Simultaneous Detection of Salmonella typhimurium and Bacillus anthracis Spores Using Phage-Based Magnetoelastic Biosensors

S. Huang[1], H. Yang[1], M. Johnson[1], I. Chen[2], V. A. Petrenko[3], and B. A. Chin[1]

1. Materials Engineering, Auburn University, Auburn, AL 36849, USA
2. Dept. of Botany & Microbiology, Auburn University, Auburn, AL 36849, USA
3. Dept. of Pathobiology, Auburn University, Auburn, AL 36849, USA

ABSTRACT

The objective of this research was to investigate simultaneous detection of two different biological pathogens using one type of sensor. The biosensors investigated are comprised of a magnetoelastic (ME) particle coated with phage as the biomolecular recognition element, and resonated by an AC magnetic field. As cells/spores are captured by the phage, the mass of the sensor increases, which results in a decrease in the sensor's resonant frequency. Two genetically engineered phages, binding with *S. typhimurium* or *B. anthracis*, were immobilized onto two separate ME particles with different dimensions, allowing simultaneous measurement of two different resonance peaks. Upon exposure to solutions containing known concentrations of each analyte, only the biosensor coated with the corresponding phage responded. SEM and TEM were used to verify and quantify interaction of each biosensor with its target analyte. Results show that the observed number of bound cells/spores corresponds closely to the number calculated from the frequency shift data.

Keywords: magnetoelastic, biosensor, multiple, phage, *Salmonella typhimurium*, and *Bacillus anthracis s*pores

1. Introduction

Every year, over 76 million Americans suffer from food-borne illnesses, out of which about 10,000 result in death. Bacterial pathogens account for more than 50% of these food-borne illnesses, of which *S. typhimurium* is one of the most common. Additionally, spores and fungi affect the quality of our food leading to spoilage of the product. Recently, fears of deliberate contamination of our food supply have become a concern, *B. anthracis* (anthrax) being a primary pathogenic spore of interest. Intensive research has been focused on developing techniques for early detection of these pathogens. The need for new technologies that can be used in the field is great since traditional laboratory based methods are time-consuming and manpower intensive, usually requiring several days to yield results [1]. Additionally, for most of the current microbiological tests, the testing procedure is complicated, equipment is expensive and qualified operators are required. Therefore, the development of portable, rapid, specific, and sensitive biosensors for real-time, field detection is now a primary need. In this research, we investigate the use of ribbons of magnetoelastic (ME) material as the sensor platform. A bio-molecular recognition probe (phage that binds *S. typhimurium* bacteria or *B. anthracis* spores) is immobilized onto the platform surface. It should be noted that in the experiments we use *Bacillus anthracis* Sterne spores, the nonpathogenic vaccine strain, which is almost identical to the pathogenic strain of *Bacillus anthracis* and has similar binding characteristics. The objective of these experiments is to investigate whether it is possible to simultaneously detect two different types of biological pathogens using this method.

2. Materials and Methods

2.1 Magnetoelastic Platform

Commercially available ME strips (2826MB Metglas™ film, $Fe_{40}Ni_{38}Mo_4B_{18}$, Honeywell) were polished to a thickness of 15μm, followed by cutting using a micro-dicing saw. Sensors were diced into two sizes of 2.0 x 0.4 mm and 1.9 x 0.4 mm in order to create two sensors with two distinct, different resonance peaks. These platforms were rinsed with fresh acetone, methanol, and finally dried in air to remove the adhesive used in the dicing process. Prior to further processing, the clean platforms were annealed to remove residual stresses, as well as any remaining trace organic residue. Thin films of chromium and then gold were deposited onto the surfaces of the ME platforms using a Denton™ Vacuum Discovery-18 sputtering system (Moorestown, NJ). The gold layer is necessary for bioactivity, as well as corrosion resistance, while chromium serves as an interlayer between the gold and the Metglas™ material for improved adhesion.

