



Direct detection of *Salmonella typhimurium* on fresh produce using phage-based magnetoelastic biosensors

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ABSTRACT

Current bacterial detection methods require the collection of samples followed by preparation and analysis in the laboratory, both time and labour consuming steps. More importantly, because of cost, only a limited number of samples can be taken and analyzed. This paper presents the results of an investigation to directly detect *Salmonella typhimurium* on fresh tomato surfaces using phage-based magnetoelastic (ME) biosensors. The biosensor is composed of a ME resonator platform coated with filamentous E2 phage, engineered to bind with *S. typhimurium*. The ME biosensors are wireless sensors, whose resonance oscillation and resonance frequency are actuated and detected through magnetic fields. The sensors used in this study were 0.028 mm × 0.2 mm × 1 mm in size. In this study, the tomato surface was spiked with *S. typhimurium* suspensions with concentrations ranging from 5×10^1 to 5×10^8 CFU/ml and then allowed to dry in air. The detection was conducted by directly placing ME measurement biosensors and control sensors on the spiked surface for 30 min in a humid environment. The control sensors were identical to the measurement biosensors, but without phage. Both measurement and control sensors were blocked with BSA to reduce non-specific binding. The resonance frequencies of both measurement and control sensors were measured prior to and after the placement of the sensors on the tomato. Shifts in the resonance frequency of the measurement biosensors were observed, while the control sensors showed negligible change. Scanning electron microscopy (SEM) was used to verify the specific binding of *S. typhimurium* to the biosensor. Results of multiple biosensor detection and corresponding analyzes showed statistically different responses between the measurement and control sensors for tomatoes spiked with *S. typhimurium* suspensions with concentrations of 5×10^2 CFU/ml and greater. This study demonstrates the direct detection of food-borne bacteria on fresh produce.

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1. Introduction

Food-borne illnesses pose an imminent threat to public health and can result in significant economic loss due to medical costs, lost productivity, recall costs and unsaleable produce. In recent years, consumption of fresh fruits and vegetables has increased rapidly due to increased awareness of the benefits of a healthy diet (Heaton and Jone, 2007). Unfortunately, along with the increase in fresh produce consumption, several high profile food-borne illness outbreaks associated with fresh fruits and vegetables have occurred (Hanning et al., 2009). Prevention of food-borne illness requires rapid detection of pathogens and identification of the contamination source. However, food-borne contamination is difficult to trace. Many factors can cause pathogen contamination on food

products throughout the production process, including pathogen populations present in soil and fertilizers, contaminated irrigation water, poor worker hygiene, and poor equipment sanitation (Johnston et al., 2005). Food products may be cleaned at the harvesting site, transported to a warehouse, and then recleaned and repackaged several times before reaching retail outlets, all of which leaves a lengthy trail that needs to be inspected and monitored. In 2008, a food-borne illness outbreak associated with fresh tomatoes, led to a confirmed 1442 persons infected with *Salmonella* in 29 of 50 states of the United States (CDC, 2008a). FDA teams collected and tested over 1700 samples of tomatoes and none were found to be the outbreak source. The strain of *Salmonella* responsible for the confirmed sicknesses was later (6 months passed) isolated to jalapeno peppers (CDC, 2008b). Despite the issuance of an “FDA safe list” of sources of tomatoes, the public ceased buying tomatoes. This led to an estimated crop loss of over \$100 million. A pathogen detection technique that can be applied on site to provide real-time contamination information is urgently needed.

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Current bacteria detection methods, such as culture and colony counting, polymerase chain reaction (PCR) (Kim et al., 2002; van Belkum, 2003) and antibody-based enzyme-linked immunosorbent assay (ELISA) (Lequin, 2005) techniques, require the collection of many samples followed by sample preparation and analysis of the sample solutions. This testing is tedious and time consuming, resulting in time delays between sample collecting and obtaining the screening results. More importantly, these methods can only provide screening information on the food products since sample collection is labour intensive and costly. Thus seldom are more than one sample analyzed from a large volume of food. The demand for a pathogen detection technique that allows true real-time and *in situ* detection on fresh food products has led to increased research in this area. Fourier transform infrared spectroscopy (FTIR) has been used to directly detect pathogens on corn and apple surfaces with minimal or no sample preparation (Irudayaraj et al., 2002; Yang and Irudayaraj, 2003). The recently developed desorption electrospray ionization (DESI) technique allows mass spectroscopy to be conducted in air, which makes possible the direct characterization and identification of pathogens on food products. This technique has been used to directly detect *Escherichia coli* and *Salmonella* without sample preparation (Jackson et al., 2008; Song et al., 2007, 2009). In spite of these advancements, low cost, real-time pathogen detection in the field remains a challenge.

