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Detection of airborne *Bacillus anthracis* spores by an integrated system of an air sampler and a cantilever immunosensor

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Abstract

A near real-time, sensitive and reliable method of detecting airborne *Bacillus anthracis* spores by air sampling (wet mode) and then exposing the collected sample to an antibody-functionalized piezoelectric-excited millimeter-sized cantilever (PEMC) sensor is presented. Using a commercial air sampler, a 10 min air sample at 267 L/min captured the airborne particulates containing *Bacillus anthracis* (BA) spores and concentrated them into 5 mL of phosphate buffered saline (PBS). This sample was then injected into a flow cell containing an antibody-functionalized PEMC sensor. The resonant frequency of the PEMC sensor at 925.1 kHz decreased exponentially as the BA spores attached to the sensor surface producing a positive response well beyond the noise level in 2 min and reached a steady state value in 20 min. In liquid phase, the sensor response correlated well ($R^2 = 0.99$) with spore concentration and was shown to follow: [Response in Hz] = (0.0637) × (spore concentration in #/mL). Our results show that detection of 38 BA spores/L of air is achievable in near real-time with an estimated lower limit of detection of ∼5 spores/L of air in the configuration tested.

Keywords: Resonance; Size distribution; Resonant-frequency; Sterne strain

1. Introduction

Detection of airborne *Bacillus anthracis* (BA) spores has gained significant interest since the anthrax spore mailing in Fall of 2001. The common performance parameters of the detection methods currently in use and/or under development include time to detect, lower limit of detection (LOD), repeatability and reliability of detection. Current detection methods can be grouped into two broad categories—culture enrichment followed by either PCR-based detection or antibody-based detection. Both methods require either dry or wet mode air sampling to concentrate the airborne particulates prior to subjecting them to detection. Prior to recent advancement in advanced detection methods, the time-consuming culture enrichment method was required to identify biowarfare agents [1]. Several sensing platforms under development that are capable of providing reliable identification of air-borne biowarfare agents include PCR-based measurements [2–4], and antibody-functionalized systems such as RAPTOR [5], various test kits [6] and the piezoelectric-excited millimeter-sized cantilever (PEMC) sensor [7–9]. All of these methods require an air sampler to collect airborne BA prior to detection and have limitations that tradeoff cost, detection time, detection limit and repeatability. For PCR-based techniques, their high selectivity is offset by their limitations of high sample purity requirements, cost and detection time where detection of a single pathogen can take up to several hours [2]. The commercial RAPTOR system depends on both an immobilized capture antibody and a fluorescence-labeled antibody for detection. It requires long preparation time and shows an LOD on the order of 10^4–10^5/mL [5]. In a separate study, Tims and Lim have described the detection of *Bacillus anthracis* spores from various powders using RAPTOR at an LOD of 3.2×10^5 spores/mg of powder. To the knowledge of the authors, three test kits are available for detecting anthrax spores [6]: Anthrax Bio-Threat Alert (BTA), BioWarfare Agent Detection Devices (BADD), and Anthrax SMART II. Their disadvantages include a high LOD (10^5–10^6 spores), cost, and questionable repeatability [6]. All of the preceding methods' limitations (cost, time to detect, selectivity and LOD) are overcome by the antibody functionalized PEMC sensor. Recent

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developments for PEMC sensors have shown both very low detection levels of 300 spores/mL (over 3 orders of magnitude improvement) and high selectivity where antibody immobilized PEMC sensors are selective to BA spores in presence of varying amounts of *Bacillus thuringiensis* (BT) spores [8]. We showed that while the presence of BT increases the sensor response time, the magnitude response was unaffected. In the current method, two advantageous features reduce non-specific binding. Measurement under sample flow conditions (~0.1 cm/s) combined with active vibration of sensing surface gives unparalleled selectivity in complex fluid environment. Previous work showed selective detection of *Bacillus anthracis* at 330 spores/mL in the presence of two *Bacillus* species at 330,000 spores/mL [10] and the detection of *Escherichia coli* O157:H7 in ground beef samples at 10 cells/mL [11]. The high degree of specificity in complex fluids reduces false negatives, thereby improving reliability.

In this paper, we show system level detection (air sampling, sample transfer and subsequent detection of the captured airborne BA spores) in less than 20 min at an airborne concentration as low as 38 spores/L of air.