2.2 Bacteriophage Immobilization

To form the biosensor, the two different sizes of sputtered ME platforms were immersed into two genetically engineered phage solutions (produced by Dr. Petrenko [2,3] of Auburn University's college of Veterinary Medicine) for 1 hour, allowing the phage to attach to the sensors' surface. One phage is for binding with *S. typhimurium* and the other is for binding with *B. anthracis*. Afterwards, the loose binding of phage, as well as the salts contained in phage solution, were subsequently rinsed in distilled water, and

then transferred to cleaned PCR tubes. BSA solution (1% (w/v)) was then added into these tubes and allowed to sit for 40 min., followed by another distilled water rinse. The purpose of BSA is to serve as a blocking agent to prevent nonspecific binding during exposure to the analyte solutions. At this point, the ME biosensors are ready for the simultaneous testing of *S. typhimurium* bacteria and anthrax spores. In this research, a control sensor (no phage immobilization, but blocked with BSA) was 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 Analyte Binding Measurement

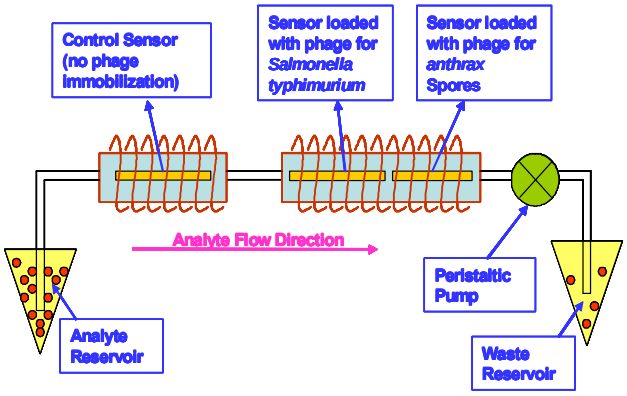

Fig.1 Setup for multiple ME biosensors measurement

Experiments were conducted where an analyte containing only *S. typhimurium or B. anthracis* spores at a concentration of 5×10^8 cfu/ml was passed through the detection chamber by the flowing system. A peristaltic pump was used to achieve a flow rate of 50µl/min, which was chosen to ensure laminar flow conditions existed over the ME sensors. Fig.1 shows the experiment setup. Two treated sensors, one loaded with phage for *S. typhimurium* with the size of 2.0 x 0.4 mm, while the other one loaded with phage for *B. anthracis* spores with the size of 1.9 x 0.4 mm, were placed together in one measurement chamber. The control sensor with the size of 2.0 x 0.4 mm was placed in a second measurement chamber. These two chambers were connected using a capillary tube. All biological test solutions were prepared and tested on the same day. Test solutions were stored at 4 °C and equilibrated to room temperature prior to testing.

2.4 Principle of Operation

ME materials, a subset of magnetostrictive materials, are generally soft amorphous ferromagnetic materials. The magnetostriction phenomenon is found in most of these materials. When the ME materials are exposed to an external magnetic field, the materials undergo a change in dimensions. For a thin (i.e. length is much larger than the thickness), ribbon-shaped sensor of length L, a time-varying AC external magnetic field can be used to resonate this ME biosensor. The ribbon shaped sensor will vibrate mainly along the length direction at its fundamental resonant frequency. This frequency is given as equation (1) [4]:

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

where L is the length of the sensor, E, ρ and σ are Young's modulus of elasticity, density, and the Poisson ratio of the sensor material, respectively. The resonance frequency is dependent on geometry as well as mass. With an additional mass load on the sensor surface, a shift in the resonance frequency will result as described by equation (2) [5]:

$$\Delta f = -\frac{f}{2}\frac{\Delta m}{M} \tag{2}$$

where M is the initial mass of the ME material, Δm is the mass change on the ME material, f is the fundamental resonant frequency, and Δf is the shift in the resonant frequency of the sensor. Recently, by measuring Δf, Grimes et al. have demonstrated the use of ME materials as mass sensitive detectors [6-11].

