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Sequential detection of *Salmonella typhimurium* and *Bacillus anthracis* spores using magnetoelastic biosensors

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ABSTRACT

Multiple phage-based magnetoelastic (ME) biosensors were simultaneously monitored for the detection of different biological pathogens that were sequentially introduced to the measurement system. The biosensors were formed by immobilizing phage and 1 mg/ml BSA (blocking agent) onto the magnetoelastic resonator's surface. The detection system included a reference sensor as a control, an E2 phage-coated sensor specific to *S. typhimurium*, and a JRB7 phage-coated sensor specific to *B. anthracis* spores. The sensors were free standing during the test, being held in place by a magnetic field. Upon sequential exposure to single pathogenic solutions, only the biosensor coated with the corresponding specific phage responded. As the cells/spores were captured by the specific phage-coated sensor, the mass of the sensor increased, resulting in a decrease in the sensor's resonance frequency. Additionally, non-specific binding was effectively eliminated by BSA blocking and was verified by the reference sensor, which showed no frequency shift. Scanning electron microscopy was used to visually verify the interaction of each biosensor with its target analyte. The results demonstrate that multiple magnetoelastic sensors may be simultaneously monitored to detect specifically targeted pathogenic species with good selectivity. This research is the first stage of an ongoing effort to simultaneously detect the presence of multiple pathogens in a complex analyte.

1. Introduction

Freestanding magnetoelastic (ME) biosensors have been explored for successful detection of foodborne pathogens (e.g., *Salmonella* and *E. coli*) (Guntupalli et al., 2007; Lakshmanan et al., 2007a; Ong et al., 2006). In addition, these sensors have been used to measure or monitor various physical properties (e.g., pH and pressure) and blood coagulation behavior (Grimes et al., 2002; Puckett et al., 2003). ME technology utilizes an ME strip as the sensor platform and an immobilized biomolecular recognition element (antibody, phage, enzymes, etc.) as the receptor for specific target agents (bacteria, spores, viruses, etc.). The operating principle of this sensor is based upon the magnetoelastic nature of the ME material. When subjected to a time-varying external magnetic field produced by an exciting coil, the sensor platform undergoes an oscillating change in dimensions, effectively converting the applied magnetic energy into mechanical vibration. When the frequency of the applied field is the same as the characteristic frequency of the ME platform, resonance will occur with the largest amplitude and the greatest mechanical energy. This, in turn, causes a change in the current passing through a pickup coil, and thus the vibration can be remotely detected. In our work, the driving coil and pickup coil are one in the same. This enables the measurement to be performed remotely and wirelessly due to the lack of any physical connection between the sensor and the detection system. This also makes it possible to perform measurements in liquids, such that the sensor may be used for *in-situ* and *in-vivo* monitoring. The sensitivity of these sensors is generally enhanced by decreasing the size of the sensor platforms.

In order to detect the resonance frequency of ME sensor, a time-varying (AC) magnetic field is used to induce resonance in magnetoelastic the ribbon-shaped sensor platform. The resonance frequency is dependent upon the resonator's material properties and geometry. For a thin ribbon-shaped ME sensor of thickness t and length L ($\ll t$), the characteristic fundamental resonance

