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# Piezoelectric-excited millimeter-sized cantilever (PEMC) sensors detect *Bacillus anthracis* at 300 spores/mL

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**Abstract**

Piezoelectric-excited millimeter-sized cantilever (PEMC) sensors consisting of a piezoelectric and a borosilicate glass layer with a sensing area of 2.48 mm$^2$ were fabricated. Antibody specific to *Bacillus anthracis* (BA, Sterne strain 7702) spores was immobilized on PEMC sensors, and exposed to spores (300 to $3 \times 10^6$ spores/mL). The resonant frequency decreased at a rate proportional to the spore concentration and reached a steady state frequency change of $5 \pm 5$ Hz ($n = 3$), $92 \pm 7$ Hz ($n = 3$), $500 \pm 10$ Hz ($n = 3$), $1030 \pm 10$ Hz ($n = 2$), and $2696 \pm 6$ Hz ($n = 2$) corresponding to 0, $3 \times 10^2$, $3 \times 10^3$, $3 \times 10^4$, and $3 \times 10^6$ spores/mL, respectively. The reduction in resonant frequency is proportional to the change in cantilever mass, and thus the observed changes are due to the attachment of spores on the sensor surface.

Selectivity of the antibody-functionalized sensor was determined with samples of BA ($3 \times 10^6$/mL) mixed with *Bacillus thuringiensis* (BT; $1.5 \times 10^9$/mL) in various volume ratios that yielded BA:BT ratios of 1:0, 1:125, 1:250, 1:500 and 0:1. The corresponding resonance frequency decreases were, respectively, 2345, 1980, 1310, 704 and 10 Hz. Sample containing 100% BT spores ($1.5 \times 10^9$/mL and no BA) gave a steady state frequency decrease of 10 Hz, which is within noise level of the sensor, indicating excellent selectivity. The observed binding rate constant for the pure BA and BT-containing samples ranged from 0.105 to 0.043 min$^{-1}$ in the spore concentration range 300 to $3 \times 10^6$/mL. These results show that detection of *B. anthracis* spore at a very low concentration (300 spores/mL) and with high selectivity in presence of another *Bacillus* spore (BT) can be accomplished using piezoelectric-excited millimeter-sized cantilever sensors.


*Keywords:* PEMC; *Bacillus anthracis*; *Bacillus thuringiensis*; Resonance; Selectivity

## 1. Introduction

*Bacillus anthracis* (BA), a spore forming rod-shaped Gram positive and non-motile bacterium, is the etiology agent of *anthrax*. Under favorable growth conditions, the bacteria exist as vegetative cells. If the growth conditions deteriorate (extreme temperatures and/or nutrient deprived environment) the vegetative cells sporulate: forming intracellular endospores (Hunter et al., 1989; Stragier and Losick, 1996; Turnbull, 1990). As the vegetative cells die the endospores are released as spores. Spores are biologically dormant structures that are highly resistive to extreme temperatures, physical damage, desiccation, and harsh chemicals. These properties allow the bacterial spores to survive for years in soil. Spores remain dormant until they encounter an ideal growth environment in which they germinate into their vegetative state. Spores are also airborne and cause respiratory infection, as was seen in the bioterrorism *anthrax* spores mailed in the United States of America in the Fall of 2001. There are three forms of human *anthrax* known to date: gastrointestinal, cutaneous, and inhalation *anthrax*. *B. anthracis* spores can enter the body by ingestion, through the skin, and by inhalation. The inhalation *anthrax* is the most severe one because 99% casualties occurred in individuals who were not treated before symptoms developed (James et al., 1998).

The threat of *anthrax*-causing *B. anthracis* spores as a bioterrorism agent has created an urgent need for a rapid real-