Recently, free-standing, phage-based magnetoelastic (ME) biosensors have been investigated as a novel wireless biosensor system for real-time pathogen detection (Huang et al., 2008b,c, 2009; Lakshmanan et al., 2007a,b; Wan et al., 2007a,b). The ME biosensors have been successfully shown to detect various pathogens, such as *Salmonella*, *Bacillus anthracis* spores, and *E. coli* (Huang et al., 2009; Lakshmanan et al., 2007a,b; Lu et al., 2009; Shen et al., 2009; Wan et al., 2007a,b), and monitor various biological processes (Pang et al., 2008; Roy et al., 2008a,b; Xiao et al., 2008). A phage-based ME biosensor is composed of an ME resonator platform that is coated with filamentous phage. The phage is the bio-molecular recognition element and is genetically engineered to bind with the target pathogen. The ME resonator platform is made of a magnetoelastic material, which possesses the property that it elongates or contracts along the direction of an applied external magnetic field. Under an applied alternating magnetic field, the ME resonator undergoes a corresponding oscillating shape change that gives rise to a mechanical vibration with a characteristic resonance frequency. The oscillation of the ME resonator results in an emission of a magnetic signal that can be remotely detected using a pick-up coil. The characteristic resonance frequency of a ME resonator depends on its dimensions and the material's properties. For a thin, strip-shaped ME resonator of length L , width w and thickness t ($\ll L, w$), its fundamental characteristic resonance frequency, f_0 , of the longitudinal oscillation is expressed as (Landau and Lifshitz, 1986; Liang et al., 2007):

$$f_0 = \frac{1}{2L} \sqrt{\frac{E}{\rho(1-\nu)}} \quad (1)$$

where E , ρ , and ν are the Young's modulus, density, and Poisson ratio of the material, respectively.

Addition of a small mass ($\ll$ mass of the sensor) on the resonator surface causes a change in the resonance frequency (Δf). Δf is proportional to the initial frequency f_0 , reciprocal of initial resonator mass M and the mass added (Δm) (Grimes et al., 1999). Hence, the sensitivity (S_m) of a strip-shaped ME resonator is given as follows:

$$S_m = \frac{\Delta f}{\Delta m} = -\frac{1}{4L^2wt} \sqrt{\frac{E}{\rho^3(1-\nu)}} \quad (2)$$

The negative sign means the resonance frequency of the ME resonator decreases with an increase of the mass load. Thus, the mass

load on the ME resonator can be easily detected by simply measuring the shift in the resonance frequency. To form a biosensor, a bio-molecular recognition element, E2 phage in this study, is immobilized on the resonator platform surface. When the ME biosensor comes into contact with the target pathogen, the bio-molecular recognition element will bind/capture the target pathogen. This adds an additional mass load on the biosensor. This additional mass causes a drop in the resonance frequency of the biosensor. Therefore, the presence of any target pathogen can be identified by monitoring for a shift in the biosensor resonance frequency.

Due to its wireless nature, ME biosensors can be used for remote detection and real-time *in situ* monitoring. A large number of ME biosensors can be deployed and monitored simultaneously. More importantly, the binding of target pathogens on one out of many ME biosensors can be detected. Therefore, utilizing multiple ME biosensors potentially enables the identification of a small number of pathogens in large volumes of food. Previously investigations using ME biosensors analyzed samples taken of food products (Huang et al., 2009; Lakshmanan et al., 2007a,b; Shen et al., 2009; Wan et al., 2007a,b). In these investigations, a water rinse was used to wash the foods to generate the sample for testing or samples of liquid foods were analyzed. The ME biosensors were placed in the water/liquid food solution or the water/liquid food solution was passed over the ME biosensors for detection. These tests showed that the phage-based ME biosensors exhibited a real-time detection capability with high sensitivity and specificity (Huang et al., 2009; Lakshmanan et al., 2007b; Wan et al., 2007b). In this paper, the direct detection of *Salmonella* on tomato surfaces using ME biosensors was demonstrated. ME biosensors coated with E2 phage (engineered specifically to bind *S. typhimurium*) were used in the detection. The tomato surface was spiked with *Salmonella* cells. The ME biosensors were placed directly on the tomato surface and *Salmonella* was detected, eliminating the water rinse/sampling step.

