AUTONOMOUS SENTINELS FOR THE DETECTION OF INVASIVE PATHOGENS

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Abstract: This paper describes the results of a research project to investigate and develop an autonomous pathogen detection and capture system that mimics the function of naturally occurring biological defensive systems, such as white blood cells. The autonomous sentinel system is envisioned to have the capability of seeking out invasive pathogens in liquid environments, detecting and capturing them. Once detected and captured the invasive pathogens can be removed, by retrieving the sentinels using a magnetic field. The sentinels are composed of two main parts: a magnetoelastic resonator whose motion and detection functions is actuated and monitored using magnetic fields; and a bio-probe that is immobilized onto the resonator surface and captures specific target pathogens. The freestanding sentinels require no on-board power for motion or to signal detection of a target pathogen. Upon contact with the target pathogen, the bio-molecular recognition element on the sentinel will bind with the target cell. This will cause a mass change of the sentinel, which results in a change in the sentinel's resonant frequency and the instantaneous detection of the target pathogen. Similar to white blood cells, the autonomous sentinels when placed in a liquid analyte will move through the analyte, capture and disable the target pathogens and signal their detection. The objective of this paper is to demonstrate proof-in-principal of the concept of autonomous sentinels.

1 INTRODUCTION

For centuries, humankind has attempted to mimic the designs of Nature to develop new engineering materials and systems. The human blood system is an excellent example of one of Nature's amazing creations that inspires us in this work. The human blood contains many components that work synergistically to keep us healthy. As part of the immune system, white blood cells are the main defensive mechanism against pathogenic invaders. There are a variety of white blood cell types (neutrophil, eosinophil, lymphocytes, etc.) that target different pathogens. This capability serves as the model for a bio-inspired system of autonomous sentinels for the capture and detection of invasive pathogens described in this paper (Figure 1). To provide proof-in-principal of the concept, research results for autonomous sentinel detection in liquid analytes are presented in this paper. Potential short term applications include the capture and detection of bacteria in urine and liquid food products such as water, juices and milk.

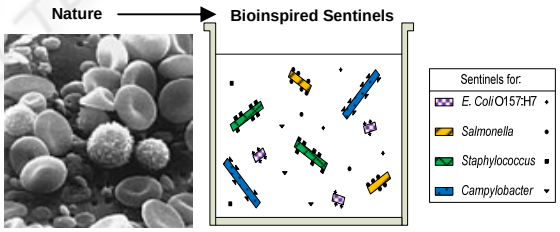

Figure 1: Bio-inspired sentinels will target different types of bacteria (*E. coli, Salmonella* Typhimurium, etc.) mimicking white blood cells that target different invasive pathogens (Wetzel and Schaefer, 1982).

2 THEORY OF THE SENTINEL

A biosentinel is constructed of a freestanding magnetoelastic (ME) resonator (transducer platform) that is coated with a biorecognition layer (bacteriophage) that specifically captures or binds a single type of pathogen. The magnetoelastic resonator investigated in the paper is strip-shaped, a rectangular, flat piece of material. The resonator is constructed from an iron-based, amorphous alloy

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with magnetostrictive properties. Magnetostrictive materials undergo a change in shape when subjected to an applied magnetic field. If the magnetic field is varied at the proper frequency aligned along the length direction of the resonator, the structure can achieve resonance. The detection principle of the ME sentinels is shown in Figure 2. The freestanding ME resonator serves as the transduction platform, actuated into resonance by the application of an alternating magnetic field. Upon contact with the specific target bacteria, the bio-molecular recognition element on the sentinel's surface captures the target bacterial cells, causing the overall sensor mass to increase which results in a decrease in the resonant frequency. The resonant frequency is remotely and wirelessly measured using a pick-up coil. No onboard power is required by a sentinel.

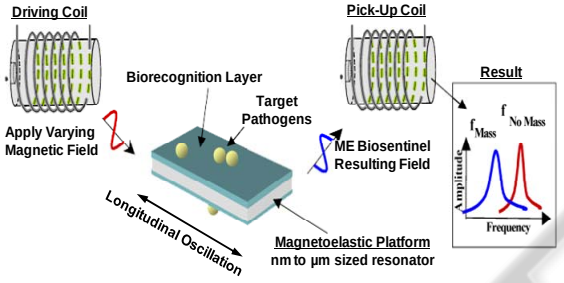

Figure 2: Detection principle of a magnetoelastic (ME) biosentinel. A driving coil generates a modulated magnetic field that drives the ME resonator into vibrational resonance. Binding of the target bacteria to the bio-molecular recognition layer immobilized onto the resonator increases the mass of the sensor resulting in a decrease in resonant frequency.