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time, highly selective and sensitive technique to detect the presence of *anthrax* spores. In response to the *anthrax* threat, various detection techniques capable of providing reliable identification of *anthrax* spores are currently under development. These detectors include, evanescent wave fiber-optic biosensors (Tims and Lim, 2004), Love-wave biosensors (Branch and Brozik, 2004), real-time PCR (Fasanella et al., 2001; Kim et al., 2005; Makino and Cheun, 2003; Ramisse et al., 1996; Sjöstedt et al., 1997; Wang et al., 2004), phage display (Turnbough, 2003), membrane-based on-line optical analysis systems (Floriano et al., 2005), electrostatic precipitator (Mainelis et al., 2002), electrochemiluminescence (Bruno and Kiel, 1999; Gatto-Menking et al., 1995), and quartz crystal microbalance (QCM) (Lee et al., 2003). When confronted with the requirement of low concentration detection, traditional approach involves the growing of the micro-organism on selective media for at least 24 h, followed by morphological and biochemical analysis (Davey and Kell, 1997; Francis et al., 2001). The 24-h incubation time is for too limiting, particularly in the context of public safety. Hence, there is a need for a simple and inexpensive method for the detection of *B. anthracis* spores in real time. Furthermore, the United State Postal Service (USPS) has required the development of a rapid detection method, which is cost effective, for the identification of bioterrorism threat agents (Fox et al., 2002). In a similar manner the Department of Transportation (DOT) requires a detection system that will identify bioterrorism agents within 20 min of exposure.

In this paper, we explore the application of the piezoelectric-excited millimeter-sized cantilever (PEMC) sensors for detection of *anthrax*-causing *B. anthracis* spores at a concentration as low as 300 spores/mL in the liquid phase. The development of biosensors has been significantly enhanced over the past decade by biomedical (Liu et al., 2003) and chemical applications (Alvarez et al., 2003). However, the growing interest of millimeter-sized cantilever biosensors for biological detection is due to its high performance characteristics: high sensitivity, short response time, robustness, selectivity, resonance stability, and surface regeneration capabilities. It is worth noting that a complete biosensor system for the control and prevention of a bioterrorism attack requires a three-step process: a detection step, an identification step, and finally a communication step (Morris and Sadana, 2005). Here, we employ the PEMC sensor to the first step (detection).

The PEMC sensors are a composite structure of two layers: a lead zirconate titanate (PZT) and a borosilicate glass layer of a few millimeters in length. The PZT layer acts both as an actuating and a sensing element. The detection of biological entities requires the immobilization of a recognition molecule, such as an antibody or a receptor molecule, on the sensor surface (Campbell and Mutharasan, 2005a). When the target of interest, in this case spores, binds to the cantilever's sensing surface the effective mass of the cantilever increase which alters the cantilever's resonant frequency. The resonant frequency change with time is used to provide quantitative measurement of the spore concentration.

## 2. Cantilever physics

The theoretical analysis of the resonant frequency of an oscillating rectangular cantilever in air is well documented in literature as (for example see: Elmer and Dreier, 1997; Naik et al., 2003):

$$f_n = k_n \sqrt{\frac{K}{M_e}} \tag{1}$$

where $k_n = 0.1568$, 0.9827, 2.7517, and 5.3923 corresponding to the first four eigen values for a rectangular cantilever (Elmer and Dreier, 1997). The parameter $K$ is the effective spring constant and depends on the thickness, density and modulus of the cantilever material, namely both glass and PZT. $M_e$ is the effective tip mass of the cantilever in air. Upon immersing a cantilever in a liquid sample, the mass of liquid adjacent to the cantilever oscillates with the cantilever becoming part of its effective mass. When target pathogens attach to the cantilever surface, it adds mass to the cantilever resulting in Eq. (1) being modified as

$$f'_{nf} = k_n \sqrt{\frac{K}{M'_e + \Delta m}} \tag{2}$$

In the above, $M'_e$ is the effective mass of the cantilever under liquid immersion condition, and $\Delta m$ is the equivalent mass of pathogen attached to the oscillating cantilever surface. A more detailed development is available in Campbell and Mutharasan (2005a). From the above, one gets

$$f_{nf} - f'_{nf} = \frac{1}{2} f_{nf} \frac{\Delta m}{M'_e} \tag{3}$$

where $(f_{nf} - f'_{nf})$ is the change in resonant frequency of the $n$th mode in fluid due to pathogen attachment. Therefore, a change in resonant frequency represented by the left-hand side of Eq. (3) is linearly dependent on the change in cantilever mass, at the resonant mode of interest. In this paper, we measure the change in resonant frequency $(f_{nf} - f'_{nf})$ under various test conditions.

## 3. Fabrication

The PEMC sensors were manually fabricated as a composite structure of two layers: a 127 μm thick PZT single sheet (Piezo Systems Inc., Cambridge, MA) and a 160 μm thick cover glass slip (Fisher Scientific); for details see Campbell and Mutharasan (2005b). The cantilevers tips were designed with the PZT layer, 1.5 mm × 1 mm (length × width), bonded to the glass layer, 3.5 mm × 1 mm (length × width), with a non-conductive adhesive such that a 2 mm length of the glass