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DETECTION OF *SALMONELLA* ON SPINACH LEAF USING PHAGE-BASED MAGNETOELASTIC BIOSENSORS

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ABSTRACT In 2008, a foodborne illness outbreak associated with tomatoes, led to a confirmed 1442 persons infected with Salmonella in 29 of 50 States of the United States. FDA teams collected and tested over 1700 samples of tomatoes and none were found to be the outbreak source. Despite the issuance of an "FDA safe list," the public ceased buying tomatoes, resulting in an economic loss to growers of over $100 million. This presentation describes an investigation into the direct detection of Salmonella on fresh spinach, using phage-based magnetoelastic (ME) biosensors. The wireless biosensors are composed of a ME resonator platform that is coated with filamentous E2 phage (genetically engineered to bind with Salmonella). An alternating magnetic field is used to drive the biosensors into resonance and a pick up coil measures the sensors' resonance frequencies. Upon contact with a target pathogen, the phage binds the pathogen to the biosensor, causing an increase in the biosensor's mass and a corresponding decrease in resonance frequency. Spinach surfaces were spiked with a known number of Salmonella cells and then allowed to air dry. The biosensors were placed on the spinach leaves and measured after 30 minutes. Shifts in the resonance frequency of the measurement biosensors were observed due to binding of Salmonella while reference sensors showed negligible change. The specific binding of Salmonella to the biosensors was verified by Scanning Electron Microscopy (SEM). The results indicate that this methodology may provide real-time contamination information on fresh produce in the agricultural field.

Keywords: Biosensor, Wireless, Magnetoelastic, Salmonella detection, Fresh food produce.

INTRODUCTION Food-borne illnesses pose an imminent threat to the public health and result in a great economic loss. Prevention of food-borne illness requires rapid detection of pathogens and identification of the contamination source. However, foodborne contamination is difficult to trace because products may be cleaned at the harvesting site, transported to a warehouse, and then recleaned and repackaged several times before reaching retail outlets. This leaves a lengthy trail that covers many states and

often more than one country. In 2008, a foodborne 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 1,700 samples of tomatoes and none were found to be the outbreak source. The strain of *Salmonella* responsible for the confirmed sicknesses was later (six 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.

Current bacteria detection methods, such as polymer 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 samples. These methods are time consuming. More important, these methods can only provide selective information about the food products since sample collection requires intensive labor and costs.

Recently, free-standing phage-based magnetoelastic (ME) biosensors have been investigated as a novel wireless biosensor system for possible pathogen detection on fresh fruits and vegetables (Huang et al. 2008a; Huang et al. 2008b; Huang et al. ; Lakshmanan et al. 2007a; Lakshmanan et al. 2007b; Li et al. 2008; Wan et al. 2007a; Wan et al. 2007b). The ME biosensors have been successfully shown to detect various pathogens, such as *Salmonella Typhimurium* and *B. anthracis* spores (Huang et al. 2009; Lakshmanan et al. 2007a; Lakshmanan et al. 2007b; Shen et al. 2009; Wan et al. 2007a; Wan et al. 2007b), and the changes in various material properties (Grimes and Kouzoudis 2000; Grimes et al. 1999; Jain et al. 2000). The magnetoelastic biosensor is composed of a ME resonator platform that is coated with filamentous phage. The phage is the biomolecular recognition element and is genetically engineered to bind with the target pathogen. Utilizing magnetoelasticity, the ME resonator oscillates with a characteristic resonance frequency under an applied alternating magnetic field. The oscillation of the ME resonator results in the emission of a magnetic signal that can be remotely detected using a pick-up coil. Due to its wireless nature, ME biosensors can provide real-time *in-situ* detection remotely. 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 enables identification of pathogen contamination over large volumes of food produce.
Previously investigations of ME biosensors were based on the sampling method (Huang et al. 2009; Lakshmanan et al. 2007a; Lakshmanan et al. 2007b; Shen et al. 2009; Wan et al. 2007a; Wan et al. 2007b). Samples were taken from food products by using a water rinse technique. The ME biosensors were then placed in the solutions or solutions passed over the ME biosensors for detection. In this paper, the direct detection of *Salmonella* on spinach leaves using ME biosensors was demonstrated. ME biosensors coated with E2 phage (engineered specifically to bind *Salmonella Typhimurium*) were used in the detection. The spinach leaves were spiked with *Salmonella* cells. The ME biosensors were placed directly on the leaves and *Salmonella* was detected without using a sampling step.

PRINCIPAL OF OPERATION: THE ME BIOSENSOR The magnetoelastic sensor platform is a resonator fabricated from a magnetoelastic material. A magnetoelastic

material possesses the property that it elongates or contracts along the direction of an applied external magnetic field. Thus, under an alternating magnetic field, the magnetoelastic material undergoes a corresponding oscillating shape change that gives rise to a mechanical vibration with a characteristic resonance frequency. This mechanical vibration consequently causes an emission of magnetic flux from the sensor platform. A pickup coil can be used to measure this magnetic flux and therefore, the resonance frequency of the magnetoelastic sensor platform can be measured remotely and wirelessly.