ME sentinels have unique advantages that stem from both the magnetoelastic resonator platform and the phage biorecognition layer. The sentinels are wireless devices, enabling in-situ remote detection of multiple target pathogens (Figure 3). Due to its wireless nature, a large number of sentinels can be deployed simultaneously, which significantly enhances the probability of binding with a target pathogen. More importantly, the binding of target pathogens on only **one out of many** sentinels can be easily detected. By taking advantage of these properties and capabilities of phage-coated ME resonators, a system of sentinels that mimics the functions of white blood cells can be built and deployed for enhanced medical diagnostics, food safety, or water quality applications.

One of the key parameters of these Fe based, bio-sentinels is the minimum detection limit. At low bacterial concentrations, the odds of detection are

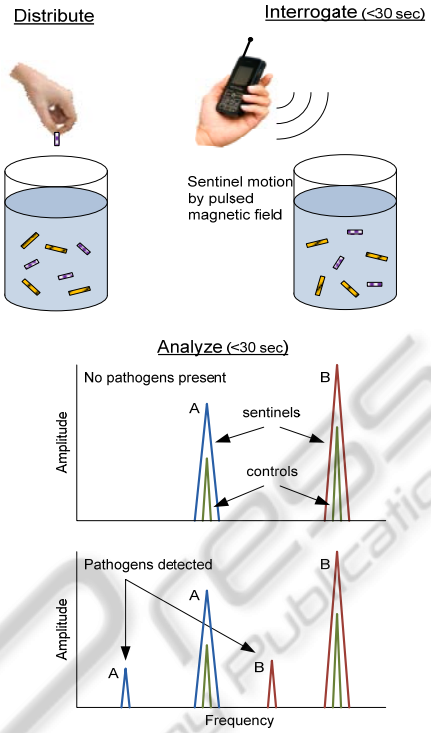

Figure 3: A large number of sentinels targeting different pathogens may be mixed together and interrogated simultaneously for pathogen detection. Different pathogens may be detected simultaneously since the sentinels for different pathogens are designed to operate in different frequency ranges.

improved either by increasing the number of ME sentinels deployed or by exposing the sentinels to a dynamic environment. For detection in liquid media, dynamic exposure can be achieved by flowing the media past the immobilized sentinels or by moving the sentinels around within the media. While flow cells are a viable option, a simpler approach to achieve greater exposure is to harness the magnetic field that is currently used only for pathogen detection to provide the forces for sentinel motion (Figure 4). A nonuniform magnetic field can be used to propel and steer the sentinels.

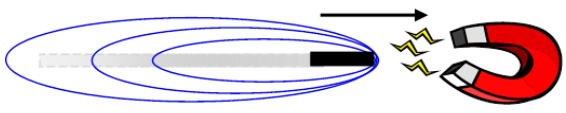

Figure 4: ME sentinel motion. A nonuniform magnetic field generated by the detection system can be used to induce movement in strip shaped sentinels.

2.1 The Resonator Platform

Acoustic resonators as sensor platforms have been widely investigated (Ballantine et al., 1997). Quartz crystal microbalances, microcantilevers, surface acoustic wave devices, and magnetoelastic particles are all examples of acoustic resonators. Acoustic resonators are mass sensitive devices where a change in the mass load on the sensor surface causes a change in the sensor's resonant frequency. Acoustic resonators are characterized by two important parameters: 1) the sensitivity (S_m) which represents the shift in the initial resonant frequency ($\Delta f = f - f_0$) due to the attachment of a unit mass load ($\Delta m = m - m_0$) as shown in Equation 1 (Grimes et al., 1999); and 2) the resonance performance (Q factor), which is defined as the ratio of the energy stored in the resonant structure to the total energy losses per oscillation cycle. In an amplitude-frequency spectrum, a measure of the Q factor is given by the resonant frequency f divided by the 3 dB frequency bandwidth. A higher Q factor means a sharper resonant peak and thus better resolution in determining the resonant frequency. The minimum detectable mass (Δm_{min}) for an acoustic sensor platform depends on the ability to resolve resonant frequency shifts as a result of the mass loading.

$$S_m = \frac{\Delta f}{\Delta m} \approx -\frac{1}{2}\frac{f_0}{m_0} \qquad (\Delta m << m_0) \qquad (1)$$

