Direct Detection of Bacteria on Fresh Produce

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Abstract: This paper presents a revolutionary method of bacterial detection that directly detects and quantifies the presence of specific bacteria on the surfaces of fresh produce without sample preparation (water rinse, soak, stomaching) and/or enrichment. The speed of detection is from 2 to 10 minutes with a limit of detection in a range of 10^2 to 10^4 cfu/mm^2. The specificity of detection is 2 in 10^6 background bacteria. This technology was awarded a $20,000 prize in the first United States Food and Drug Administration (FDA) Food Safety Challenge. The method combines wireless magnetoelastic (ME) biosensors and a surface-scanning detector for rapid determination of bacterial contamination. Tests were conducted on tomatoes and grapes spiked with different concentrations of *Salmonella* Typhimurium. The resonant frequency changes of the biosensors were found to be dependent on the surface concentration of *Salmonella*. Detection limits were found to be affected by the surface roughness of the food. A 90-second video of a test for *Salmonella* on tomato can be viewed at http://eng.auburn.edu/food-safety. The method presented in this paper is envisioned for use at ports of entry for the swift screening of foods.

1 INTRODUCTION

The past decades have been marked by a global increase in the outbreaks of food poisoning and associated illnesses. These public health problems are caused by the accidental supply and consumption of contaminated food, largely due to improper safety knowledge, perspectives, and practices of food producers as well as insufficient consumer awareness. Although substantial progress on food safety regulations has been made worldwide, up to 30% of the population even in industrialized countries suffer from foodborne illnesses each year (WHO, 2007). In the United States, approximately 48 million cases of foodborne illnesses are estimated to occur annually, resulting in 128,000 hospitalizations, 3,000 deaths, and $51.0 to $77.7 billion economic losses (Scallan et al., 2011a; Scallan et al., 2011b; Scharff, 2012).

Salmonella is one of the most pervasive food problems today. In the past years in the United States, $1.1 billion was lost, and over 3,000 individuals were confirmed sick due to *Salmonella* food contamination (2008 tomato, 2009 peanut butter, and 2010 egg outbreaks). Since foodborne illnesses spread so easily, rapid, on-site detection can play an important role in preventing the spread of contamination. However, current detection methods of *Salmonella* can take up to several days, causing delays in contacting consumers, removing food items from the marketplace, and preventing producers from selling products while the commodity remains stored in warehouses. In this work, we present a revolutionary bacterial detection method that allows for testing of contamination to be completed in only a matter of minutes without sample preparation (water rinse, soaking, stomaching, concentration, etc.) and/or enrichments in the testing process. The method combines phage-coated magnetoelastic (ME) biosensors and a surface-scanning detector, which can be used on site at ports of entry, food processing facilities and in agriculture fields for food inspection and outbreak investigation.

2 MATERIAL AND METHODS

2.1 Fabrication of ME Biosensor Platforms

Strip-shaped ME biosensor platforms (length $\times$ width $\times$ thickness: 1 mm $\times$ 0.2 mm $\times$ 30 μm) were fabricated by dicing a commercially available Metglas 2826MB ribbon (Metglas, Inc.). The diced biosensor platforms were, then, coated successively with thin

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layers of chromium (90 nm in thickness) and gold (150 nm in thickness) by electron-beam induced deposition. The chromium layer acts as an adhesive interlayer. The gold layer provides corrosion resistance as well as a ready surface for the immobilization of a phage probe. Before and after the metal deposition, annealing was performed in vacuum at 220 °C for 2 hrs to relieve residual internal stresses and correct any surface defects from the fabrication processes (Chai et al., 2012; Chai et al., 2013c; Chai et al., 2013b; Chai et al., 2013a; Li et al., 2010; Horikawa et al., 2011; Horikawa et al., 2014b; Horikawa et al., 2014a).

2.2 Preparation of E2 Phage and *Salmonella* Typhimurium

E2 phage was derived from a landscape f8/8 phage library (Sorokulova et al., 2005) and used as the biomolecular recognition element for the ME biosensor. E2 phage possesses 10- to 1,000-fold greater binding affinity for *S.* Typhimurium over other bacteria. The specificity of detection has been reported to be 2 in 10^6 background bacteria (Lakshmanan, 2008).