For a thin strip-shaped ME sensor of length L, width w and a thickness t (<< L, w), its fundamental characteristic resonance frequency, *f*, of this longitudinal oscillation is expressed as: (Landau and Lifshitz 1986; Liang et al. 2007)

$$f = \frac{1}{2L}\sqrt{\frac{E}{\rho(1-\upsilon)}} \quad (n = 1, 2, 3\ldots) \tag{1}$$

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

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

$$S_m = \frac{\Delta f}{\Delta m} = -\frac{1}{L^2 wt}\sqrt{\frac{E}{\rho(1-\upsilon)}} \tag{1}$$

The negative sign means the resonance frequency of the MSP decreases with an increase of the mass load. Thus, the mass load on the MSP can be easily obtained 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 platform surface. When the ME biosensor comes into contact with the target pathogens, 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 pathogens can be identified by monitoring for a shift in the biosensor resonance frequency.

MATERIALS AND METHODS

Fabrication of magnetoelastic sensor platform Magnetoelastic strip-shaped sensor platforms of size of 2x0.4x0.028 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 particles 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 hours in a vacuum (10^{-3} Torr) to remove residual stresses. After annealing, two layers of thin films (Cr & Au) were sputtered onto both sides of the sensor platforms.

The Cr layer was deposited first in order to improve the adhesion between the ME platform and the Au layer. The Au layer provides a corrosion protection layer for the sensor platform, and a biological compatible surface for the phage immobilization.

E2 phage immobilization The filamentous E2 phage for binding to *S. typhimurium* was provided by Dr. James M. Barbaree's lab in the Department of Biological Sciences at Auburn University. The magnetoelastic sensor platforms were placed in vials containing 200 μL of phage E2 (≈10^{11} virions/mL). The immobilization of phage on the sensor surface was done by placing these vials on a rotor (running at 8 rpm) for 1 hour. These sensors were then washed three times with Tris-Buffered Saline (TBS) solution and two times with sterile distilled water in order to remove any unbound or loosely bound phage. The phage-based ME biosensors were ready to use.

In order to prevent nonspecific binding during exposure to multiple analytes, 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 at least one hour, followed by a distilled water rinse. To calibrate the effects of environmental changes, such as temperature and non-specific binding, control sensors are also used in the experiment. The control sensor is identical to the measurement biosensor except it lacks the E2 phage coating. The control sensors were also treated with BSA to block nonspecific binding.

Direct detection of *S. Typhimurium* on dpinach leaves 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.

Spinach leaves were purchased from the local grocery store. To simulate the pathogen contamination on the spinach leaves, the leaves were spiked with *S. typhimurium*. Pipetted volumes of *S. typhimurium* suspension (each volume contained 30 μL of suspension) were dropped on the spinach leaf. The leaf was then allowed to dry in air (RH=30%) at room temperature. The resonance frequencies of both control and measurement ME biosensors were measured prior to placement of the sensors onto the contaminated leave surface. The ME biosensors and control sensors were then placed on the leaf. The leaf was then placed in a humidity controlled chamber (RH>50%). The ME biosensor and the control sensor were allowed to sit on the leaf for 30 minutes. The resonance frequencies of the sensors were then measured again. The resonance frequencies of the sensors before and after the placement on the leaf were compared.

RESULTS AND DISCUSSIONS An SEM image of the area on the spinach leaf spiked with *S. typhimurium* suspension with concentrations of $5x10^8$ cfu/mL is shown in Figure 1. The SEM images show the amount and the distribution of the *S. typhimurium* cells on the spinach leaves. As shown in Figure 1, *S. typhimurium* cells covered almost the whole drop area due to the high concentration. When the E2 phage coated ME biosensors were placed on the leaf, the biosensor and the E2 phage coated on the sensor surface made contact with the *S. typhimurium* cells.

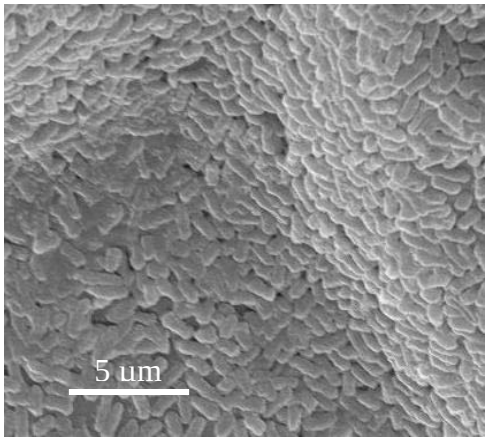

Figure 1. Spinach leaf surfaces spiked with *Salmonella typhimurium* suspension with the concentration of $5x10^8$ cfu/mL.

