Rapid Detection of Bacterial Spores Using a Quartz Crystal Microbalance (QCM) Immunoassay

Sang-Hun Lee, Desmond D. Stubbs, John Cairney, and William D. Hunt

***Abstract*—Weaponized spores of a pathogenic bacterium such as *Bacillus anthracis* are a new critical threat to mankind. The occurrences in New York and south Florida in 2001 showed the potential capability of the spores to be used for mass destruction. Due to their stealthiness during the infection and resistance to harsh environment, an early and prompt detection of the spores before they endanger the population is a significant issue. In this paper, we present a method of instant identification of *Bacillus subtilis* (nonpathogenic simulant for *Bacillus anthracis*) spores by constructing a dual quartz crystal microbalance (QCM) immunosensing system. A set of 10-MHz AT-cut QCMs operating in thickness shear mode are employed in an enclosed flowcell. Specificity is maintained through the use of an immuno-sensing layer consisting of monoclonal antibodies raised against spores of a single *Bacillus* species. The fidelity of sensing parameters is ensured by the presence of a reference device coated with an antibody that is not specific for the target antigen. Associating the QCM response signature with the specific binding of a particular species of *Bacillus* spore to an antibody has implications for future identification of pathogenic substances.**

***Index Terms*—Acoustic wave sensor, bacterial spore detection, immunoassay, quartz crystal microbalance (QCM).**

I. Introduction

THE genus *Bacillus* is of particular importance because it can adapt by changing to a dormant life form known as an endospore, which is capable of surviving severe environmental conditions, and stringent decontamination procedures that would destroy a vegetative cell. These spores may contaminate food, water, or may be transmitted as aerosols via air conditioning, in either case they can be the source of animal and human infections. More recently, pathogenic endospores such as *B. anthracis*, the causative agent of anthrax, have become feared as a potential threat for biological warfare and bio-terrorism. The availability of monoclonal antibodies specific to a single spore type (recognizing particular epitopes on the endospore protein coat) along with the design of a quartz crystal microbalance (QCM) system forms the building blocks for a highly sensitive, highly specific biosensor. It is known that the monoclonal anti-spore antibodies do not cross react with the vegetative cells from which they are derived and are species specific; spores of other species are not recognized [1]. Early detection of spores in, for example, public water systems, is important since it permits rapid responses that can isolate sources of contamination and, thus, prevent the dissemination of a pathogen. Current immuno-detection methods require periodic sampling and assay by an investigator, with results emerging in minutes or hours. Monitoring by this method is, thus, discontinuous and limited by the frequency of sampling. Given the swiftness with which sporophytic pathogens can be administered to water supplies, populations would be at risk in the intervening periods. Rapid detection implies that the existence of target is identified without time-consuming biochemical post-process such as enzyme-linked immunosorbent assay (ELISA) or microscopy analysis. Further, the presence of an appropriate nanobiosensor in a water system would provide *continuous* monitoring for pathogens.

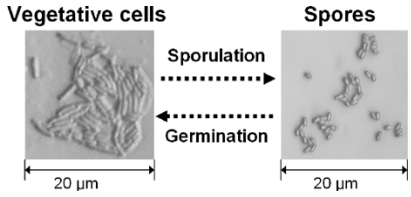

Fig. 1. *Bacillus subtilis*: A benign "surrogate" for *Bacillus anthracis*. Optical microscope images taken from Hunt Laboratory, Georgia Institute of Technology.

We have employed the specificity of antibodies raised against the spores of *B. subtilis*. *Bacillus subtilis* is a Gram-positive, spore-forming bacterium commonly used as a nonpathogenic simulant for *Bacillus anthracis*. Fig. 1 shows the microscope pictures of *Bacillus subtilis* cells and spores taken from our laboratory. The size of a spore and a vegetative cell is approximately 1 and 4 μm, respectively. Because the biological behavior and physical characteristics of *B.subtilis* are very similar to anthrax, the protocols used for the detection of *B.subtilis* can be applied for the detection of anthrax spores.