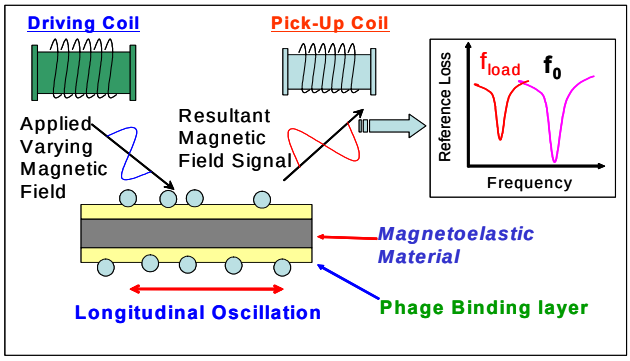

Fig.2. Schematic drawing illustrating the wireless nature of magnetoelastic biosensor and the basic principle for detecting spores or cells.

In our research, an AC time varying magnetic field is used to resonate the sensors. As *S. typhimurium* or *B. anthracis* spores are captured by the specific phage on the sensors, the mass of the sensor increases, which results in a decrease in the ME sensors' resonant frequencies. The measurements are performed remotely and wirelessly. Fig.2 illustrates the wireless nature of the individual sensor and the basic principle for detecting spores or cells.

2.5 Microscopic Analysis

A JEOL-7000F scanning electron microscope (SEM) was used to confirm and quantify the binding of target

NSTI-Nanotech 2007, www.nsti.org, ISBN 1420061836 Vol. 2, 2007

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, followed by coating with 60 nm thickness of Au to provide a conductive surface for SEM imaging. A PHILIPS-301 transmission electron microscope (TEM) was used to verify the interaction of bacteria and spores with the corresponding phage. The TEM samples were prepared on 400 mesh formvar/carbon coated nickel grids. The grid was floated on a drop of sample solution for one hour. Upon removal from the drop, excess fluid was drained from the grid by touching its edge to filter paper. Then the grid was washed gently with one drop of stain solution (PTA) and floated on another drop of stain solution for 3 min to obtain a negative stain of the sample. The grid was allowed to dry before examination.

3. Results and Discussion

3.1 Response Curve

Fig.3 shows the simultaneous response of the multiple phage-based ME biosensors to water and a high concentration of *S. typhimurium* and *B. anthracis* spore solutions. Curves A, B, and C represent the control sensor, the sensor loaded with phage for *S. typhimurium* bacteria, and the sensor loaded with phage for *B. anthracis* spores, respectively. For the first 20 min, filtered water was pumped through the two chambers continuously at a flow rate of 50µl/min until the frequencies for the three ME biosensors stabilized. Then the high concentration of *S. typhimurium* solution was introduced into the system and passed across the sensors for 20 min under the same flowing conditions. A clear drop in resonance frequency was measured (curve B) within two minutes of exposure to *S. typhimurium* solution. This indicates that cells of the bacteria *S. typhimurium* have bound to the phage on the ME sensor, resulting in an increase in mass and a corresponding decrease in resonance frequency. During this time, the other two sensors showed no change, indicating no binding to the control (A) or *B. anthracis* sensor (C) had occurred. Saturation of the *S. typhimurium* sensor occurred approximately 10 minutes after the introduction of the concentrated analyte of *S. typhimurium* as shown by the sensor's resonance frequency that stabilized to a steady state. *B. anthracis* spore solution was then introduced and allowed to flow for 20 min. A similar response can be seen in curve C, which shows a sudden drop as soon as the *B. anthracis* spore solution is passed across the sensor loaded with phage for *B. anthracis* spores. No significant change in the resonance frequency for the control sensor (A) and the *S. typhimurium* sensor (B) was observed. This again indicated negligible non-specific binding to the control and *S. typhimurium* sensor. During the entire test, the frequency of the control sensor (A) exhibited no appreciable change regardless of the composition of analyte introduced. This showed that non-specific binding had been blocked during testing. Also, the sensor showed good environmental stability in the flowing liquid system, as evidenced by no corrosion. The testing results can be summarized by the following conclusions: 1) multiple sensors can be used to detect multiple targets simultaneously in real time; 2) non-specific binding can be blocked using standard blocking agents; 3) the phage shows good binding affinity and specificity to the targets; 4) ME biosensors show acceptable environmental stability in liquid analytes; and 5) the sensor loaded with phage for *S. typhimurium* bacteria, had a frequency shift of 862 Hz, while the sensor loaded with phage for *B. anthracis* spores had a frequency shift of 755 Hz after testing.