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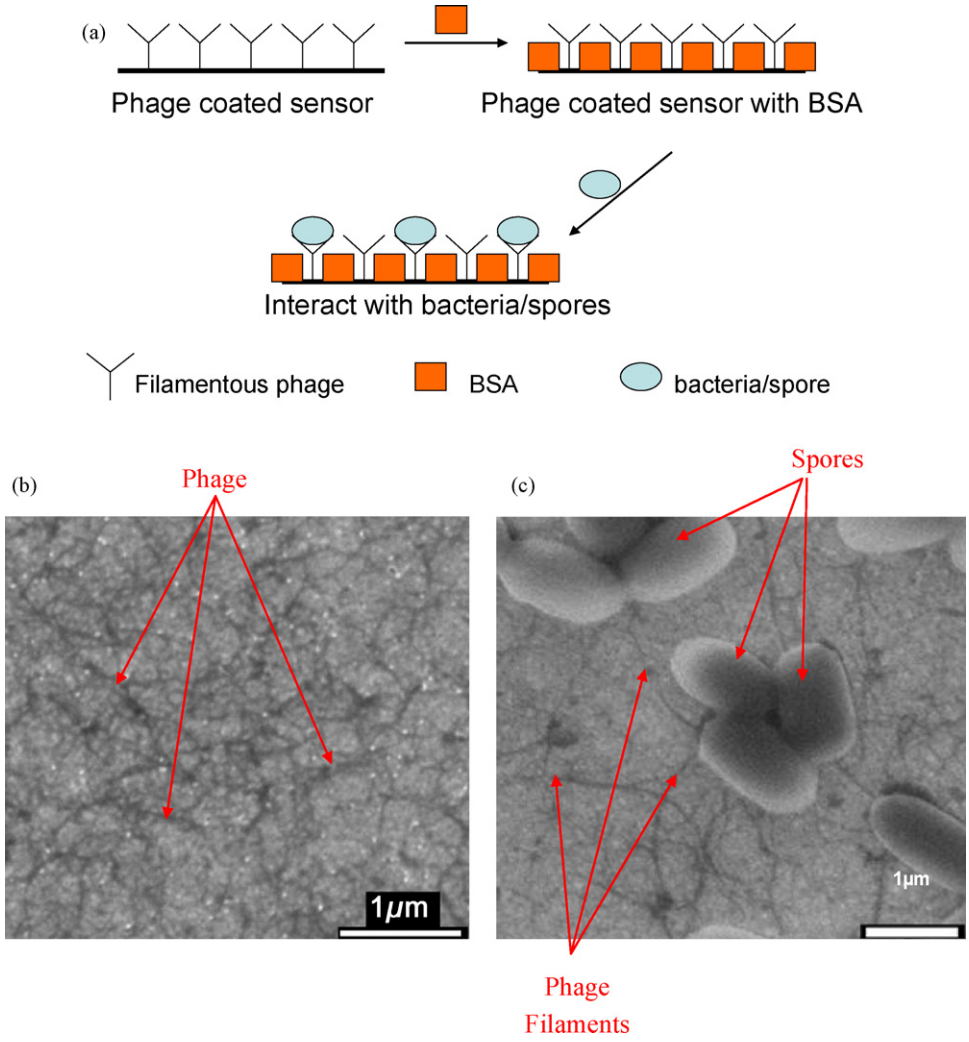

Fig. 1. Schematic representation of the detection of bacteria/spores using ME biosensor (a) the coating procedure; SEM pictures show (b) the distribution of phage; (c) the interaction of spores with bacteriophage.

frequency f, is (Liang et al., 2007):

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

where E, ρ, and ν are the Young's modulus of elasticity, density, and the Poisson's ratio of the sensor material, respectively. Liang recently demonstrated that plane-stress conditions dominate for thin, slender ribbons resonating mainly in the longitudinal direction, thus the term $E/(1-\upsilon)$ in Eq. (1). As target agent cells are captured by the biorecognition layer coated on the sensor, the additional mass results in a decrease in the ME sensors' resonance frequency. With any additional non-magnetoelastic mass load on the sensor surface, a shift in the resonance frequency, Δf, will occur as described by (Stoyanov and Grimes, 2000):

$$\Delta f = -\frac{f}{2}\frac{\Delta m}{M} \quad (2)$$

where Δm ($\ll M$) is the mass added to the ME material, and M is the initial mass of the ME material.

Previously, the focus of our efforts has been towards the detection of a single target species (for example *Bacillus anthracis* spores) using a single ME biosensor (Wan et al., 2007a; Johnson et al., 2008; Lakshmanan et al., 2007b). In this reported effort, we investigate the detection of two different biological pathogens (*Salmonella typhimurium* bacteria and *B. anthracis* spores) using two different ME biosensors with separate characteristic resonance frequencies and different biorecognition elements though monitored simultaneously. *S. typhimurium* is one of the most common causes of foodborne illnesses, while *B. anthracis* is a possible bioterrorism agent that could be used to deliberately contaminate our food supply. The reason these two quite different species were chosen was to demonstrate that multiple, targeted, phage-based magnetoelastic biosensors can be simultaneously monitored to detect and discriminate between different pathogens. It should be noted that in all experiments reported in this paper, *B. anthracis* Sterne strain spores were used. *B. anthracis* Sterne strain is a nonpathogenic, veterinarian vaccine strain that has an identical exterior make-up to the pathogenic strain of *B. anthracis*, and so the two are expected to have similar binding characteristics.