II. Background: Signature of a QCM Immunosensor

The sensing mechanism of QCM is based on perturbation of the sensor surface when the detection occurs that leads to the change of the resonant frequency. The perturbation arises from a mass attached on the sensor surface or changes of physical properties of the contacting medium, or both. In 1959, Sauerbrey [3] derived the following equation describing the linear relationship

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of frequency change (Δf) and mass loading (Δm) on a QCM surface:

$$\Delta f = -K f_0^2 \frac{\Delta m}{A} \tag{1}$$

where f_0 is the resonance frequency, K is a constant, and A is the sensing area. This is a fundamental equation for the QCM based sensors but it can only be used for a rigid film with negligible thickness and in vacuum environment.

In 1985, Kanazawa and Gordon [4] derived a similar relationship of QCM in contact with liquid

$$\Delta f = -f_0^{3/2} \left(\frac{\eta \rho}{\pi \mu_q \rho_q} \right)^{1/2} \tag{2}$$

where f_0 is again the resonance frequency, η and ρ are the viscosity and absolute density of the solution, respectively, and μ_q and ρ_q are the shear stiffness and density of the quartz crystal, respectively. This equation introduces another term, the viscosity of the medium, in addition to the mass.

As the coating layer becomes complex, however, additional physical factors need to be involved to describe the characteristic of sensor responses. Fig. 2 represents a side cross-sectional view of a QCM in thickness shear mode and with a selective layer, an antibody film here, attached on it. H_q and h_f are the thickness of the quartz plate and the film, respectively. In the case of a QCM immunosensor coated with antibody layer and/or other biomolecules, the profile of the sensor response does not always follow (1) or (2). To examine more detailed behavior of the frequency responses in this particular situation, we review the partial differential equation for the frequency shift of QCM based sensors developed by Hunt *et al.* [7] from the complex reciprocity relation and time-dependent perturbation theory

$$t\frac{\partial \Delta\omega}{\partial t} + \Delta\omega = \frac{\omega_u h_f}{\pi\sqrt{\rho_q \mu_q}} \times \left\{ -\omega_u \left[\Delta\rho - \frac{\Delta\mu}{V_s^2} \right] + j \left[\frac{\partial \Delta\rho}{\partial t} - \frac{1}{V_s^2} \cdot \frac{\partial \Delta\mu}{\partial t} \right] \right\} \tag{3}$$

where V_s is the acoustic velocity across the thickness; ρ is the density of the film; μ is the stiffness of the film; Δ is the difference between perturbed and unperturbed (denoted by subscript u) quantities. Assuming that neither $\Delta\omega$, $\Delta\rho$, or $\Delta\mu$ changes with time, we get

$$\Delta f = -\frac{2 f_u^2 h_f}{\sqrt{\rho_q \mu_q}} \left[\Delta\rho - \frac{\Delta\mu}{V_s^2} \right]. \tag{4}$$

Noting that μ_q and ρ_q are constants and the mass loading can be expressed as $\Delta m = \Delta\rho A h_f$, (4) is essentially the Sauerbrey (1) with an additional term describing changes in the mechanical stiffness of the film. This equation predicts that mass loading will lower the frequency but increase in stiffness will incur a positive frequency shift. Note that neither (1) nor (2) can explain a positive frequency shift. An example for this will be in Section V-A.

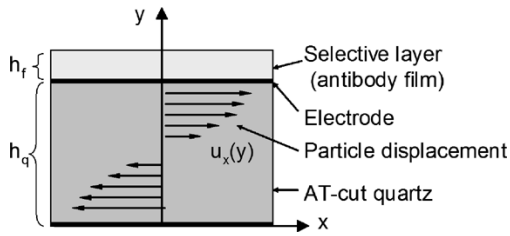

Fig. 2. Side cross-sectional diagram of a QCM with biofilm coated (not drawn to scale).

