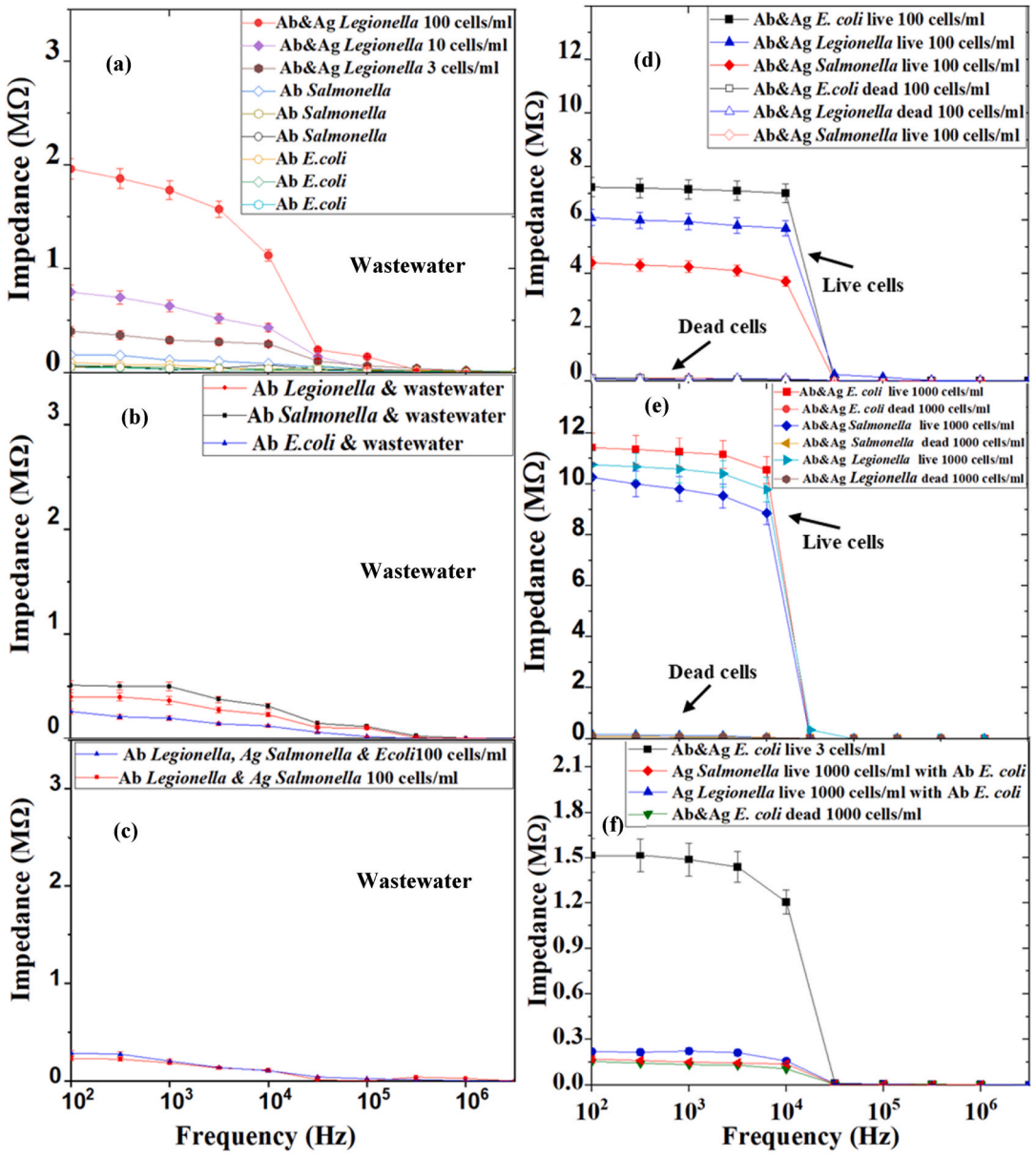

Fig. 6. (a) Wastewater samples were spiked with *legionella* with different concentration (100,10 and 3 cells/ml). (b) Negative wastewater samples. (c) Specificity testing, wastewater samples were spiked with *E. coli O157 and Salmonella* with concentration (100 cells/ml) with *Legionella* antibody. Comparison between (d) live and dead *legionella, E. coli O157: H7 and Salmonella* using spiked water Samples, (e) live and dead *E. coli O157: H7, Legionella and Salmonella* using spiked water samples. (f) low concentration of live *E. coli O157: H7* (3 cells/ml), dead *E. coli O157: H7* cells with high concentration (1000 cells/ml), and non-specific binding of *Salmonella* and *Legionella* with high concentrations of (1000 cells/ml) to *E. coli O157: H7* antibody using tap water samples.

the water before use until it tests negative. Thus, a rapid testing will reduce the waiting time. The simultaneous detection, low LOD, and rapid testing made the impedance biosensor very competitive to current commercial technologies used for detection of waterborne as well as foodborne pathogens.

4. Conclusions

A microfluidic based impedance biosensor was investigated for rapid simultaneous detection of *Salmonella*, *Legionella*, and *E. coli O157:H7* in tap water and wastewater samples. The biosensor was designed as a disposable device for one-time use in the field or in laboratories. The device has a wide range of applications including testing tap water, recreational water, cooling towers, and wastewater. The addition of two sets of focusing electrode arrays increases the detection sensitivity via concentrating the bacteria in the sample by removing more than 90% of the fluid. The three sets of IDE arrays coated with specific antibodies enable simultaneous detection of three pathogens, with a high sensitivity, selectivity and specificity. The microfluidic channel with a fluidic flow control enables antibody coating without contaminating the control and focusing electrodes. The device was tested against one, two and three waterborne pathogens with a LOD of 3 bacterial cells/ml. The overall detection time was 30 to 40 min, including 10 min for antibody-bacterial antigen binding and detection, and 20 to 30 min for washing the microchannel. The experimental results demonstrated that the addition of two sets of focusing electrodes enhanced the detection sensitivity by a factor of 5 to 6.2 times. The biosensor was able to differentiate live and dead bacteria even at a low concentration of live *E. coli O157: H7* (3 cells/ml) and a high concentration of dead *E. coli O157: H7* (1000 cells/ml). The biosensor was also specific as evidenced by no or minimal impedance changes when *Salmonella* and *Legionella* at a high concentration of 1000 cells/ml were applied to electrodes coated

with anti- *E. coli O157: H7* antibodies.

CRediT authorship contribution statement

Sura A. Muhsin: Investigation, contributed to Investigation, Validation, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Muthana Al-Amidie:** Investigation, Data curation, contributed to Investigation and Data Curation. **Zhenyu Shen:** Investigation, Validation, contributed to Investigation, and Validation. **Zahar Mlaji:** Investigation, contributed to Investigation. **Jiayu Liu:** Conceptualization, contributed to Conceptualization. **Amjed Abdullah:** contributed to Investigation. **Majed El-Dweik:** Resources, contributed to Resources. **Shuping Zhang:** Conceptualization, contributed to Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. **Mahmoud Almasri:** Conceptualization, contributed to Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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