2. Materials and methods

2.1. Fabrication of the magnetoelastic resonator platform

Magnetoelastic strip-shaped resonator platforms of size of $0.028 \text{ mm} \times 0.2 \text{ mm} \times 1 \text{ mm}$ were fabricated from METGLAS® 2826MB alloy, obtained from Honeywell International. The as-received alloy was in the shape of a roll of ribbon. The ribbon was diced into rectangular shaped platforms with the desired sizes using a computer controlled automatic micro-dicing saw. The sensor platforms were ultrasonically cleaned, first in acetone, and then in ethanol, followed by annealing at 220°C for 2 h in vacuum (10^{-3} Torr) to remove residual stresses. After annealing, two layers of thin films (Cr and Au) were sputtered onto all sides of the sensor platforms. The Cr layer was deposited first in order to improve the adhesion between the ME resonator platform and the Au layer. The Au layer provides a corrosion protection to the resonator (Huang et al., 2008a), and a biological compatible surface for phage immobilization.

2.2. E2 phage immobilization

The filamentous E2 phage for binding to *S. typhimurium* was affinity selected from a landscape f8/8 phage library and provided by Dr. James M. Barbaree's lab in the Department of Biological Sciences at Auburn University (Petrenko and Sorokulova, 2004). The clone E2 phage used in this work has been studied and verified to be highly specific and selective towards *S. typhimurium* (Sorokulova et al., 2005). The phage was immobilized on the ME resonator platform surface using physical adsorption. Each ME resonator platform

was placed in a vial containing 300 μL of phage E2 suspension (5×10^{11} vir/ml in $1 \times$ Tris-Buffered Saline (TBS)). These vials were then rotated and incubated on a rotor (8 rpm) for 1 h. After the immobilization process, the sensors were washed three times with $1 \times$ TBS solution and two times with sterile distilled water in order to remove salt and any unbound or loosely bound phage.

In order to prevent non-specific binding during exposure to analytes, bovine serum albumin (BSA) solution was then immobilized on the sensor surfaces to serve as a blocking agent. The ME biosensors were immersed into 1 mg/ml BSA solution for 1 h, followed by a distilled water rinse. The phage-based ME biosensors were ready to use.

In this study, control sensors were fabricated and used to compensate for the effects of environmental changes, such as temperature, humidity and non-specific binding. The control sensor is identical to the measurement biosensor except it lacks the E2 phage coating. Both the control and measurement sensors were treated with BSA to block non-specific binding.

2.3. Direct detection of *Salmonella* on tomato surface

The *S. typhimurium* (ATCC13311) culture used in this work was provided by Dr. James M. Barbaree's lab in the Department of Biological Sciences at Auburn University, Auburn, AL. The cultures obtained from Dr. Barbaree's lab were provided in the form of a suspension at a concentration of 5×10^8 CFU/ml. The suspensions were serially diluted in water to prepare bacterial suspensions with the concentrations ranging from 5×10^1 to 5×10^7 CFU/ml. All test solutions were prepared on the same day as the biosensor testing. The test solutions were stored at 4°C (during transfer and storage) and equilibrated to room temperature in a water bath prior to the experiments.