If $\Delta\rho$ and $\Delta\mu$ were known as function of time, the solution of (3) would be

$$\Delta\omega(t) = \frac{1}{t} \left(\int_t \frac{\omega_u h_f}{\pi\sqrt{\rho_q \mu_q}} \left\{ -\omega_u \left[\Delta\rho(\tau) - \frac{\Delta\mu(\tau)}{V_s^2} \right] \right\} d\tau + j \left[\Delta\rho(t) - \frac{\Delta\mu(t)}{V_s^2} \right] + C \right). \tag{5}$$

Though $\Delta\rho$ can be extracted from kinetic approximations, $\Delta\mu$ is a complete unknown. This equation represents a mathematical tool for extract conformational change data from real-time QCM measurements by recording $\Delta\omega(t)$ [7].

III. Antibody Immobilization Protocol

Antibodies were attached to the QCM gold electrode using commonly used SAM (self-assembled monolayer) method (Fig. 3). The sulfur in thiol group has high affinity to a gold surface and the other terminal of the hydrocarbon chain binds to the stem (Fc region, or carboxyl terminal end) of the antibody through a coupling agent. QCMs were cleaned using Piranha solution [30% H_2O_2 and conc. H_2SO_4 in 3:7 (v/v) mixture]. The crystals were air dried. 1 mg of 3,3'- dithiopropionic acid was dissolved in 100% ethyl alcohol to make a 1-mM alcoholic solution. The monolayer was applied to the QCM gold electrodes and allowed 2 h to self assemble. The crystals were then washed with 95% ethanol followed by aliquots of deionized water before allowing them to air-dry. A mixture of 1-Ethyl-3-(3-Dimethylamino-propyl) Carbodiimide (EDC) (0.0133 mg) dissolved in 0.1 mL of 1 × TAE (Tris-Acetate-EDTA) buffer and 13.5 mg of NHS (N-hydrocylsulfo-succinimide) dissolved in 0.1 mL of buffer was used to activate the carboxyl groups on the dithiopropionic acid for the subsequent coupling reaction with the antibody. The crystals were washed with deionized water and allowed to dry. Antibodies (3–5 μl) were diluted in 1 × TAE buffer, and then allowed to incubate on the crystals' surfaces overnight before the final washing steps to remove unbound material. Ethanol-amine (0.05 M) was added to the surface to block all unbound sites. The crystals were then mounted in a flowcell for sampling.

IV. Sensor System Setup

The QCM sensor system we used herein consists of three main parts (Fig. 4): injection, sensing, and data collection. The injection part has high precision syringe pumps from Harvard Apparatus, loaded with medium and target solution. The sensing part includes a dual QCM flow cell and two oscillators that drive

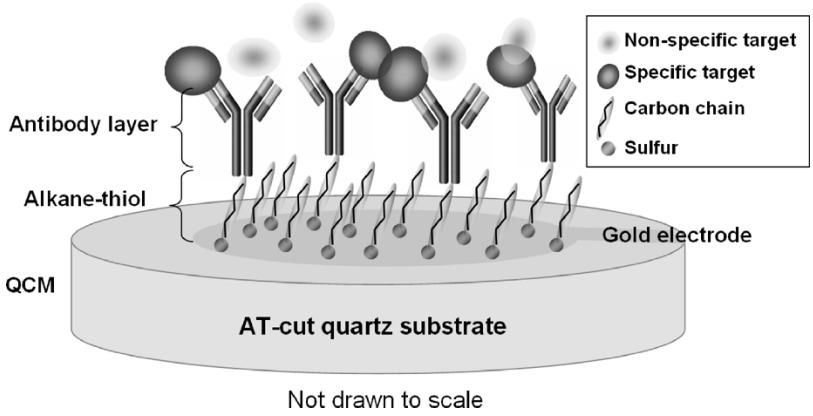

Fig. 3. Schematic of the antibody immobilization on the Au electrode using a self assembled monolayer (SAM) as a cross linker.