The multiple detection system presented in this work uses a reference sensor (devoid of phage) to calibrate for environmental changes (e.g., temperature, pressure, viscosity, non-specific binding) that may influence the measured resonance frequencies, thus improving the accuracy of the technique. In addition, the ability to analyze for multiple agents significantly increases the possibility of application in real food matrices, where more than one species of bacterium is often present at the same time. These novel features, along with the low-cost, wireless, and disposable nature of

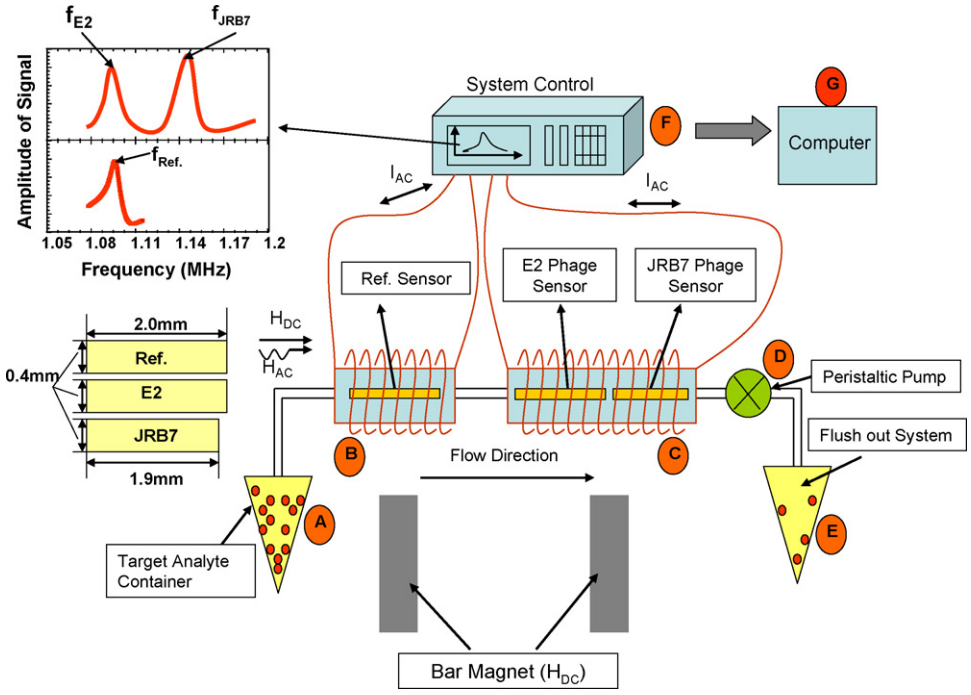

Fig. 2. Scheme of multiple ME biosensors measurement approach.

these sensors, make this technique ideal for detecting the presence of pathogenic bacteria in solutions.

2. Materials and methods

2.1. Magnetoelastic platform

In this work, there were three different sensors (one reference sensor and two measurement sensors) that were investigated. They were fabricated directly from commercially purchased ME ribbons (2826 MB Metglas™ film, $Fe_{40}Ni_{38}Mo_4B_{18}$, Honeywell) according to a procedure reported previously by Huang et al. (2008). In short, the ME ribbons were polished (about 15 μm) and diced by auto-controlled precision micro-dicing saw to final dimensions. These platforms were cleaned with acetone for 30 min to remove the adhesive used in the dicing process, and then sputter coated with Cr followed by Au on both sides of sensors to prepare the sensor platforms for immobilization of the biomolecular recognition coating. Prior to phage immobilization, all sensors were annealed in a vacuum oven at 200 °C for 2 h to remove defects and residual stress produced during dicing and sputtering.

2.2. Fabrication of measurement and reference sensors

The filamentous phage clones E2 (specific to *S. typhimurium*) and JRB7 (*B. anthracis* spores), with a concentration of 1.06×10^{13} vir/ml, were provided by Dr. Barbaree of the Department of Biological Sciences at Auburn University and have been respectively shown to be highly specific and selective to *S. typhimurium* and *B. anthracis* (Petrenko and Smith, 2000; Petrenko et al., 1996; Olsen et al., 2005; Wan et al., 2007b; Sorokulova et al., 2005). Phage was immobilized on the sensor surface by incubating the ME particles in the phage solutions (300 μl) for 1 h. The immobilization method is based on physical adsorption (Nanduri et al., 2007). To form the measurement sensor for the detection of *S. typhimurium*, an ME sensor platform was immersed into E2 phage solution (5×10^{11} vir/ml in 1× TBS), followed by washing with distilled water to remove any loosely-bound phage and residual salts. To form the other measurement sensor for the detection of *B. anthracis* spores, an ME platform was immobilized with JRB7 phage (5×10^{11} vir/ml in 1× TBS) using the same procedure as above.