Fig. 1 shows the process of direct detection of *Salmonella* on the tomato surface. Tomatoes were purchased from a local grocery

store and spiked with a known volume of known concentration of *Salmonella*. Before the tomatoes were spiked with *Salmonella* suspensions, the tomatoes were rinsed with distilled water once and then dried using a nitrogen gas stream. Pipetted volumes of *Salmonella* suspension (each volume contained 20 μL of suspension) were then placed on the tomato surface. Due to the hydrophobicity of the tomato surface, a 20 μL *Salmonella* suspension formed a drop with a diameter of ~ 3 mm. Therefore, each drop of the suspension covered and spiked an approximate area of 7.065 mm^2 of the tomato surface. The drops on the tomato were then allowed to dry in air (RH = 35%) at room temperature. The resonance frequencies of both measurement biosensors and control sensors were measured prior to placement of the sensors onto the contaminated tomato surface. The measurement biosensors and control sensors were then placed on the spiked tomato. For each area spiked by a drop, three ME measurement sensors and one control sensor were placed on the surface. The tomato was then placed in a humidity controlled chamber (RH = 85%). The ME biosensors and the control sensors were allowed to sit on the tomato for 30 min. The resonance frequencies of the sensors were then measured again. The resonance frequencies of the sensors before and after the placement on the tomato were compared.

The resonance frequencies of the sensors were measured using an HP network analyzer 8751A with S-parameter test set (Huang et al., 2009; Wan et al., 2007b). The ME sensor was placed in a tube, outside of which a pick-up coil was wound and connected to the network analyzer. The analyzer scanned, measured and recorded the resonance frequency spectrum of the ME sensor.

A JEOL-7000F scanning electron microscope (SEM) was used to confirm the binding of *Salmonella* on the phage-coated ME biosensor. After the detection, the ME biosensors were exposed to osmium tetroxide (OsO_4) vapour for 45 min. The biosensors were then mounted onto aluminum stubs and examined using the SEM.

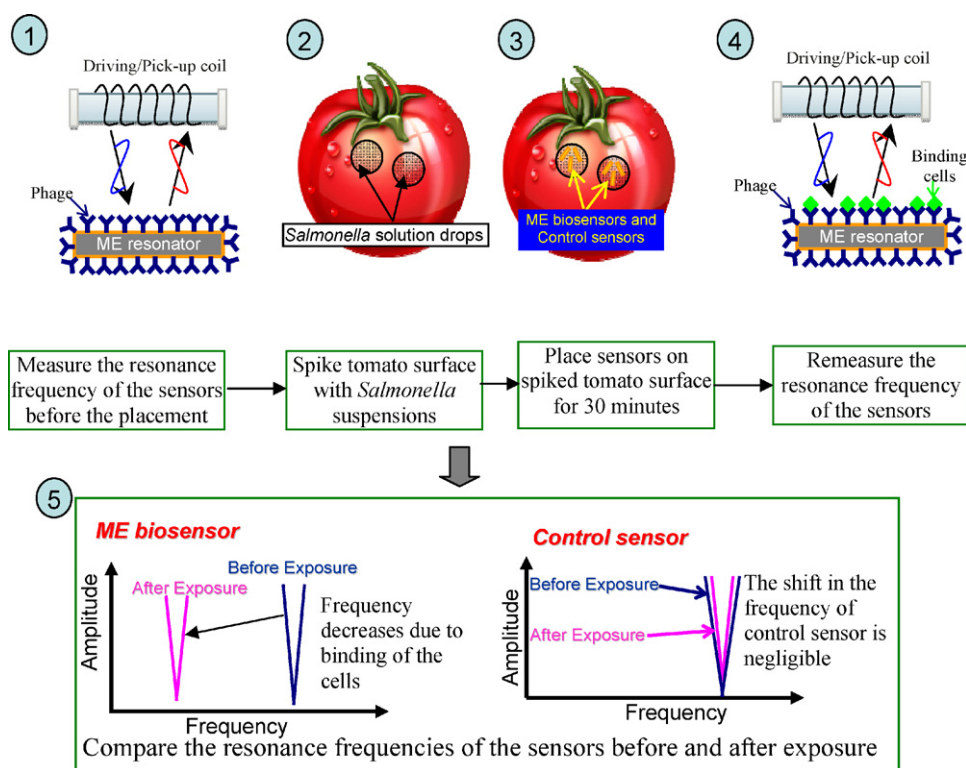


Fig. 1. Scheme of the process used for the direct detection of *Salmonella typhimurium* on tomato surfaces using ME biosensors.