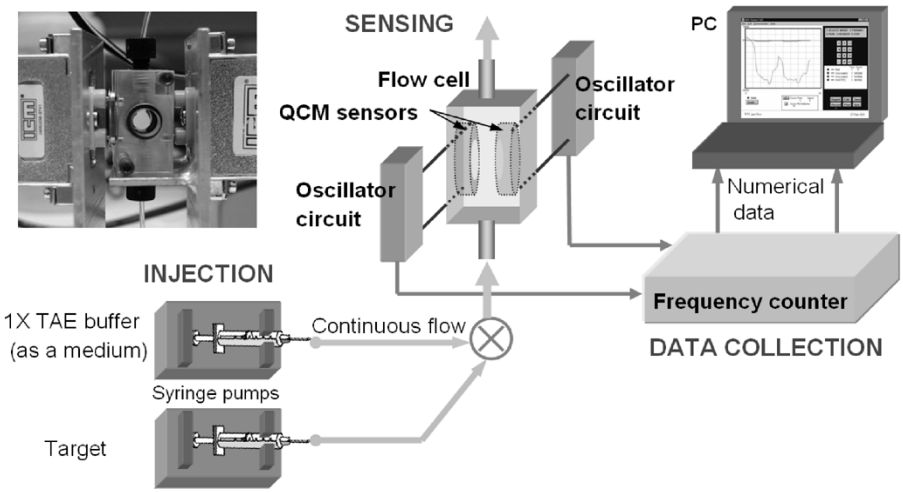

Fig. 4. Schematic of the dual QCM sensor system setup. The upper-left picture shows the actual sensing part; flowcell with the QCMs and oscillators mounted.

the QCMs. The photograph in the upper-left corner of the Fig. 4 shows the actual image of the sensing part. The data collection part has a frequency counter and a laptop PC. The sensor data is transferred from the frequency counter to PC thru RS232C serial ports and collected in real-time by LabVIEW program installed on the PC. The sampling period and the resolution of the frequency counter are 0.5 s and 0.1 Hz, respectively. All the experiments were done at room temperature.

The specification of the QCM used in this experiment is shown in Fig. 5. The oscillator units and 10-MHz QCMs with AT-cut quartz were from International Crystal Manufacturing Co, Inc. The electrode (active sensing area) of the QCM is 0.22 cm^2, which gave us sensitivity of ~ 4.5 ng/Hz-cm^2. This sensitivity is calculated from the result of our experiments and the fact that one spore weighs about one picogram. We have seen that 18 000 spores on 0.22 cm^2 electrode area made 20 Hz of frequency shift which results in about 4.5 ng/Hz-cm^2.

Two notable aspects of our QCM sensing system are as follows.

- First, we designed a liquid flowcell (Fig. 4) that can hold two QCM sensors facing each other so that these two QCM sensors can undergo virtually same physical changes, i.e., hydraulic pressure, temperature variation, and external vibrations. Also, one sensor is coated with antibodies specific to the target and the other with antibodies that do not recognize the target. This enables us to exclude all the unnecessary signals that are not related to the target detection, as well as to subtract nonspecific ligand-antibody interactions from genuine binding interactions and, thus, to perceive a real signature of the detection.
- Second, we inject a continuous stream of $1 \times$ TAE buffer solution into the flowcell at a small flow rate of 0.1 mL/min throughout the experiment and the targets are added onto this buffer stream. The role of this *continuous* buffer stream is to efficiently remove unbound and/or nonspecific accumulation of analytes near the QCM surface that can hinder the immunoreactions between

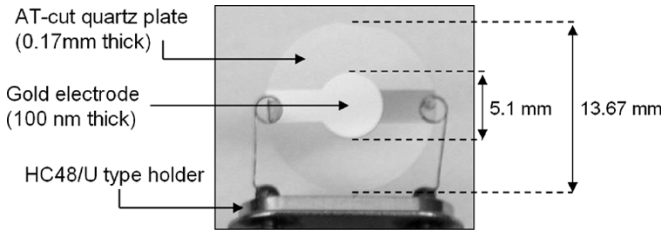

Fig. 5. A 10-MHz AT-cut QCM resonator used for the experiments.