Suspensions of E2 phage (1×10^{12} virions/ml in TBS) and *S.* Typhimurium cells (ATCC 13311, 5×10^8 cfu/ml in sterile distilled water) were prepared and provided by Dr. James Barbaree's group at Auburn University, Auburn, Alabama, U.S.A. The concentrated *Salmonella* suspension was diluted with sterile distilled water as desired prior to use.

2.3 Phage Immobilization and Surface Blocking

The fabricated biosensor platforms were individually immersed in 330 μL of the E2 phage suspension in a polypropylene PCR tube. The tubes were, then, rotated with a Barnstead LabQuake tube rotator (Fisher Scientific, Inc.) at 8 rpm for 1 hr. In this way, the phage was allowed to uniformly attach to the biosensor platforms via physical adsorption. Finally, a water rinse was performed to remove loosely attached phages and TBS buffer residues from the platform surfaces.

In order to reduce non-specific binding of *S.* Typhimurium on biosensor surfaces, surface blocking was performed. The phage-coated biosensors, or what we call "measurement sensors," were individually immersed in a 330-μl solution of SuperBlock Blocking Buffer (Thermo Fisher Scientific, Inc.) in a PCR tube. After 2 hrs of tube rotation at 8 rpm, the biosensors were collected from the solution and thoroughly rinsed with sterile distilled water to be ready for use. "Control sensors," which are not coated with E2 phage but only surface-blocked with the blocking buffer, were also prepared and used for determination of the limits of detection.

$$\Delta f \approx -\frac{\Delta m}{4L^2WT}\sqrt{\frac{E}{\rho^3(1-\nu)}}, \tag{1}$$

2.4 Principle of Detection

The ME biosensor used in this work is made of Metglas 2826MB, a magnetostrictive alloy (Li et al., 2012). Hence, the biosensors can be placed into mechanical resonance when subjected to an externally applied time-varying magnetic field at the right frequency. In this study, the fundamental resonant frequency of longitudinal vibration, f, is of interest, and thus, an external magnetic field was applied in the direction parallel to the length of the biosensor to excite such a mode of vibration. For a freestanding, strip-shaped biosensor, f can be expressed by (Liang et al., 2007)

$$f = \frac{1}{2L}\sqrt{\frac{E}{\rho(1-\nu)}}, \tag{2}$$

where L, E, ρ, and ν denote the length, modulus of elasticity, density, and Poisson's ratio of the biosensor, respectively. When this biosensor comes into contact with *S.* Typhimurium, E2 phage that is coated on the biosensor binds specifically with the bacteria, thereby increasing the total mass of the biosensor by Δm. This change in mass causes a corresponding decrease in the biosensor's resonant frequency. The mass-induced resonant frequency change, Δf, can be approximated by (Grimes et al., 2011)

where W and T represent the width and thickness of the biosensor, respectively. By recording Δf as a function of time, real-time monitoring of the presence of *Salmonella* on food surfaces can be performed. Unlike a measurement sensor (with E2 phage), a control sensor (without E2 phage) does not bind specifically with *S.* Typhimurium, which gives a background resonant frequency shift in the testing environment.

2.5 Construction of the Surface-Scanning Detector

The surface-scanning detector is a microelectronically fabricated planar coil that serves as: (1) a driving coil to magnetically excite the longitudinal vibration of the biosensor and (2) a pick-up coil to read the resonant frequency of the biosensor. The detector was fabricated using standard microelectronic fabrication techniques. As shown in Fig. 1, the coil turns, leads,