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 correct concentration of BSA in solution is the main variable at this stage of the process. Proper blocking not only eliminates nonspecific binding, but also maintains the optimum binding affinity of the biosensors to bacteria cells or spores for which it is designed. Three different concentrations (1, 5, and 10 mg/ml) of BSA solution were prepared, into which the sensors were immersed for at least 1 h, followed by a distilled water rinse. At this point, the ME biosensors were ready for bacteria/spores detection. A depiction of the coating procedure is shown in Fig. 1a. Based on the binding numbers of the bacteria/spores on the sensor's surface (like shown in Fig. 1c), the optimum concentration of BSA solution was determined and used as the standard for subsequent sensor processing for both reference sensor and measurement biosensors.

In this research, a reference sensor (blocked with BSA only, with no phage immobilized on the surfaces) was also manufactured and used as a reference to eliminate the effects of nonspecific binding with possible contaminants in the analyte, varying flow conditions, and variable temperature conditions.

2.3. Multiple detection system

Schematics of the multiple detection system and the principle of ME biosensor operation are shown in Fig. 2. The system was composed of a target analyte container, a peristaltic pump, two measurement chambers (connected by a capillary tube), a flush out container, three ME biosensors for detection, and a network analyzer system for data acquisition and control. Two sensors, an E2

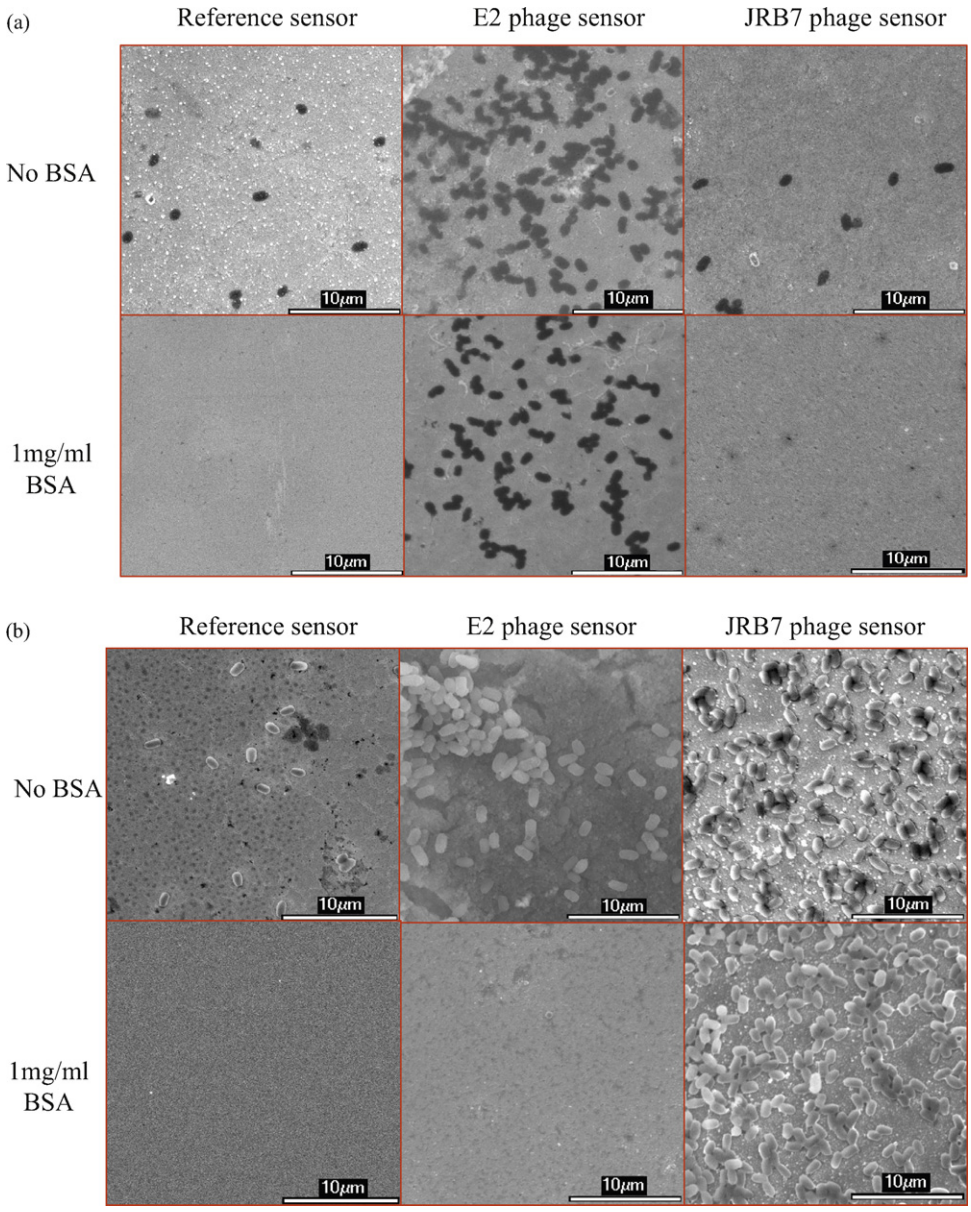

Fig. 3. SEM binding images of the sensors with and without 1 mg/ml BSA after exposure to (a) *S. typhimurium* suspension (5×10^8 cfu/ml), (b) *B. anthracis* spores suspension at a concentration of 5×10^8 cfu/ml.