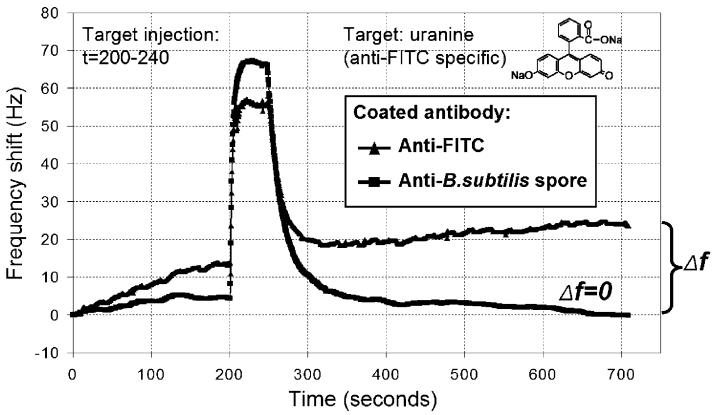

Fig. 6. Response of the antibody coated QCM sensors to 1 ng of uranine.

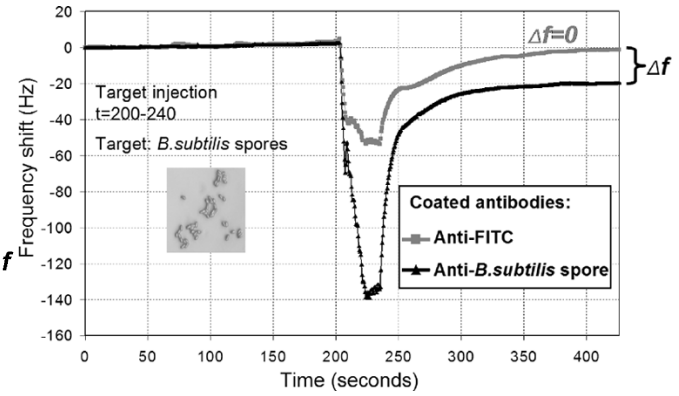

Fig. 7. Response of the anti-FITC and anti-*B.subtilis* spore coated QCM sensors to the *B. subtilis* spore injection.

incoming targets and the antibodies. The buffer stream also eliminates the flushing steps at the end of injection which facilitates faster analysis of the results. Further, a moving, rather than stationary sample medium more closely approximates the state of a "real world" medium in which the biosensor would be deployed—within a turbulent reaction vessel or continuously sampling from a larger reservoir.

Commonly used liquid phase immunosensing systems employ a flowcell where a target solution is injected into at a certain flow rate. Because the size of a spore is too large ($\sim 1\ \mu$m) compared to an antibody (a few nanometers), the flow rate of target injection is important. A high flow rate will result in the spores being swept out without having a chance to bind to antibodies. Too low a flow rate will incur unnecessary accumulation of unbound analytes near the sensor surface, which hinders the binding events. Of course, the choice of optimal flow rate depends on the structure of a flowcell and the size of tubes. After a couple of tests, we found out a combined flow rate of 0.5 mL/min would be acceptable for our system.

V. Experiment Results and Comments

A. Response to a Control Target

Before proceeding to the spore detection experiment, we conducted an experiment to see how the anti-spore antibody coated sensor reacts to a target that is known to be nonspecific to the anti-spore antibody. As mentioned above we use two sensors in a flowcell which are subjected to a same environment to extract the real signature from the sensor responses. To achieve this there needs be further requirement; the surface condition or the characteristic of the coating on each QCM before subject to the target should be same. For example, if one QCM is coated with antibodies specific to a target, the other also should be coated with antibodies using the same immobilization method. As a control antibody, we used mouse monoclonal anti-FITC (fluorescein isothiocyanate) antibodies from Sigma Chemical Company. Because FITC is not water soluble, we selected uranine (a.k.a., fluorescein sodium salt) as a FITC analyte. Uranine is water soluble and binds to anti-FITC antibodies [2].