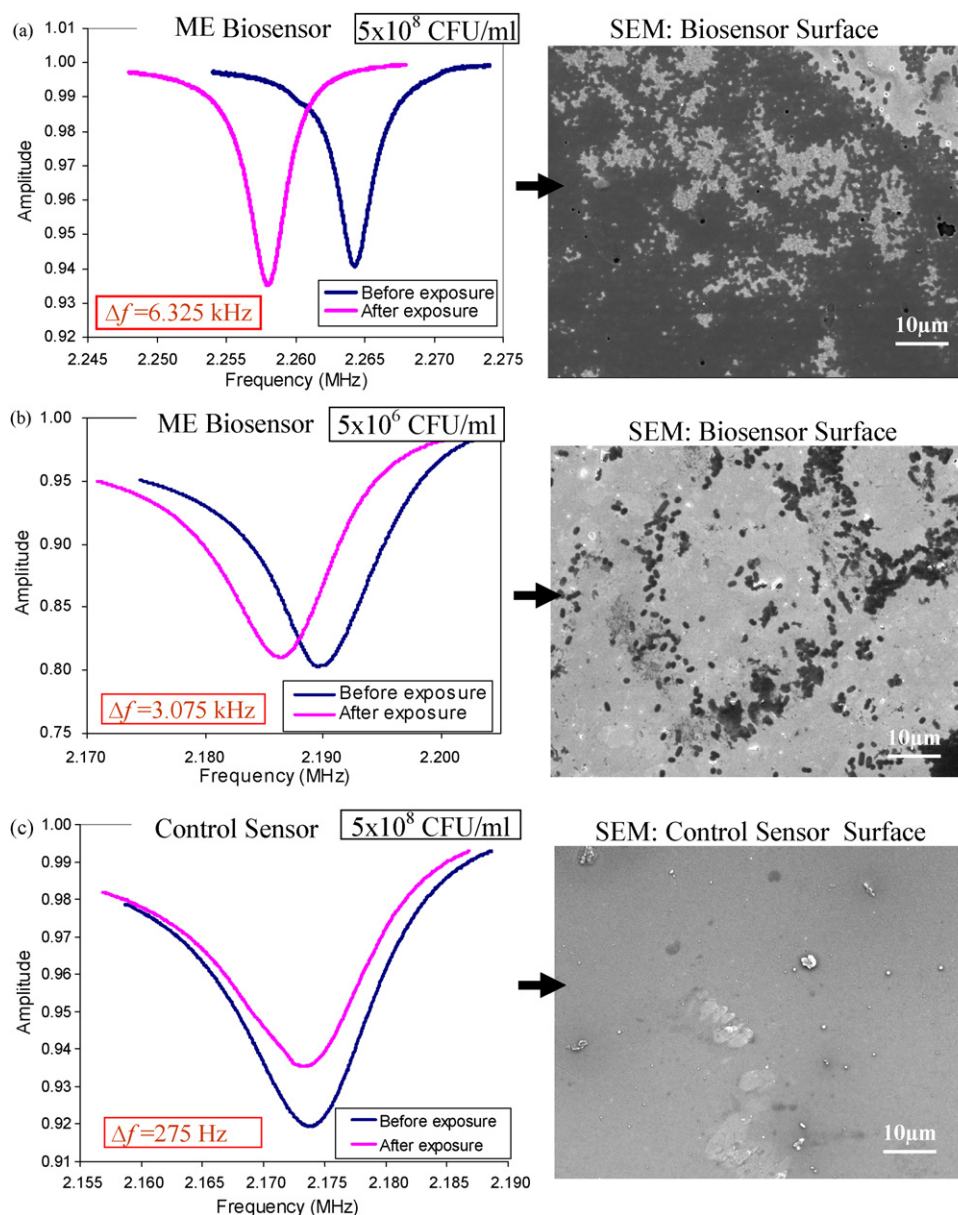


Fig. 2. Resonance frequency curves for ME biosensors and control sensor after exposure to spiked tomato surfaces and corresponding SEM images of biosensor surfaces: (a) tomato spiked with 5×10^8 CFU/ml. Note SEM image shows large number of bound *Salmonella* cells to sensor surface. (b) Tomato spiked with 5×10^6 CFU/ml. Note SEM image shows smaller number of bound *Salmonella* cells to sensor surface. (c) Control sensor exposed to tomato spiked with 5×10^8 CFU/ml. Note very few bound cells.