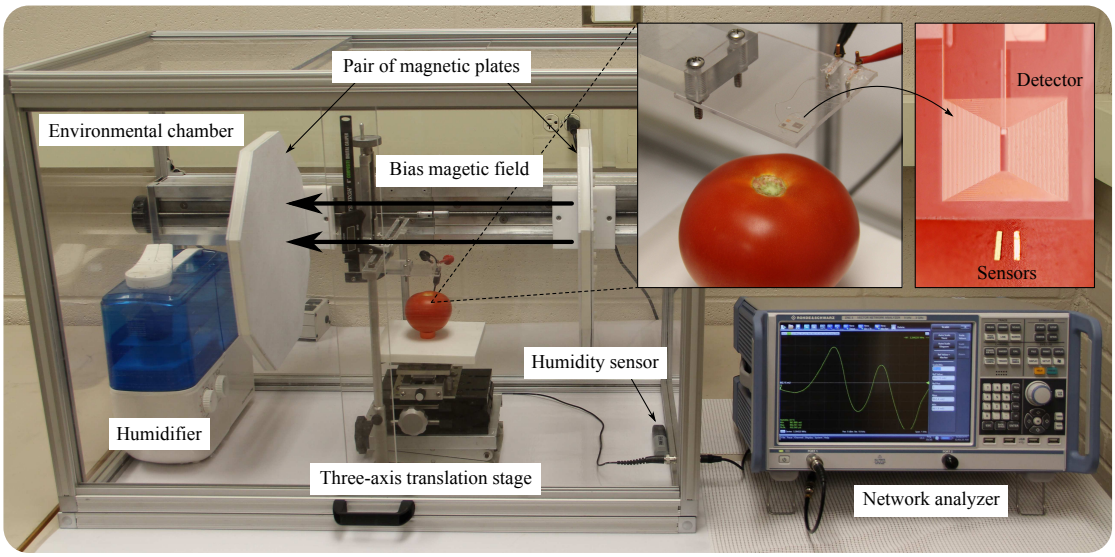

Figure 2: Measurement setup for the direct detection of *S.* Typhimurium on food.

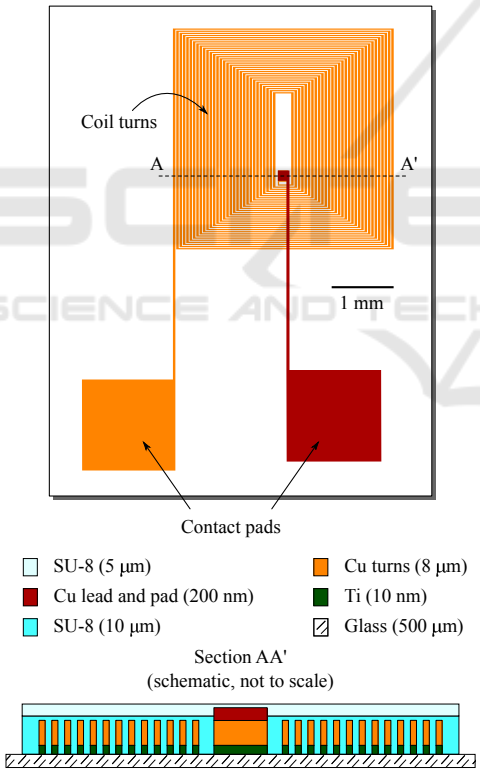

Figure 1: Design of the surface-scanning detector.

and contact pads are made of copper, and they reside on a glass substrate. To promote good adhesion of the coil to the substrate, titanium was used as the interlayer. In addition, SU-8 3005 (MicroChem Corp.), an epoxy-based photoresist, was used as the insulating filler and topmost layer.

2.6 Spiking of Fresh Produce with *S.* Typhimurium

Fresh tomatoes and grapes were purchased from a local grocery store (Kroger) and used as-received. 20-μl drops of *S.* Typhimurium with various concentrations (5×10^5 to 5×10^8 cfu/ml) were spot-inoculated on the surfaces of the foods. *Salmonella* were, then, allowed to dry in air for 2 hrs. Finally, the area of the *Salmonella*-spiked spots were measured, which allows conversion of cfu/ml into cfu/mm^2 (i.e., surface density of *S.* Typhimurium).