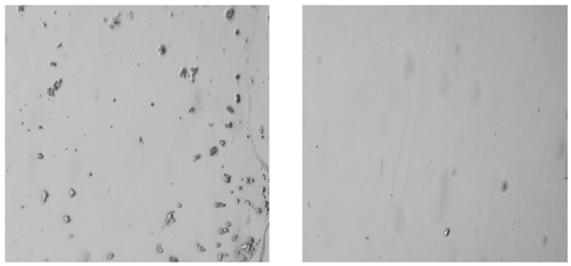

Fig. 8. Optical microscope images of QCM surface after spore detection experiment. Anti-*B.subtilis* spore coated QCM (left) and anti-FITC coated QCM (right).

A solution containing approximately 1 ng of uranine was injected (Fig. 6). The anti-*B.subtilis* spore coated QCM reacted during the injection period but returned to the initial frequency, indicating "no false-alarm," as the analytes are swept out by buffer stream. Anti-FITC coated QCM which is specific to uranine made a noticeable positive baseline shift. We assume the positive frequency shift as opposed to the negative one that can be predicted from the well known QCM [(1) and (2)] is attributed to the fact that the mechanical stiffness [which is essentially $\Delta\mu$ in (4)] of the antibody layer has increased while the antibodies undergo conformational changes before and during the molecular recognition event and it is dominant over the mass loading effect. Such a positive frequency shift has been reported by several research groups [5]–[7]. The conformational change of the antibody was proposed in[8].

B. Response to B.subtilis Spores

1) Signature of the Spore Detection: Purified *B.subtilis* spore suspension purchased from STERIS Company was diluted in $1\times$ TAE buffer making approximately 18 000 spores per injection.

0.3 mL of target solution at a flow rate of 0.5 mL/min was added to a stream of $1\times$ TAE buffer solution that is continuously flowing through the flowcell at a flow rate of 0.1 mL/min.

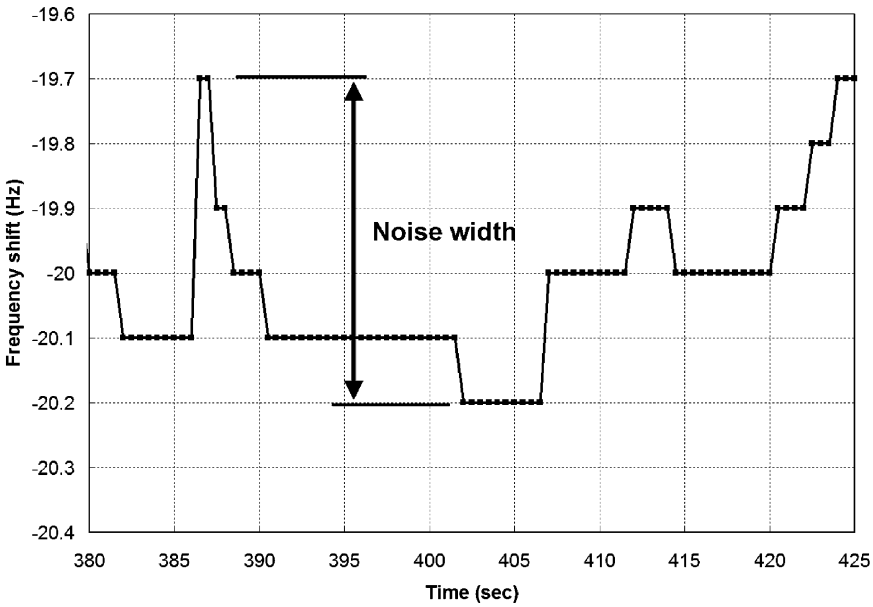

Fig. 9. Frequency fluctuation when the system is idle; defining the noise level of the sensor.