2. Sensing principle

PEMC sensors are immunosensors that have mass change sensitivity in the range of picograms to sub-femtograms [9,10,12,13,14]. They are constructed from a piezoelectric ceramic (lead zirconate titanate, PZT) and a fused silica layer of different lengths to provide bending at resonance [7]. The method of measurement is by monitoring its resonant frequency as the sensor is exposed to liquid sample containing the desired target analyte. It is well established in the literature that the frequency at which a cantilever-based sensor resonates is inversely proportional to the square root of its mass. For small changes of cantilever mass, its resonant frequency decreases linearly with the attached mass. Therefore, quantitative measurements of the bound spores can be determined by tracking the change in resonant frequency.

Piezoelectric-excited millimeter-sized cantilever (PEMC) sensors, comprising of a piezoelectric layer bonded to a non-piezoelectric layer (glass) and anchored at one end, exhibit high-order mode resonance at ~900 kHz that shows mass change sensitivity in the range of 0.3–2 fg/Hz [10,13,14]. The piezoelectric layer acts both as an actuating as well as a sensing element while the glass provides the surface for antibody immobilization. PEMC sensors are used in resonance mode. Binding of the target antigen to the sensor surface causes change in mass that results in resonance frequency decreases which is measured using an impedance analyzer.

3. Experimental

3.1. Apparatus

The test apparatus shown in Fig. 1 was located inside a biological hood. It consists three major sections—aerosol generator, air sampler and detector. Aerosol generation utilizes a nebulizer (LT2 Nebulite, GF Health Products, Inc. GA) and 0.2 μm filtered