phage-coated sensor and a JRB7 phage-coated sensor, were placed together in measurement chamber (C). These two sensors were fabricated with different lengths to obtain separated characteristic resonance frequencies that can be used to distinguish each target sensor. For instance, the larger size sensor could be used for detection of *S. typhimurium* and the smaller size sensor for detection of *B. anthracis*. Therefore, detection of both *B. anthracis* (by JRB7 phage-coated sensor) and *S. typhimurium* (by E2 phage-coated sensor) may be conducted simultaneously, as shown in Fig. 2. The reference sensor was placed in the second measurement chamber (B). Each chamber was connected to the network analyzer (F) via its own separate channel. The analyzer is able to measure and display the signals from both channels simultaneously. This enables the simultaneous measurement, display and recording of the three sensors' resonance frequencies in real time. For the set of sensors, the length of the E2 phage and reference sensors was 2 mm, which produced a resonance frequency of about 1.8 MHz; while the JRB7 phage-coated sensor was constructed with a length of 1.9 mm, giving a resonance frequency of about 1.14 MHz. These values are approximate since slight variations in size and shape of the sensor platforms, due to variances in the fabrication process, will affect the exact resonance frequency of each individual sensor. The experiment was conducted by using the peristaltic pump (D) to drive an analyte containing only *S. typhimurium* or *B. anthracis* spores at a concentration of 5×10^8 cfu/ml from container (A) through the detection chambers. When the target bacterium/cells bind to a corresponding ME biosensor, a frequency shift was observed to confirm the interaction, while the frequencies of the other two biosensors remained relatively stable. A flow rate of 50 μl/min was used in order to ensure laminar flow over the ME sensors. Resonance frequencies were continuously monitored and recorded by the analyzer (F) and computer system (G). Finally, the waste analyte was collected in the flush-out container (E) for disposal. Both *S. typhimurium* and *B. anthracis* Sterne suspensions were obtained