2.7 Measurement Setup and Experimental Procedure

The measurement setup for the direct *Salmonella* detection on food consists of a surface-scanning detector, a 3-axis translation stage, a network analyzer (Rohde & Schwarz ZNC3), a humidifier, a pair of magnetic plates, and an environmental chamber as shown in Fig. 2. ME biosensors were first placed on a *Salmonella*-spiked spot on food. The food was, then, placed between the magnetic plates in the environmental chamber. At this time, the biosensors on the food can spontaneously align parallel to the direction of the external magnetic field, which is perpendicular to the magnetic plates. The 3-axis translation stage was next used to position the biosensors under the surface-scanning detector. The detector is connected to the ZNC3 network analyzer, operated in the S_{11} reflection mode. An incident AC power is applied across the detector to magnetically excite the longi-

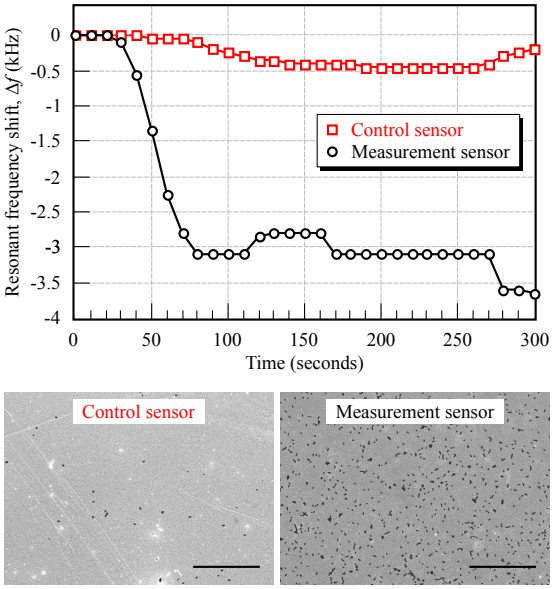

Figure 3: Resonant frequency shifts of biosensors on a tomato that is spiked with 5.6×10^4 cfu/mm^2 *S.* Typhimurium (open squares: control sensor and open circles: measurement sensor). Scanning electron micrographs confirm the resonant frequency shift results. *Salmonella* cells are shown as the black spots in the micrographs (scale bars: 50 μm).

tudinal vibration of the biosensor, and the resultant reflected power is compared to the incident power over a selected range of frequencies. In this manner, frequency-dependant reflection coefficient, S_{11}, can be determined for the circuit. At the resonant frequency of the biosensor, the largest change in the reflected power of the circuit occurs, which is visible as a downward peak in the network analyzer output. To enhance the magnitude of the resonance peak, a proper bias magnetic field was applied to the biosensor by adjusting the distance between the magnetic plates. The measurement was conducted at 23 °C and 85% relative humidity. Data were collected every 10 seconds (power: 0 dBm, bandwidth: 1 kHz, frequency span: 100 kHz, number of date points: 2001, averaging: 10 times, and smoothing aperture: 1 %).

3 RESULTS AND DISCUSSION

3.1 Direct Detection of *S.* Typhimurium on Food

Resonant frequency shifts of ME biosensors placed on the *Salmonella*-spiked foods were recorded every 10 seconds using the measurement setup described in Subsection 2.7. Typical test results for a tomato