For biological detection, the surface of the ME sentinels is coated with a biorecognition element, such as an antibody or phage. This biorecognition element is designed to specifically bind the target of interest. When the ME biosentinel comes into contact with the target pathogens, the biorecognition element will capture/bind the target pathogen creating an additional mass load on the sentinel resulting in a decrease in the resonant frequency. Therefore, the presence and concentration of any target pathogens can be identified by monitoring the resonant frequency shifts of the sentinel. For a thin strip-shaped ME resonator of length L, the largest vibrations will occur along the length direction. The fundamental resonant frequency of this longitudinal oscillation is given as (Landau and Lifshitz, 1986, Liang et al., 2007):

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

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

The sensitivity of the ME biosentinel is compared with cantilevers in Figure 5. For ME biosentinels and cantilevers fabricated from the same material and of the same size, the ME sensor exhibits an S_m about 100 times better than the cantilever. Advanced microfabrication processes will enable the optimization of the resonance performance of the ME sentinels which will lead to improved pathogen detection capabilities. Different shapes, structures, and/or material compositions are parameters that affect sentinel motion, sensitivity and resonance performance.

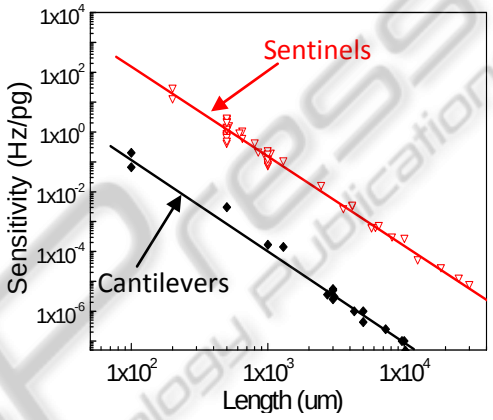

Figure 5: Sensitivity vs. length for cantilever and sentinels. Sentinels are 100 times more sensitive.

2.2 Fabrication of ME Resonators

The ME resonators were fabricated using standard microelectronic fabrication techniques of photolithography and physical vapor deposition (sputtering). The process used to fabricate the resonators is shown schematically in Figure 6. Binary alloy magnetoelastic resonators are fabricated on a patterned wafer by co-depositing iron and boron at controlled rates under vacuum. The resonators are coated with gold that provides oxidation protection for the alloy and a bioactive surface to immobilize the phage. The resonators are freed from the wafer by lift-off using an acetone rinse and collected using a magnet. Fabrication of the sensor platform begins by coating a 100 mm plain silicon test wafer with a layer of chromium, and then gold, each at a thickness of 30−40 nm. This is accomplished using a Denton Vacuum Discovery-18™ magnetron sputtering system, which employs three cathodes (each holding a 3 inch diameter target) aimed off-axis at a circular, rotating substrate platform, along with DC and RF power supplies. The gold layer is needed to adhere the next deposited film (also gold) to the wafer, while the

chromium merely serves to act as a bond between the silicon and the gold. Next, a layer of photoresist is applied to the gold surface of the wafer by spin coating such that the resultant thickness is at least twice that of the desired magnetoelastic film to be deposited later. This photoresist is then UV exposed using a positive mask comprised of evenly-spaced rectangles, which are the desired length and width of the magnetoelastic sentinels. The wafer is then developed in a 2:1 solution of de-ionized water and AZ-400K developer, rinsed, dried, and then inspected for pattern integrity and thickness.

The magnetoelastic film is then deposited onto the patterned wafer using the same sputtering system as before. First, the wafer is loaded into the deposition chamber, along with a gold, iron, and boron target for each of the three cathodes, and then the chamber is pumped down to 7×10^{-7} Torr in order to minimize residual oxygen in the film. Next, a gold layer is deposited onto the patterned wafer to a thickness of about 30–40 nm. The magnetoelastic layer is formed by co-depositing iron (DC) and boron (RF) simultaneously using a dual-cathode method. This method differs somewhat from the usual procedure for co-sputtering iron and boron, which typically involves using a specially made composite target. The advantage here is that the power of each cathode can be tuned separately such that the film has the desired composition at a reasonable deposition rate. Thickness of this film depends on process conditions, and is generally limited by the thickness of the photoresist layer, but highly magnetostrictive films of up to about 7 μm have been obtained using this dual-cathode method. Finally, another gold layer, using the same processing conditions as before, is applied on top of the iron-boron film, such that the magnetostrictive particles will be completely enclosed in gold. From an 8" wafer, approximately 40,000 sentinels can be fabricated. The cost of fabrication of a single 8" silicon wafer of sentinels is approximately $28.00. Hence the cost of a single ME sensor is less than 1/1000 of a cent.