2.4. Statistical analysis

Multiple ME biosensors were applied to detect *Salmonella* on the tomato surface. All sensors were assumed to have the same physical properties. Statistical analysis was conducted on the responses of ME biosensors exposed to tomato surfaces spiked with different concentrations of *Salmonella*. For each concentration, a one tail unpaired Student's *t*-test was performed to compare the response of E2 phage-coated ME biosensors with the response of control sensors.

3. Results and discussion

The typical resonance frequency changes of the ME biosensors after the biosensors were placed on the tomatoes spiked with *Salmonella* suspensions with concentrations of 5×10^8 and 5×10^6 CFU/ml are shown in Fig. 2(a) and (b), respectively. SEM images of the ME biosensor surfaces after the detection are also

shown in Fig. 2(a) and (b). These photomicrographs confirm that the measured decreases in the resonance frequencies are due to the attachment of *Salmonella* cells to the measurement biosensors. The typical response of the control sensor and the corresponding SEM image of the control sensor surface after exposure of the control sensor to 5×10^8 CFU/ml of *Salmonella* on the tomato surface are shown in Fig. 2(c). The photomicrograph in Fig. 2(c) shows negligible attachment of *Salmonella* cells. As shown in Fig. 2(a), a large shift (6325 Hz for this sensor) in the resonance frequency of the ME biosensor was observed. SEM analysis showed that the biosensor surface was densely covered with bound *Salmonella* cells. As the concentration of the *Salmonella* suspension spiked on the tomato surface decreased, the shift in the resonance frequency of the biosensors decreased and the corresponding SEM images showed binding of a smaller number of *Salmonella* cells, as shown in Fig. 2(b). On the other hand, the control sensors showed no significant changes and little if any cell binding. As shown in Fig. 2(c), only a 275 Hz resonance frequency shift was observed for the con-

control sensor even after the control sensor had been exposed to a tomato surface spiked with *Salmonella* with a concentration of 5×10^8 CFU/ml. The SEM examination showed only a few cells, dust and residual salt attached to the surface of the control sensor. The response of the measurement/control ME sensors and the corresponding SEM images confirm that the shifts in the resonance frequency are due to the binding of the *Salmonella* cells on the biosensor surface. More importantly, the results demonstrated that the binding reaction of the E2 phage with the *Salmonella* cells occurs in humid air. Upon contact with *Salmonella* cells on the tomato, the E2 phage binds the *Salmonella* to the surface of the ME biosensors and changes in the resonance frequency can then be measured wirelessly and remotely. No longer is a sampling technique (such as water rinse or stomaching) needed.

The response of an individual biosensor is strongly dependent on how many cells the sensor contacts upon being placed on the tomato surface. Unlike detection in sampling solutions, where bacterial cells are uniformly suspended in the solution, the distribution of *Salmonella* cells on the spiked tomato surface is not uniform. When placed on the food during the spiking process *Salmonella* bacteria can rapidly migrate and move along the food surface and aggregate at regions containing nutrients and water needed to sustain life. Representative SEM images of the areas on the tomato surface spiked with *Salmonella* suspensions with concentrations of 5×10^8 , 5×10^6 , and 5×10^3 CFU/ml are shown in Fig. 3(a)–(c), respectively. The SEM image of a fresh tomato surface is shown in Fig. 3(d). For the surface spiked with 5×10^8 CFU/ml *Salmonella* suspension, *Salmonella* cells covered almost the whole drop area due to the high concentration of bacteria in the liquid. With a decrease in the suspension concentration, the number of *Salmonella* cells on the surface decreased and the distribution of cells over the surface became more non-uniform. As the water of the spiking solution evaporated, the *Salmonella* cells aggregated to areas of residual moisture and formed clusters. Therefore, the distribution

of *Salmonella* cells became highly non-uniform on the tomato surface as the concentration of *Salmonella* in the spiking solutions was decreased. Additionally, variance in the roughness of the tomato, curvature of the tomato surface, punctures, defects and wounds contributed to a non-uniform distribution of *Salmonella* on the tomato surface.