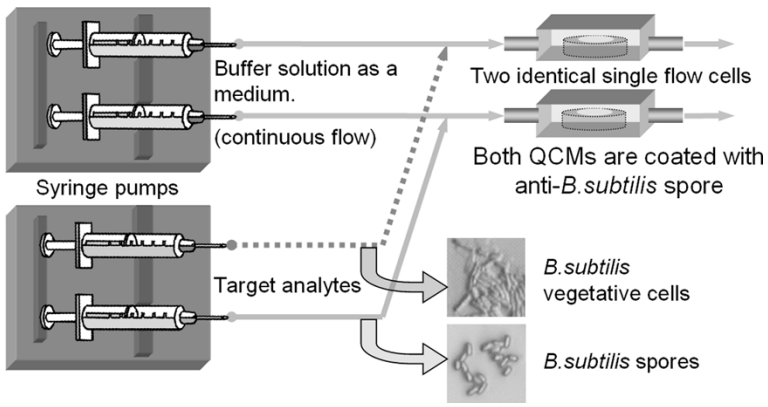

Fig. 10. Modified QCM sensor system setup for the "spores versus vegetative cells" experiment.

After the target injection was done and the system was stabilized, the anti-*B.subtilis* spore coated sensor made a permanent frequency shift about 20 Hz while the anti-FITC coated sensor returned to the baseline. We believe this baseline shift is a signature of the spore detection. Contrary to the case of uranine injection in Section V-A, the frequency shift is negative which indicates that the mass loading effect was dominant in the (4). Uranine is a very small molecule ($\sim$ 300 Dalton) compared to a 1 μm-sized spore.

2) Independent Confirmation of Antibody Capture of Spores: To further verify the result, we take microscope images for the QCM devices used in this experiment. After *B.subtilis* spores detection experiment, the quartz plate of each QCM was disconnected from package and rinsed again with DI water and air dried for microscope observation. A noticeable amount of spores were still attached on the gold electrode surface of anti-spore coated QCM (Fig. 8, left) while spores can be hardly seen on the surface of anti-FITC coated QCM (Fig. 8, right). This result is indicative of the antibody-antigen binding as well as the specificity of the antibody to the *B.subtilis* spore.

3) Estimation of the Lowest Detection Limit: Even the sensor system has been stabilized long after the target injection, we can still observe a small random fluctuation of the frequency which we consider as a noise. This is from the internal noise of the oscillator or frequency counter, or from the mechanical noise of the syringe pump (note that the syringe pump keeps injecting the medium through the flowcell). Actually, this fluctuation is superimposed on the sensor response over the entire sampling period. In order to be distinguished from the noise, the amount of frequency shift of the sensor should be greater than the amplitude of the noise, and this defines the lowest detection limit.

In Fig. 9, the QCM response in Fig. 7 is zoomed-in near the end of the sampling period ($\mathrm{t} = 380 - 425$). To estimate the lowest detection limit of our spore detecting sensor system, we map the final frequency shift in Fig. 7 (20 Hz for the 18 000

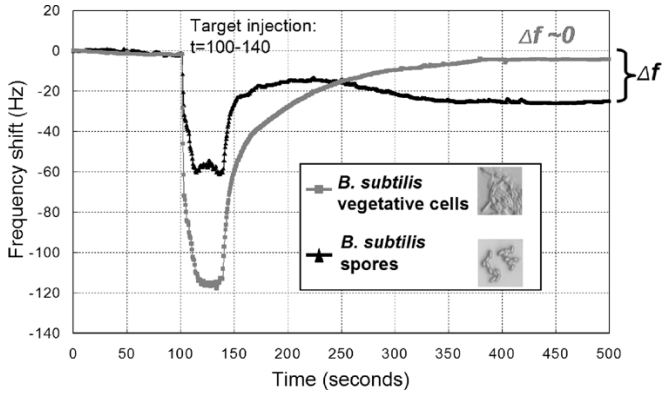

Fig. 11. Response of the anti-*B. subtilis* spore coated QCM sensors to the *B. subtilis* spores and vegetative cells.

spores) to the observed noise level in Fig. 9 (0.5 Hz), which gives an approximation of the lowest detection limit to be 450 spores. As a reference, the U.S. Army estimates the number of anthrax spores that can infect people to be 8 000–50 000, although there is no supporting theory or background for this estimation.