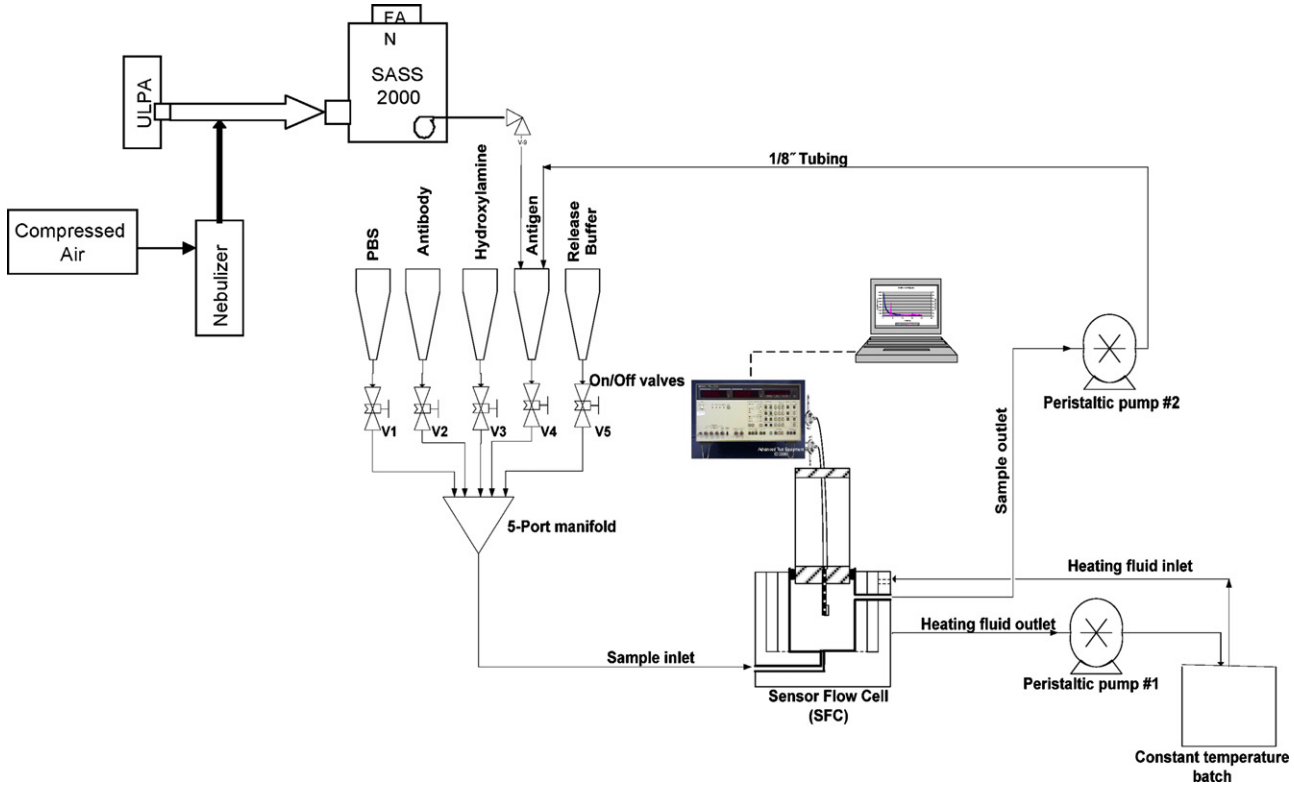

Fig. 1. Schematic of detection flow circuit. The aerosol that is generated by the nebulizer is captured by the SASS 2000. The collected SASS sample output is connected to the sensor flow circuit by a shutoff valve.

compressed air. The major components for air sampling include an inlet ULPA filter (99.99% <0.3 μm, Flanders Precisionaire, FL), and a commercial air sampler (SASS 2000, Research International Inc., WA). The flow tube connects the ULPA filter and the nebulizer to the inlet of the SASS 2000 air sampler. The SASS 2000 is a wetted wall cyclone that samples 267 L/min of air for a user defined time period producing a 5 mL liquid sample containing the captured airborne particulates. During the sampling, an internal liquid reservoir replenishes the evaporated water maintaining a constant 5 mL liquid. The detection subsystem includes the PEMC sensor installed vertically into a PBS-filled sensor flow cell (SFC) and measurement equipment. The SFC and the flow circuit contain a hold-up of 4 mL and were kept at a constant temperature of 25 °C. The cantilever electrodes were connected to an impedance analyzer (Agilent, HP 4192A) which is interfaced to a data acquisition PC with LabVIEW® application that provided the temporal impedance and phase angle measurements.

3.2. Experimental methods

Prior to use, the SASS was rinsed twice with filtered (0.2 μm) PBS. For the control sample, a 10 min run, 5 mL background sample was collected by nebulizing 4 mL of filtered PBS in flowing air (267 L/min). This was followed by introducing a known BA spore sample (10 mL) into the nebulizer for a 10 min run. The liquid sample (5 mL) output of the SASS 2000, the sample remaining in the nebulizer (6 mL), and the initial nebulizer charge (10 mL) were measured volumetrically, and for particle size distribution (PSD). The PSD measurements were carried out using the Coulter Multisizer II analyzer (Beckman Coulter Inc., CA) with a 30 μm orifice tube, 1 M NaCl electrolyte and a 100 μL sample. As shown in Fig. 1, a 2 mL liquid sample from the SASS output was directly transferred into the sensor flow circuit where all detection experiments were carried out in a recirculating flow configuration at flow rates of 0.5 and 1.0 mL/min. The operation of the sensor flow circuit was reported earlier [7]. Briefly, the antibody to BA spores was immobilized on the sensor followed by introducing the sample from the SASS 2000 for detection. To confirm that the sensor response was due to spore attachment, release response was obtained at pH 1.85. The latter protocol was established by the authors in a previous report [8].

3.3. Sample preparation

Bacillus anthracis (BA) spores, Sterne strain 7702, and Protein A purified Rabbit polyclonal antibody (anti-BA) were the kind contribution from Professor Richard Rest (Drexel University College of Medicine, Philadelphia, PA). A master stock of BA spores (2×10^5 BA/mL) in phosphate buffered saline (PBS) (10 mM, pH 7.4) was diluted to 3×10^4, 5×10^4, and 1×10^5 BA/mL. The number and particle size distribution (PSD) of the stock BA spore suspensions were determined using the method described above. For each series of runs, a background PSD was obtained. Each test sample was diluted 1:2 using filtered 2 M NaCl solution prior to measurement.

3.4. Immobilization

The method used for antibody immobilization on sensor surface was the same as in our previous studies [8]. Briefly, the sensor was cleaned, and then silanylated with 0.4% 3-aminopropyl-triethoxysilane (APTES) in deionized water at pH 3.0. The antibody was activated with zero length cross linker, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and promoted by sulfo-*N*-hydroxysuccinimide (sulfo-NHS), and subsequently reacted with the amine group on the sensor glass surface. Hydroxylamine was used to convert the activated carboxyl groups on the antibody that did not react with the sensor surface, back to normal carboxyl groups. All chemicals were from Sigma–Aldrich.