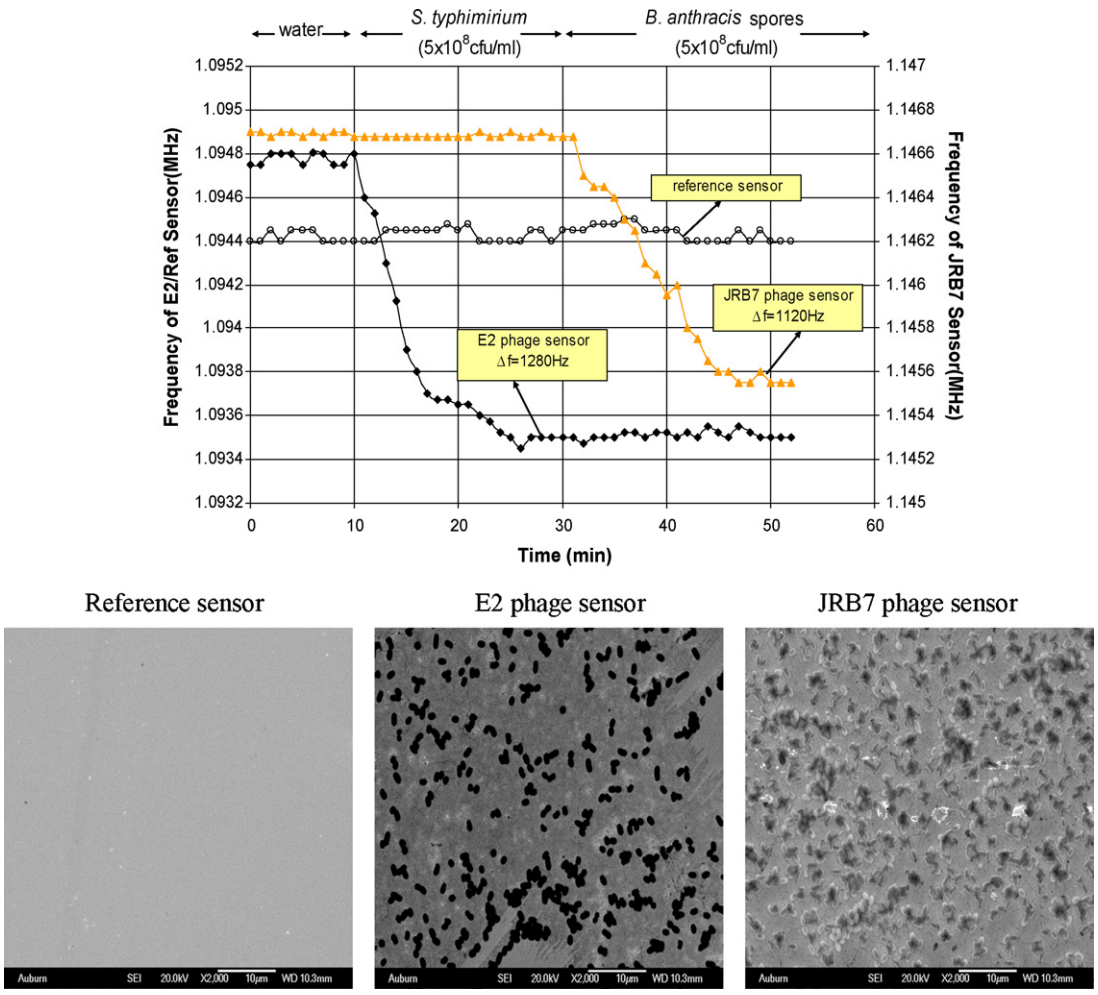

Fig. 4. Response curves for three diced ME biosensors tested simultaneously when exposed to the bacterial/spores suspension with the concentrations of 5×10^8 cfu/ml.

in a concentration of 5×10^8 cfu/ml from the Department of Biological Sciences at Auburn University. All solutions were stored at 4 °C and then equilibrated to room temperature prior to testing.

2.4. Sensitivity tests

In order to measure the sensitivity of this multiple detection system, the sensors were also separately exposed to increasing concentrations of *S. typhimurium* (5×10^1–5×10^8 cfu/ml) and *B. anthracis* spores (5×10^1–5×10^8 cfu/ml) suspensions in water. The flow rate was 50 μl/min and, for each concentration, a 1 ml suspension was used for detection.

2.5. Electron microscopy analysis

A JEOL-7000F scanning electron microscope (SEM) was used to confirm and quantify the binding of target antigens to the phage-coated ME biosensors. In preparation for SEM observations, the tested biosensors were washed with distilled water and then exposed to osmium tetroxide (OsO_4) vapor for 40 min. Afterwards, the sensors were mounted onto aluminum stubs using double-side carbon tape, and then placed into the SEM sample holder. SEM micrographs of the sensor surfaces were taken and the number of bacteria/spores bound to each sensor surface counted.

3. Results and discussion

3.1. BSA as a blocking agent

Scanning electron microscopy was used to determine the most favorable BSA concentration by comparing the binding affinity of the sensors coated with different concentrations of BSA solution. Fig. 3 shows the results of a series of cell binding experiments that were obtained after exposure to the highest concentration (5×10^8 cfu/ml) of analytes. Clear differences in the attached numbers of bacteria/spores to sensors were observed depending on the concentration of the BSA coating solution. In the case of the sensors coated with either 5 or 10 mg/ml BSA, only a few cells was observed to bind to the surface of the sensors. It is inferred from this result that the concentration of BSA was too great, covering not only the bare gold areas unoccupied by filamentous phage, but also the phage layer itself, resulting in very few available binding sites on the sensor surfaces. When the reference and JRB7 phage-coated (*B. anthracis*) sensors without BSA coating were exposed to the *S. typhimurium* suspension, there was a small degree of nonspecific binding as can be seen in Fig. 3(a). However, after treatment with 1 mg/ml BSA, the amount of this nonspecific binding was greatly reduced, while the E2 phage-coated (*S. typhimurium*) sensor still showed similar binding characteristics to the sensor without BSA coat-

ing. Similarly, with exposure to *B. anthracis* spores as shown in Fig. 3(b), the nonspecific binding was effectively blocked by pre-coating the sensors with 1 mg/ml BSA while the JRB7 phage-coated (*B. anthracis*) sensor was relatively unchanged. A BSA solution at a concentration of 1 mg/ml was chosen for use in preparation of the blocking agent for subsequent experiments.