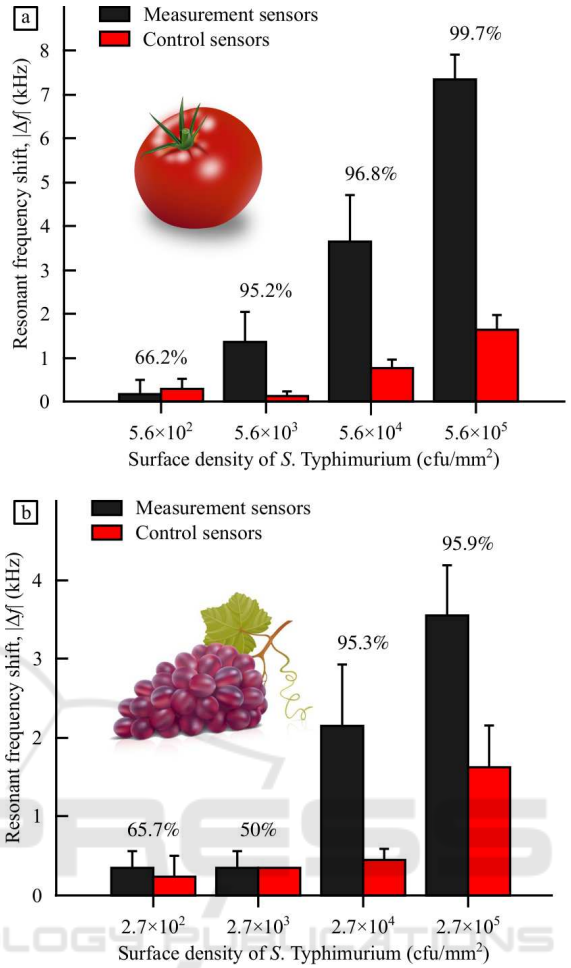

Figure 4: Dose-response results for (a) tomatoes and (b) grapes. The numbers shown above the bars are the confidence levels of difference between the measurement and control sensors at each *Salmonella* concentration.

spiked with 5.6×10^4 cfu/mm^2 *Salmonella* are shown in Fig. 3. The resonant frequency shift of a control sensor (open squares) was found to be negligible during the measurement. By contrast, a much larger resonant frequency shift was observed for a measurement sensor (open circles), due to the occurrence of the phage-based specific binding of the bacteria on the sensor. Depending on the surfaces of foods and concentration of *S.* Typhimurium, the rates of resonant frequency shifts and detection speeds were found to vary. In this particular example shown in Fig. 3, *S.* Typhimurium (5.6×10^4 cfu/mm^2) was detected within 2 minutes. Finally, scanning electron microscopy was used to confirm the frequency measurement results. A large cell count was found on the measurement sensor as anticipated, indicating that specific *Salmonella* binding had occurred.