3.5. Experimental design

Several scoping experiments were conducted to achieve a uniform PSD for the aerosolized nebulizer output. This entailed manipulating relative flow rate of nebulizing air and dilution air, and adjusting total air flow rate. For detection, a total of six experiments were conducted to assess the performance of the sensor and the overall system with respect to response time, level of detection, repeatability and signal to noise ratio. Table 1 summarizes the test conditions for these experiments where for the first three experiments the SFC flow rate was varied (0.5 and 1.0 mL/min) at the constant input concentration (50,000 BA/mL). This enabled the determination of the optimum SFC flow rate for detection. In order to determine the lower limit

Table 1
Experimental parameters, Coulter Counter PSD and PEMC sensor response to BA binding

Experiment	Flow rate of liquid sample (mL/min)	Nebulizer input 1–2 μm (counts/mL)	SASS output 1–2 μm (counts/mL)	BA spores corrected for background (BA/mL)	PEMC sensors response (Hz)
Control	1.0	5740	5740	0	14 ± 44
1	0.5	50000	34100	28360	1853 ± 16
2	0.5	50000	34800	29060	1917 ± 19
3	1.0	50000	36300	30560	2101 ± 53
4	1.0	30000	13300	7560	563 ± 29
5	1.0	50000	30620	24880	1697 ± 20
6	1.0	100000	67460	61720	3752 ± 59

of detection, the input spore concentration was then varied from 30,000 to 100,000 BA/mL at constant flow rate (1.0 mL/min).

3.6. Controls

Two controls were done with each detection experiment. The first control consisted of antibody-functionalized PEMC sensor being exposed at 1 mL/min to the control PBS buffer that was taken from the SASS 2000. This sample, as you will note in the next section, contained ~5700 particles/mL between 1 and 2 μm. The second control was the response of a clean PEMC sensor which was not immobilized with an antibody and was exposed to PBS buffer at 1 mL/min.

4. Results and discussions

4.1. Nebulizer characterization and spore capture determination

The spore concentration in each of the test samples was determined using the Coulter Counter analyzer. Fig. 2 compares the PDS of the input test sample (10 mL of 2.15×10^6 BA spores/mL) with particles captured by the SASS 2000 air sampler. The liquid sample that remained in the nebulizer was analyzed and is also shown in Fig. 2. The average size of a spore observed from Fig. 2 is approximately 1.2 μm, which concurs with the ESEM photomicrograph shown in the inset. The area under the curves between 1.0 and 2.0 μm gave an estimate of the total number of spores. Analysis of the sample remaining in the nebulizer gave 7.2×10^6 BA spores and the number of the spores collected by SASS 2000 was 5×10^6 BA spores. By subtracting this spore PSD from the input PSD, one can estimate the size distribution and number of the spores aerosolized. A system spore mass balance (input to nebulizer less remaining in nebulizer less captured by the SASS) indicates that approximately 1×10^7 BA spores of the aerosolized spores were either retained in the flow apparatus and/or exited the SASS 2000 exhaust. The

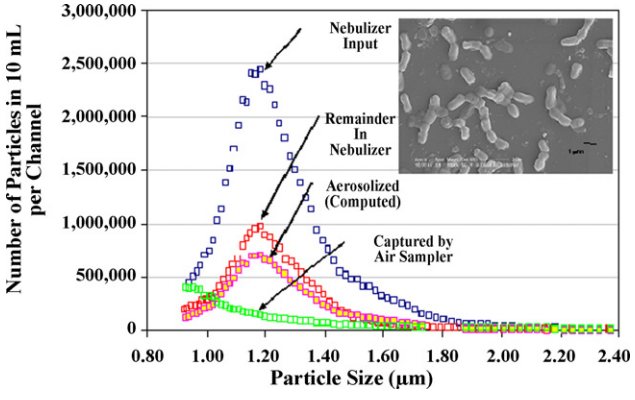

Fig. 2. A sample containing 2.15×10^6 BA spores/mL was aerosolized and the particle size distribution (PSD) was measured of the input and output enabled the characterization of the nebulizer-air sampler system. By subtracting the PSD of the beginning and ending nebulizer fluid one can estimate the PSD of the aerosolized spores. The inset BA spore photomicrograph confirms the average diameter of a spore as 1.2 μm.