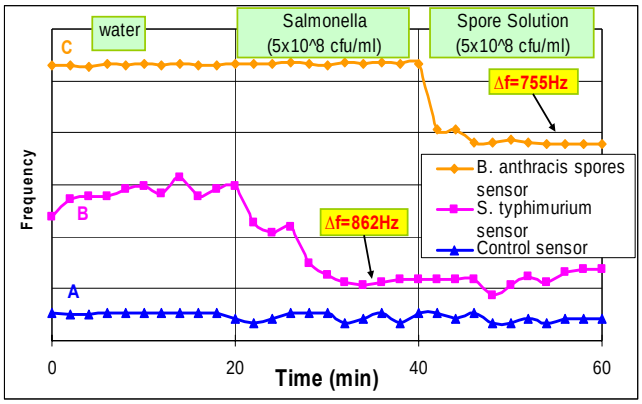

Fig. 3 Response curves for three ME biosensors tested simultaneously. Absolute scale for frequency is omitted to facilitate display of data on one chart for easy comparison.

3.2 Microscopy Analysis

Based on the experimental frequency shift data from the above, the theoretical number of cells/spores bound to the sensors can be calculated from equation (2). SEM images were used to provide visual verification of the bound bacteria or spores that contributed to the experimentally measured resonance frequency shifts. Fig.4 (a) shows the control sensor after testing. The surface showed very few bound bacteria cells or spores, which means non-specific binding was negligible. This is in agreement with a negligible shift in the control sensor's resonance frequency (Curve A in Figure 3). The sensor loaded with phage for bacteria is shown in Fig.4 (b). A uniform distribution of *Salmonella* cells was observed on the ME sensor surface. Figure 4 (c) shows the attachment of anthrax spores to the sensor surface.

The TEM images in Fig.4 (d) and (e) show the capture of *S. typhimurium* and *B. anthracis* spores respectively by the genetically engineered landscape phages immobilized on the sensor surface. In the pictures, phage bundles were observed instead of individual filaments, this is possibly due to high concentration of phage used for immobilization. Landscape phages have a constrained conformation of

foreign peptides, a multivalent display, and an extremely high local concentration of binding sites. Because of the ease of immobilizing phages on the ME platform and the high binding affinity of phages to target antigens, landscape phages show great promise for use in biosensors

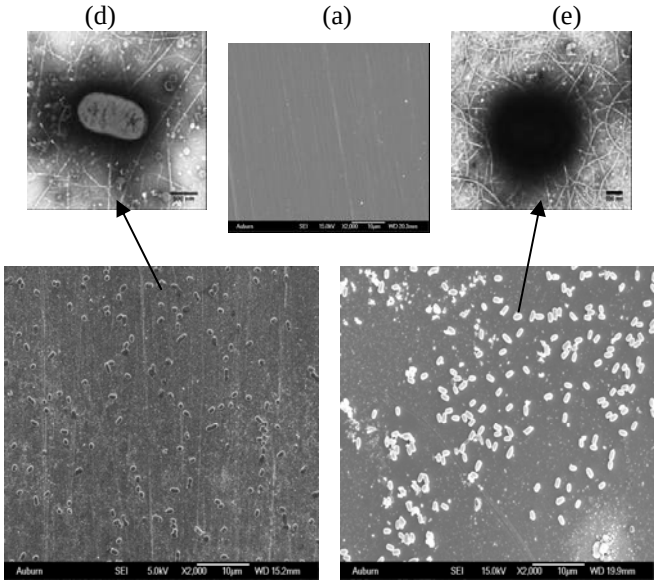

Fig.4 SEM images of sensor surfaces after phage loading and analyte exposure: (a) Control sensor; (b) Binding of *S. typhimurium*; (c) Binding of *B. anthracis* spores. TEM images: (d) capture of *S. typhimurium* by phages; (e) capture of *B. anthracis* spore phages.