2.3 Immobilization of the Bio-molecular Recognition Layer

To form functional sentinels, a bio-molecular recognition element must be immobilized onto a transducing platform to bind the specific target pathogenic species. Other investigators typically use traditional antibodies as the biorecognition element. The strengths and weaknesses of antibody binding are well known. An antibody is a relatively fragile

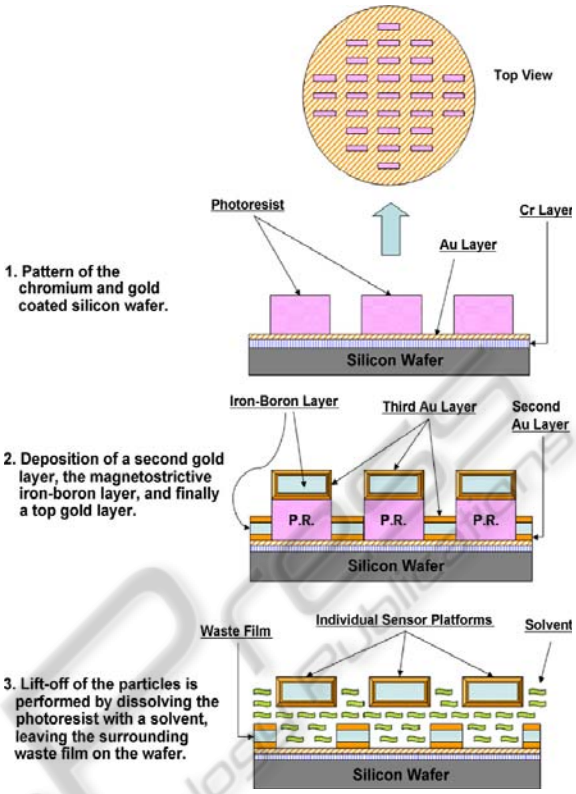

Figure 6: The ME resonator fabrication process.

species and subject to denaturation with consequential loss of sensitivity and other binding characteristics when exposed to unfavorable environments. Moreover, the quality of antibodies can vary with different animals and production variables. To be used in sentinels, antibodies require affinity purification and stabilization, which dramatically increases their cost. Monoclonal antibodies are more standard and selective, but their application in the field is hindered by their stability. The use of phage as substitute antibodies offers a stable, reproducible and inexpensive alternative (Petrenko, 2008, Petrenko and Smith, 2000). In contrast to antibodies, the phage structure is extraordinarily robust, being resistant to: heat (up to 80 °C) (Brigati and Petrenko, 2005); organic solvents (e.g., acetonitrile) (Olofsson et al., 2001), urea (up to 6 M), acid, alkali and other chemicals. Purified phage can be stored indefinitely at moderate temperatures without losing infectivity and probe-binding activity. Three major factors contribute to the high affinity binding of landscape phage to their targets: a) constrained conformation of foreign peptides; b) their multivalent display—thousands of binding sites per phage filament; and c) extremely high local concentration of binding sites. The

surface area density of the phage is 300 to 400 m^2/g, exceeding even the best-known absorbents and catalysts. The genetically engineered amino acids that form the "active receptors" of a landscape phage comprise up to 25% by weight of the phage and up to 50% of its surface area—an extraordinarily high fraction compared to natural proteins, including antibodies.

Our research team has genetically engineered filamentous phage (Figure 7**)**, to serve as a replacement for current antibody technology. The filamentous E2 phage for binding to *Salmonella enterica serovar* Typhimurium was affinity selected from a landscape f8/8 phage library and provided by 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 sensor surface using physical adsorption. Each ME sensor platform was placed in a vial containing 300 µL of phage E2 suspension (5×10^{11} vir/mL in 1 x Tris-Buffered Saline (TBS)). These vials were then rotated and incubated on a rotor (running at 8 rpm) for 1 hour. After the immobilization process, the sensors were washed three times with 1 x TBS solution and two times with sterile distilled water in order to remove salt and any unbound or loosely bound phage.

In order to reduce nonspecific binding, Bovine Serum Albumin (BSA) solution was then immobilized on the sensor surfaces to serve as a blocking agent. The ME sentinels were immersed into 1 mg/mL BSA solution for at least 1 hour, followed by a distilled water rinse. In this study, control sensors were fabricated and used to calibrate the effects of environmental changes, such as temperature and non-specific binding. 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.