In order to determine the limits of detection

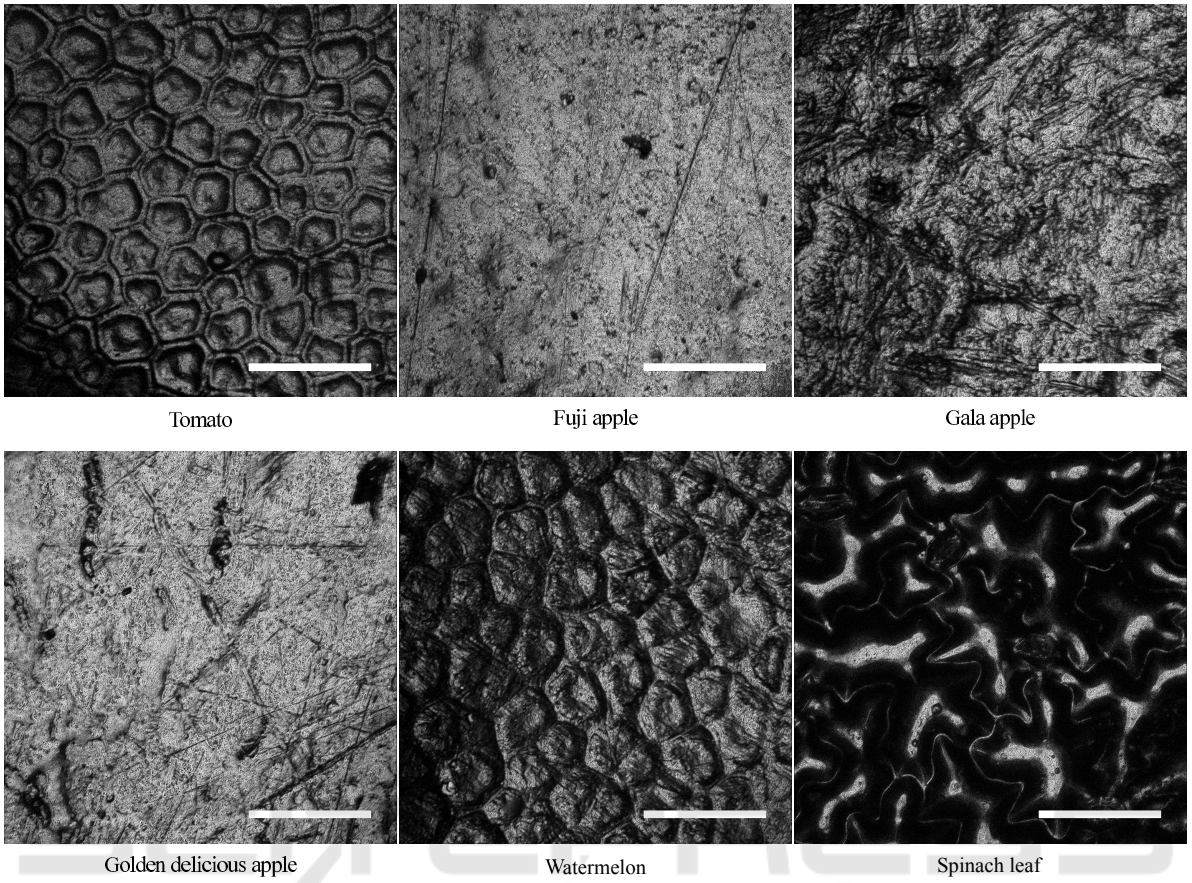

Figure 5: Surfaces of various fresh produce (scale bars: 100 μm).

(LODs), the biosensors were tested at different *Salmonella* concentrations on the food surfaces for 10 minutes. The dose-response results for tomatoes and grapes are shown in Fig. 4. As anticipated, the resonant frequency shifts of measurement sensors (black bars) were found to be larger than those of control sensors (red bars) at high *Salmonella* concentrations, which is due to the specific binding of the bacteria through E2 phage. By contrast, comparable sensor responses were observed at low *Salmonella* concentrations, indicating that the limits of detection had been reached. A one-tailed, unpaired Student's *t*-test was conducted to analyze the degree of dissimilarity between the measurement and control sensors. The confidence levels of difference were calculated and presented above the bars at each *Salmonella* concentration in Fig. 4. With a confidence level of difference higher than 95% ($p < 0.05$), the LODs were determined to be lower than: (a) 5.6×10^3 cfu/mm^2 for tomatoes and (b) 2.7×10^4 cfu/mm^2 for grapes. The method presented in this paper is a direct bacterial detection method without sample preparation (concentration, purification, washing, etc.) and/or enrichments in the testing process. The speed of detection was found to be from 2 to 10 minutes, suited for rapid, on-site pre-screening of food products.

4 FUTURE WORK

The current LODs can be improved by using ME biosensors with smaller sizes. As can be seen in Eq. 1, the mass-induced resonant frequency change, Δf, is inversely proportional to L^2WT, where L, W, and T are the length, width, and thickness of the biosensor, respectively. Smaller biosensors are, hence, more mass-sensitive and capable of detecting smaller amounts of pathogens that may be present on food surfaces. The LOD is also largely dependent on the surface topography of foods. As shown in Fig. 5, the surface pattern and roughness vary from one food to another, which necessitates the use of appropriate sizes of biosensors for improved pathogen detection. The authors have reported previously an initial model to calculate the LOD and probability of detection as a function of the size and quantity of biosensors (Horikawa, 2013). The model will be improved

and used to demonstrate a proof-in-concept in the future.

5 CONCLUSIONS

A unique, revolutionary method of bacterial detection on the surfaces of foods was presented. By combining phage-coated ME biosensors and a surface-scanning detector, *S.* Typhimurium was detected in a range of 2 to 10 minutes without sample preparation and/or enrichment in the testing process. The method presented in this paper can be used for rapid, on-site screening of food products. The pathogen-positive foods determined by this method can then be sent to lab for confirmation and identification tests.

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