Due to the non-uniform distribution of *Salmonella* cells on tomato surface, the chance of a sensor coming into contact with cells and the number of cells that bind will depend on where the sensor falls on the tomato surface. This means the response of a single biosensor placed upon the tomato surface may not give an accurate indication of whether the tomato is contaminated. Therefore, it is necessary to apply multiple ME biosensors to different regions of the tomato. Multiple biosensors and control sensors were placed on the spiked tomato surface. Fig. 4 shows the response of multiple ME measurement and control biosensors after exposure to different concentrations of *Salmonella* spiked on the tomato surfaces. The blue diamonds are the change in resonance frequencies of the measurement ME biosensors, while the red squares represent the change in resonance frequencies of the control sensors. Even though there is a large variation in the frequency changes of measurement biosensors for a given spiked concentration (due to how many cells the sensor came into contact with when placed on the tomato), the results of Fig. 4 show there is an obvious difference between the responses of the measurement and control biosensors for all concentrations of 500 CFU/ml and higher. This difference between the control and measurement biosensors indicates detection of the *Salmonella* contamination. As the *Salmonella* concentration of the solution used to spike the tomatoes increases, the difference between the measurement biosensors and control sensors increases as anticipated. Additionally the variance between control sensors was observed to increase for higher concentrations of spiked solutions (5×10^6 CFU/ml and higher). It is expected that shifts in the control sensors may be

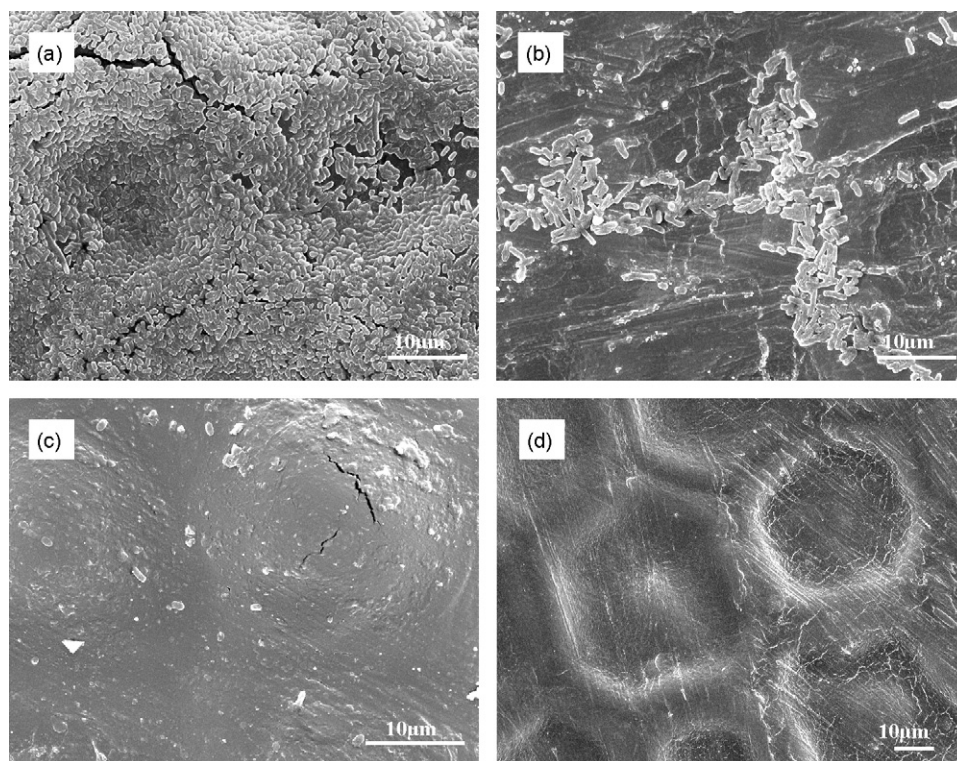


Fig. 3. SEM photomicrographs of tomato surfaces spiked with *Salmonella* concentrations of (a) 5×10^8 CFU/ml, (b) 5×10^6 CFU/ml and (c) 5×10^3 CFU/ml, and (d) fresh tomato surface. Note that the distribution of *Salmonella* cells on the tomato surface becomes highly non-uniform as the concentration decreases.

Table 1
Statistical analysis of the measured resonance frequency shifts of ME biosensors and control sensors for the direct detection of *Salmonella typhimurium* on tomato surfaces.