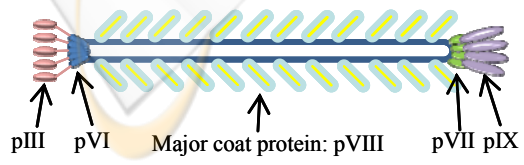

Figure 7: Filamentous Phage.

3 CHARACTERIZATION OF ME SENTINEL PERFORMANCE

3.1 Detection of Pathogens by the ME Sentinels

The *S.* Typhimurium culture (ATCC 13311) used in this work was provided by the Department of Biological Sciences at Auburn University, Auburn, AL. These cultures 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 biosentinel 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.

The resonant frequency of the sentinels was measured using an HP 8751A network analyzer with S-parameter test set. The ME sentinels (control and measurement) were placed in a tube containing pure water and the resonant frequency of the sentinel measured. The network analyzer scanned, measured and recorded the resonant frequency spectrum of the ME sensor as a function of time. After each 30 minute exposure the analyte was changed to the next highest dilution. Figure 8 shows the frequency shift measurements for ME sentinels 500 × 100 × 4 µm in size. Note that the control sensor shows a nearly constant frequency (no frequency shift), while the measurement sensor undergoes a frequency shift of nearly 120,000 Hz. As can be seen from the plot, the detection limit of the sentinel is better than 50 CFU/mL of *S.* Typhimurium in water.

A JEOL-7000F scanning electron microscope (SEM) was used to confirm and compare the binding of *S.* Typhimurium on the phage-coated measurement and control sentinels. After the detection, the ME sentinels were exposed to osmium tetroxide (OsO_4) vapour for 45 minutes. The sensors were then mounted onto aluminum stubs and examined using the SEM. Figure 9 shows the SEM micrographs for the measurement and control sentinels. The control sentinel shows only a few cells are bound to the surface while the measurement sentinel is nearly completely covered with bound *S.* Typhimurium bacteria.

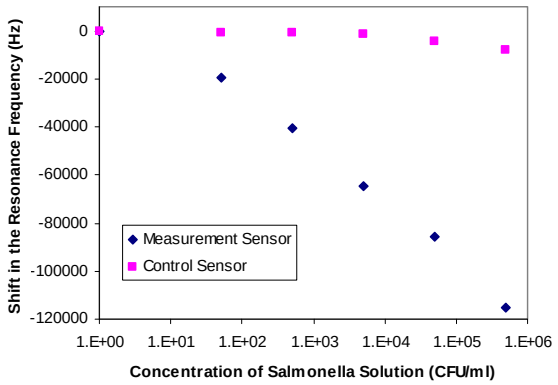

Figure 8: Response of 500 µm long biosensor exposed to increasingly higher concentrations of *S.* Typhimurium. The detection limit is less than 50 CFU/mL. The response of the control sensor (devoid of phage) is also shown.

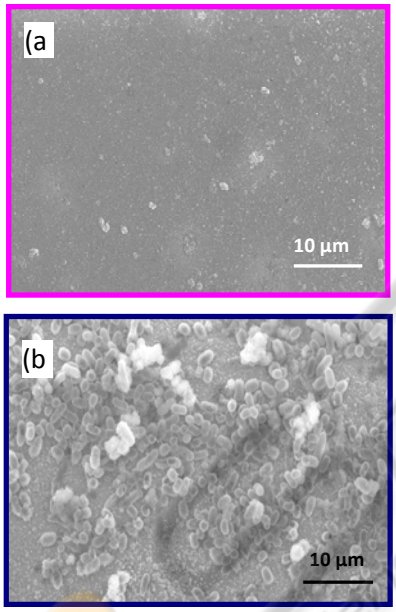

Figure 9: The SEM images show near zero binding of *Salmonella* cells to the control sentinel (a) and a large number of bound *Salmonella* cells to the measurement sentinel (b).

4 CONCLUSIONS

Proof-in-principle of the concept of autonomous sentinels for the detection of invasive pathogens has been established. Magnetoelastic strip-shaped resonators coated with a bio-molecular recognition layer can moved through a liquid using a non-uniform magnetic field and then measured remotely and wirelessly to detect the binding and capture of specific pathogenic bacteria. Because the magnetoelastic sentinels investigated in this research are iron based, they can be retrieved with a magnet and hence captured pathogenic bacteria can be removed from the system.

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