3.2. Sensor response in flowing system

3.2.1. Sensor response with S. typhimurium/B. anthracis spores

Fig. 4 shows the typical response of the multiple phage-based ME biosensors to water and different concentrations of *S. typhimurium* and *B. anthracis* spore suspensions (5×10^8 cfu/ml each). For the first 10 min, filtered water was pumped through the two chambers continuously at a flow rate of 50 μl/min until the frequencies for the three ME biosensors stabilized. Then flow was switched to the concentrated 1 ml *S. typhimurium* suspension and passed across all of the sensors for 20 min under the same flowing conditions. At the end of 20 min, the flow was switched to the concentrated 1 ml *B. anthracis* spore suspension and allowed to flow for another 20 min.

In Fig. 4, steady state response of all the sensors in water is observed during the first 10 min of the test. Then, within one minute after the introduction of *S. typhimurium*, the E2 phage-coated sensor showed a smooth decrease in resonance frequency due to the binding of these bacteria onto the sensor surface. Steady state was achieved after about 15 min for the E2 phage-coated sensor due to the saturation of the *S. typhimurium*-phage conjugates on the sensor surface. During this time, the other two sensors showed a negligible frequency change, indicating that no appreciable binding had occurred to either the reference or JRB7 phage-coated sensors.

The *S. typhimurium* suspension flow was stopped after 20 min, and a 5×10^8 cfu/ml *B. anthracis* spore solution was subsequently introduced to the system. On exposure to the *B. anthracis* spore solution, a sudden drop of the resonance frequency was observed for the JRB7 phage-coated sensor. Furthermore, no significant change in the resonance frequency of the reference or E2 phage-coated sensors, both previously exposed to water and

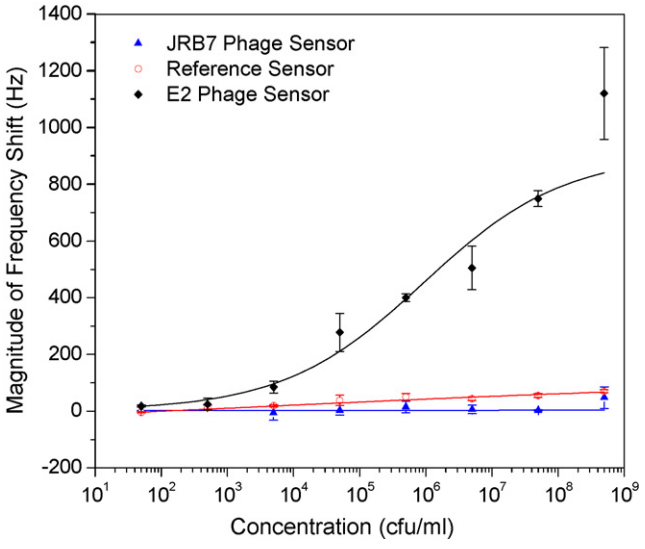

Fig. 5. The resonant frequency shifts as a function of various concentrations of *S. typhimurium* (5×10^1–5×10^8 cfu/ml). The smooth lines are the sigmoid fits of the experimental data (E2 phage sensor: $R^2 = 0.995$; JRB7 phage sensor: $R^2 = 0.95$; reference sensor: $R^2 = 0.99$).