Table 1 Comparison of the No. of bound cells/spores calculated and observed

Sensor	Calculated No. of binding	Counted No. from SEM
S. typhimurium	105,895	71,373 (67%)
B. anthracis spores	92,750	66,223 (71%)

To determine the number of bacteria cells or spores bound to the sensors, one side of each sensor was photographed entirely using the SEM. All bacteria or spores attached to the photographed surface were then individually counted. This count was then multiplied by 2 to account for both sides of the sensor. A comparison of this number obtained by SEM observations with the theoretical number of bound cells or spores as calculated from resonance frequency shifts is shown in Table 1. The numbers are in reasonable agreement. The difference between the calculated number and the counted number is mainly caused by two reasons: 1) Some spores/cells were loosely bound and were washed away by filter water when rinsed prior to SEM analysis; 2) Errors in counting could have occurred relating to the edges of the sensors, where spores and cells tend to pile up or cluster.

4. Conclusions

The use of phage based ME biosensors to detect *S. typhimurium* bacteria and *B. anthracis* spores is established. By immobilizing phage on the sensor surface using simple physical adsorption, the ME biosensor provides a simple and sensitive way to detect pathogens. Multiple sensors with a length of about 2 mm were used to simultaneously measure different targets pathogens with a response time of approximately 2 minutes. SEM observations and resonance frequency shift data confirmed the good specificity of the ME biosensors. TEM studies verify the interaction of bacteria and spores with the corresponding phage on biosensor surface.

REFERENCES

[1] E De Boer, RR. Beumer: *International J. of Food Microbiology*, 50, pp119-130, 1999.

[2] V.A. Petrenko, G.P. Smith, X. Gong, and T. Quinn: Protein Engineering, Vol. 9, No. 9, 1996, pp. 797-801.

[3] V.A. Petrenko and V.J. Vodyanoy: Journal of Microbial Methods, Vol. 53, 2003, pp. 253-262.

[4] L.D. Landau, E.M. Lifshitz, Theory of Elasticity, 3rd Edition, Vol. 7, 1986

[5] P.G. Stoyanov, C. A. Grimes, A remote query magnetostrictive viscosity sensor, Sens. Actuators, A, in press

[6] K.G. Ong, K. Zeng, X. Yang, K. Shankar, C. Ruan, C.A. Grimes, Quantification of multiple bioagents with wireless, remote-query magnetoelastic microsensors, IEEE Sens. J. 6 (2006) 514-523.

[7] C. Ruan, K. Zeng, O.K. Varghese, C.A. Grimes, A staphylococcal enterotoxin B magnetoelastic immunosensor, Biosensors and Bioelectronics 20 (3) (2004) 585-591.

[8] C. Ruan, K. Zeng, O.K. Varghese, C.A. Grimes, Magnetoelastic Immunosensors: Amplified Mass Immunosorbent Assay for Detection of Escherichia coli O157:H7, Analytical Chemistry 75 (23) (2003) 6494-6498.

[9] L.G. Puckett, G. Barrett, D. Kouzoudis, C. Grimes, L.G. Bachas, Monitoring blood coagulation with magnetoelastic sensors, Biosensors and Bioelectronics 18 (5-6) (2003) 675-681

[10] K.G. Ong, L.G. Puckett, B.V. Sharma, M. Loiselle, C.A. Grimes, L.G. Bachas, Wireless, passive, resonant-circuit sensors for monitoring food quality, Proc. of SPIE - The Int. Soc. for Opt. Eng. 4575 (2001) 150-159.

[11] K.G. Ong, J. Wang, R.S. Singh, L.G. Bachas, C.A. Grimes, Monitoring of bacteria growth using a wireless, remote query resonant-circuit sensor: Application to environmental sensing, Biosensors and Bioelectronics 16 (4-5) (2001) 305-312