Figure 2 shows the results of the detection of *S. typhimurium* on spinach leaves using the ME biosensors. The typical resonance frequency changes of the measurement ME biosensor and the control sensor after the biosensors were placed on the spiked spinach leaf are shown in Figure 2 (a) and (b), respectively. SEM images of the measurement biosensor and the control sensor surface after the detection are also shown in the Figure 2 to confirm the attachment of the *S. typhimurium* cells to the measurement biosensors. As shown in Figure 2 (a), a large shift (8462 Hz for this sensor) in the resonance frequency of the ME biosensor was observed for a spinach leaf spiked with 5×10^8 cfu/mL *S. typhimurium*. The corresponding SEM image showed dense coverage of the *S. typhimurium* cells, which confirms that the shifts in the resonance frequency is due to the binding of the *S. typhimurium* cells on the biosensor surface. The SEM images are also consistent with the amount and the distribution of the bacterial cells on the spiked spinach leaves. On the other hand, there was no significant shift observed in the resonance frequency of the control sensor and the SEM image revealed very few *Salmonella* cells attached to the surface of the control sensor, as shown in Figure 2 (b).

The results demonstrate that the binding reaction of the E2 phage with the *S. typhimurium* cells occurs in air at relative humidity levels of 30% or greater. Upon contact with *Salmonella* cells on the leaves, the E2 phage binds 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.

ME biosensor response for detecting on spinach leaf spiked with S. Typhimurium

SEM images of ME biosensor surface after detection

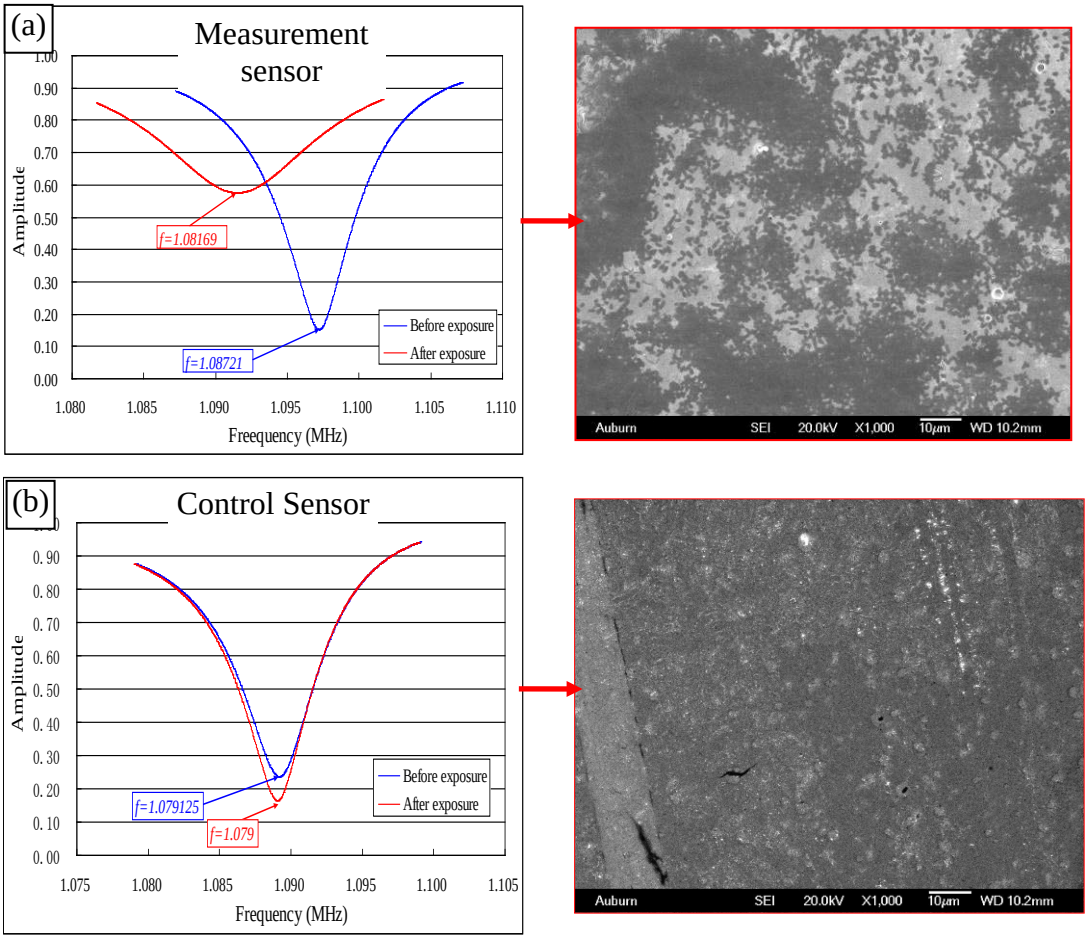

Figure 2. Typical resonance frequency shifts and the corresponding SEM images of the sensor surface for (a) the measurement ME biosensors and (b) the control sensor, as the sensors were placed on the spinach leaf spiked with *Salmonella typhimurium* suspension with the concentration of $5x10^8$ cfu/mL

CONCLUSION Direct detection of *S. Typhimurium* on spinach leaves using wireless ME biosensors was demonstrated. The spinach leaves were spiked with *S. Typhimurium* suspension with different concentrations. After placing the ME biosensors on the leaves for 30 minutes under controlled humidity, shifts in the resonance frequency of E2 phage ME biosensors were observed, while the change in resonance frequencies of control sensors were negligible. SEM images verified the specific binding of *Salmonella* to the biosensor surface. This study demonstrates the direct detection of food-borne bacteria on fresh produce.

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