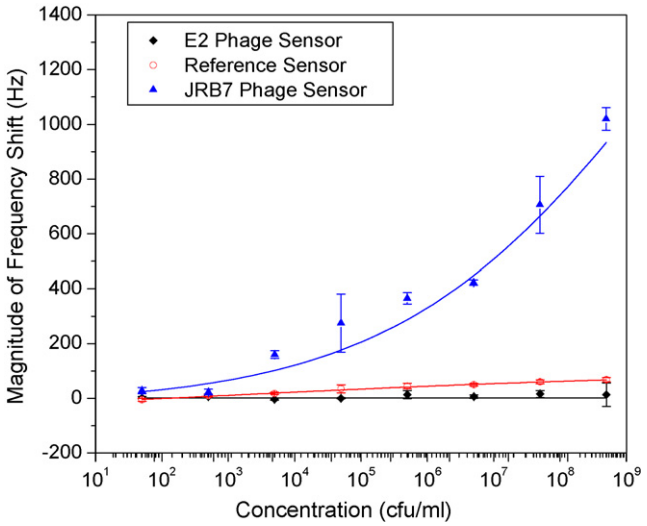

Fig. 6. The resonant frequency shifts as a function of various concentrations of *B. anthracis* spores (5×10^1–5×10^8 cfu/ml). The smooth lines are the sigmoid fits of the experimental data (E2 phage sensor: $R^2 = 0.985$; JRB7 phage sensor: $R^2 = 0.965$; reference sensor: $R^2 = 0.995$).

S. typhimurium, was observed, indicating negligible non-specific binding.

Overall, the frequency shift was about 1280 Hz for the E2 phage-coated sensor, and about 1120 Hz for the JRB7 phage-coated sensor for 5×10^8 cfu/ml analyte solutions. This interaction is confirmed by the SEM photographs of the reference and phage-coated sensors. It is visually clear that the frequency shifts were due to the spores/bacterial cells attached to the corresponding sensors. On exposure to either analyte, the decrease in frequency only occurs when bacteria cells or spores bind to the specific phage on the sensor's surface.

During the entire test, the frequency of the reference sensor exhibited no appreciable change regardless of the composition of analyte introduced. This shows that non-specific binding had been successfully blocked by the 1 mg/ml BSA pre-treatment. Also, the ME sensors showed good environmental stability in the flowing liquid system, as evidenced by a lack of corrosion. Similar trends in the measured frequency shifts were also observed for analyte concentrations of 5×10^3 to 5×10^7 cfu/ml and the magnitude of the dose response are summarized and shown in Fig. 5 and Fig. 6.

3.2.2. Dose response

The magnitude dose response of the three sensors simultaneously exposed first to *S. typhimurium* suspensions in the range of 5×10^1–5×10^8 cfu/ml is shown in Fig. 5. The detection limit for the E2 phage-coated sensor is estimated to be 5×10^3 cfu/ml, and saturation occurring at greater than 5×10^8 cfu/ml. A linear response is found between the concentrations of 5×10^3–5×10^7 cfu/ml. The reference sensor shows a slight frequency shift with increasing analyte concentration with a maximum shift of about 70 Hz at the largest concentration. There was about 30 Hz frequency shift for JRB7 phage-coated sensor due to exposure to the *S. typhimurium* suspension. This demonstrates that the non-specific binding has been blocked by the BSA coating.

A similar trend was found for the simultaneous exposure of the three sensors to *B. anthracis* spores in the range of 5×10^1–5×10^8 cfu/ml is shown in Fig. 6, immediately following the exposure to *S. typhimurium*. The detection limit estimated to be 5×10^3 cfu/ml. Again, the reference sensor exhibited a small frequency shift about 70 Hz with increasing analyte concentration

while the sensor coated with E2 phage showed no appreciable response on exposure to any concentration of the spore solution.

4. Conclusions

These results demonstrate that multiple, targeted, phage-based magnetoelastic biosensors can be simultaneously monitored to detect and discriminate between different pathogens. Immobilized phage on the sensor's surface provides the biomolecular recognition ability that insures sensitivity, specificity, and a simple method to detect multiple pathogens. This particular investigation used two widely different classes of pathogens – *S. typhimurium* bacteria and *B. anthracis* spores, to prove the concept. Future experimental work examine more difficult scenarios such as species more commonly found together in nature (e.g. a mixture of the *S. typhimurium* and *E. coli*), similar targets from the same genus (e.g. a mixture of *B. anthracis* and *B. cereus* spores), and various food products (e.g. milk, juice, meat broth, etc.) in order to determine the effect of various proteins on the sensitivity of the multiple detection system. For the currently reported ME sensor dimensions, pre-enrichment in culture media for at least 2 or 3 h might be necessary to achieve the minimum concentration to exceed the threshold detection limit of the biosensor. With further research, this ME biosensor based detection system may be extended to include hundreds to millions of individual ME biosensors. Using this approach, the statistical probability of a small number of target species interacting with one or more of a large number of ME biosensors will be greatly increased over that of interaction with a single ME sensor. This technique may therefore have significant advantages in the monitoring of large volumes of food products for a small number of biological pathogens or contaminants.

Acknowledgments

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