Salmonella concentration (CFU/ml)	Sensor type	Statistics			
		Mean (Hz)	Standard deviation (Hz)	Probability of <i>t</i> -test	Actual confidence level of different response (%)
5×10^8	Biosensor	2138.5	2730.6	0.103023	89.70
	Control sensor	281.3	352.4		
5×10^7	Biosensor	2201.4	1823.3	0.012172	98.78
	Control sensor	258.3	359.8		
5×10^6	Biosensor	2604.2	907.6	0.002664	99.73
	Control sensor	208.3	691.2		
5×10^5	Biosensor	2507.1	1511.1	0.01188	98.81
	Control sensor	−16.7	225.5		
5×10^4	Biosensor	2262.5	2299.5	0.07767	92.23
	Control sensor	104.2	191.3		
5×10^3	Biosensor	765.6	704.6	0.061436	93.86
	Control sensor	25.0	347.3		
5×10^2	Biosensor	194.4	281.3	0.134195	86.58
	Control sensor	−12.5	185.0		
5×10^1	Biosensor	67.5	237.4	0.446742	55.33
	Control sensor	93.8	150.3		

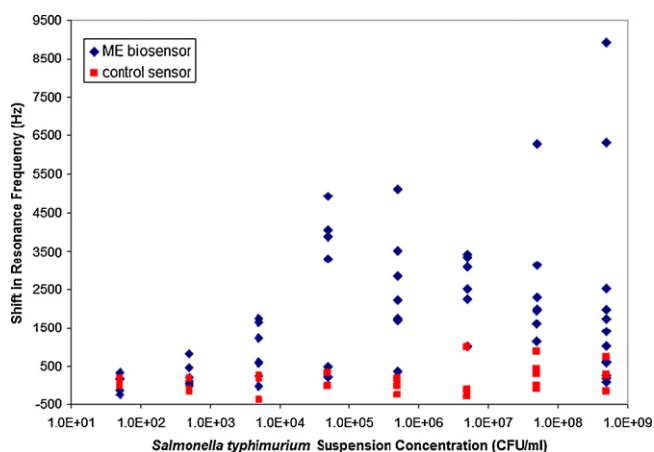


Fig. 4. Measured shifts in the resonance frequencies of ME biosensors and control sensors after exposure to tomato surfaces spiked with different concentrations of *Salmonella*. For concentrations of 5×10^2 CFU/ml and higher, a statistical difference (>80% confidence) between the control and measurement sensors was measured, indicating that contamination of the tomato has been identified.

further minimized through optimization of the BSA blocking procedure.

In order to quantitatively compare the difference between responses of the measurement and control biosensors, a one tail unpaired Student's *t*-test was conducted for each suspension concentration. The results are summarized in Table 1. Based on Table 1, the responses of the phage-based ME biosensors and control sensors showed a statistically significant difference for all concentrations of 5×10^2 CFU/ml and higher, scoring more than 80% confidence that there is a difference between the responses of the control and measurement sensor for these concentrations. This means that statistically *Salmonella* was detected in tomatoes spiked with concentrations of 5×10^2 CFU/ml and higher. Future experimentation will compare ME biosensor results with traditional pathogen detection methods, such as PCR Cell cultures and ELISA.

4. Conclusions

Direct detection of *Salmonella* on tomato surfaces using wireless ME biosensors was demonstrated. Tomato surfaces were spiked with suspensions containing *Salmonella* with concentrations from 5×10^1 to 5×10^8 CFU/ml. Due to *Salmonella* migration and aggregation, surface roughness, curvature, defects and puncture wounds,

the distribution of *Salmonella* on the tomato surface became more non-uniform as the concentration of the spiked solution was decreased. Multiple measurement and control biosensors were placed on the tomato for 30 min under controlled humidity conditions. Shifts in the resonance frequencies of the measurement biosensors were observed, while the change in resonance frequencies of control sensors was negligible. SEM images verified that the frequency shifts of the measurement sensors were consistent with the specific binding of *Salmonella* bacteria to the biosensor surfaces. Multiple control and measurement sensor frequency shifts were statistically evaluated using the Students *t*-test. The results show that *Salmonella* contamination was detected on tomato surfaces spiked with concentrations of 5×10^2 CFU/ml and higher at a confidence limit of 80% or higher. This study demonstrates the direct detection of food-borne bacteria on fresh produce using ME biosensors.

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