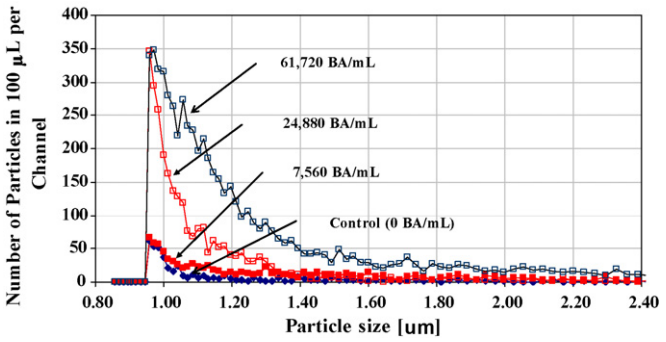

Fig. 3. The particle size distribution of the BA spore samples collected by the SASS 2000. The distribution was not symmetrical because electrical noise was dominant at the lower particle size when count was less than 50,000.

resulting system delivery/capture efficiency was 35 ± 3%. We observed that for spore counts below 5×10^5, the distribution was skewed to the lower particle size due to electrical noise. However, we were able to distinguish the BA spores from extraneous particles because the particle count of the control buffer (filtered PBS) was less than any of the BA containing samples (see Fig. 3).

In Fig. 3, we compare the PSD for the samples collected from the control buffer with those collected from experiments 4–6. By integrating particle count between 1 and 2 μm, the total number of spores can be determined, and is summarized in Table 1. Within the size range of 1–2 μm, the control buffer contained 5740 particles/mL, and experiments 4–6 contained 13,300, 30,620, and 67,460 particles/mL, respectively. By subtracting the background counts, the BA spore concentration in the three samples were estimated at 7560, 24,880, and 61,720 BA/mL. In order to determine the spore concentration in air, the total collected spore number corrected for collection efficiency was divided by the air volume flow over the sampling period. For example, in the case of 7560 BA/mL, the airborne concentration was calculated from (7560 spores/mL) (5 mL)/(267 L/min) (10 min) (0.35) = 38 spores/liter-air. In experiments 5 and 6, the estimated air sampler inlet spore concentration was 133 and 329 BA spores/L of air, respectively. By way of comparison one can apply the same measured performance characteristics of the SASS 2000 to other liquid-based sensors and arrive at an estimated LOD in air. For example, a detector with a liquid LOD of 10^5 spores/mL (such as RAPTOR) would have an approximate system LOD of 375 spores/L of air when coupled to the SASS 2000 air sampler under similar conditions [5].

4.2. Characterization of the PEMC sensor

Several PEMC sensors were fabricated and used in the experiments reported here. Each sensing experiment was repeated at least twice and the data shown are typical of the results obtained. The resonant spectrum, a plot of phase angle versus excitation frequency, in air showed dominant bending mode resonance near 176.3, 990.1, and 1807.5 kHz. Upon liquid immersion the resonant frequency at 990.1 kHz decreased by 65–925 kHz, indicating that it is a sensitive mode. Therefore, the 925 kHz resonance mode in liquid was used for in-liquid detection exper-

iments as its Q value remained high ($Q \sim 12$ in liquid) and exhibited low noise ($\leq \pm 60$ Hz) under flow conditions.

4.3. Effect of Flow rate on PEMC sensor response

Because an important consideration in detection of pathogens is the time to detect and since contacting the collected sample with sensor surface is a prerequisite for detection, attempts to shorten detection time by increasing sample flow rate were investigated. Flow rate was set at 0.5 and 1.0 mL/min for three separate runs at a nominal spore concentration of 35,000 BA/mL (34,100–36,000) plus control at 0 BA/mL. The control was an antibody-functionalized PEMC sensor exposed at 1 mL/min to the control PBS buffer that was taken from the SASS 2000. Fig. 4 presents the results of these experiments where experiments 1 and 2 were carried out at 0.5 mL/min and experiment 3 at 1.0 mL/min. The time to reach steady state frequency decreased significantly by ~80%. It is believed that at the higher flow rate used, the transport of spores to the sensor surface was enhanced. At higher flow rates, the signal-to-noise ratio decreases, and thus in the current work, we limited the flow rate to 1 mL/min. However, one can conceive a mode of measurement where rapid flow is followed by stoppage for resonance frequency measurement. Such a method can potentially further reduce the sensor response time and is currently being investigated.