C. Response to B. subtilis Vegetative Cells

We are interested in the sensors specificity to the *spore* of the target bacterium, not to the vegetative cell which is relatively easy to be eliminated with mild treatment. In this section, we verify that the antibody used in the spore detection experiment is, indeed, spore specific, i.e., it does not cross react to the vegetative cells (Figs. 10 and 11). We used a sample of *B. subtilis* vegetative cells that is not supposed to be recognized by the anti-spore antibodies.

For this experiment, the system setup is slightly changed. We use two identical single flowcells with same length of tubing to connect with the syringe pump. Both QCM sensors were coated with anti-*B. subtilis* spore antibodies and the spores and vegetative cells in separate syringes but mounted on the same syringe pump were injected simultaneously to each single flowcell. The injected amount of the spore solution is same as in the previous experiment. The *B. subtilis* vegetative cells (unknown concentration) sample was obtained from the School of Biology, Georgia Institute of Technology, Atlanta.

The larger frequency shift in the response of vegetative cells during the injection period seems to be attributed to their large size and/or mass compared to spores. As vegetative cells are swept away by buffer stream, the frequency returns close to the base line. Because the sample is not purified vegetative cells, a small amount of sporulated cells that might have existed in the sample resulting in the baseline not completely returning to zero. In the case of spore injection, the response was similar to that in Fig. 7; the spores are bound on the QCM surface, more exactly, captured by anti-spore antibodies and made a permanent frequency shift about 24 Hz.

VI. Summary and Conclusions

The data from a series of experiments presented a detection of bacteria spores by utilizing monoclonal antibodies specific to the target spore. The target was instantly identified without time-consuming post process by using a dual QCM sensor with an estimated detection limit of 450 spores. The QCM sensor was responding only to the target spores not to the negative controls, and could distinguish the form of the potential pathogen, discriminating between the relatively benign vegetative stage of *Bacillus* and the potentially virulent spore form. The results were further verified by taking optical microscope images of QCM surfaces that had just undergone the experiment. Also we discussed the non-Sauerbrey behaviors of the antibody coated QCM sensor by considering additional properties such as conformational changes of the antibody and resulting stiffness changes of the coating. We conclude that real-time detection of large molecules such as bacterial spores using a QCM liquid phase immunoassay is possible upon availability of the antibodies and proper setup of the sensor system.

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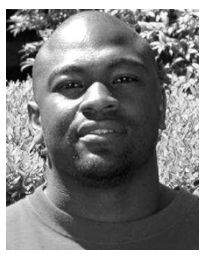

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William D. Hunt received the B.S. degree from the University of Alabama, Tuscaloosa, in 1976, the M.S. degree from the Massachusetts Institute of Technology (MIT), Cambridge, in 1980, and the Ph.D. degree from the University of Illinois, Urbana-Champaign, in 1987, all in electrical engineering.

Prior to his entrance into MIT's graduate program, he was an Engineer with Harris Corporation. Following completion of the M.S. degree, he joined the engineering staff at Bolt Beranek and Newman Corporation. He joined the electrical engineering faculty at the Georgia Institute of Technology, Atlanta, following completion of the Ph.D. degree. His area of expertise is in the area of microelectronic acoustic devices for wireless applications, as well as chemical and biological sensors based on this technology. He has published over 70 papers in refereed journals and conference proceedings. He holds four U.S. patents and five provisional patents.

Dr. Hunt received the NSF Presidential Young Investigator Award in 1989, the DuPont Young Faculty Award in 1988, and the University of Alabama Distinguished Engineering Fellowship in 1994. He was a Rhodes Scholar Finalist in 1975.