In Fig. 4, the three experiments containing nearly the same number of spores ($29{,}330 \pm 1120\ \mathrm{mL}^{-1}$) gave nearly the same PEMC sensor response of 1957 ± 129 Hz. The maximum noise level of measurement in these three experiments was 53 Hz and thus the variance of sensor response for the three samples is less than three times the noise level. That is, the PEMC sensor provides repeatable measurement considering the fact that in each experiment, the sensor surface was prepared starting with cleaning followed by amination and fresh antibody immobilization.

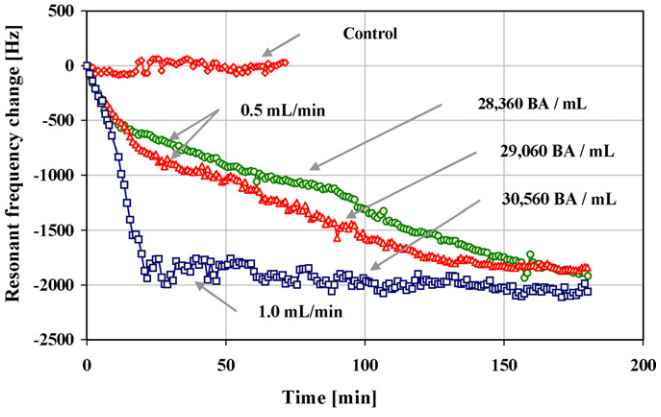

Fig. 4. The spore attachment to sensor surface is measured by resonance frequency change. Flow rate affected the total sensor response as shown above. For samples from experiments 1 and 2, flow rate of 0.5 mL/min and for sample from experiment 3, 1 mL/min was used. The three samples contained approximately the same BA concentration. We note that while the total resonance frequency changes are similar, the binding rate was more rapid at the higher flow rate. The control response shown is the response of the antibody-immobilized sensor to the sample collected with plain DI water. As noted in Table 1, the control contained particulate in the 1–2 μm range of 5740 mL^{-1}.

Given that 2 mL sample was introduced into the sensor circuit, approximately 60,000 spores were exposed sensor surface. A 2 mm^2 sensor can accommodate 2 million spores if we assume close packing of the spores and cross sectional area of a single spore as ~1 $\mu\mathrm{m}^2$. That is, if all the spores in the sample were to attach to the sensor surface, coverage would be ~3%.

Based on the results given in Fig. 4, we conclude that 1 mL/min SFC flow rate reduces detection time while not affecting the steady state response significantly. Hence, subsequent detection experiments were conducted at 1 mL/min.

4.4. Effect of varying BA concentration on PEMC sensor response

An important parameter in detection methods is the limit of detection. Hence, a series of experiments were conducted where the spore concentration in the nebulizer was systematically varied. In Fig. 5 we summarize the results obtained in experiments 4–6. As the spore concentration increases, the rate of spore attachment increases. In all experiments the sensor took less than 20 min to reach a steady state frequency value which is consistent with the results of the flow rate tests discussed above. As noted in Table 1 increasing the spore concentration from 7560 to 24,880 to 61,720 BA/mL, the sensor response showed a corresponding increase in total frequency change (563 ± 29, 1697 ± 20, and 3752 ± 59 Hz, respectively). Note that the change in sensor response (3× for sample 1 to 2 and 7× for samples 1–3) is in approximate agreement with the change in BA spore concentrations (3× and 8×, respectively). For the negative control samples, the resonant frequency change fluctuated around zero with an average value of 14 ± 44 Hz showing no significant change in resonant frequency with a minimum signal-to-noise across all measurements of 19. While Figs. 4 and 5 show the transient response of the PEMC sensor to solutions containing BA spores of varying concentration, Fig. 6 shows the steady state response of the sensor. Least squares fit with forced zero intercept gave an excellent correlation ($R^2 = 0.99$) indicating that proportional response to BA containing samples was obtained. It

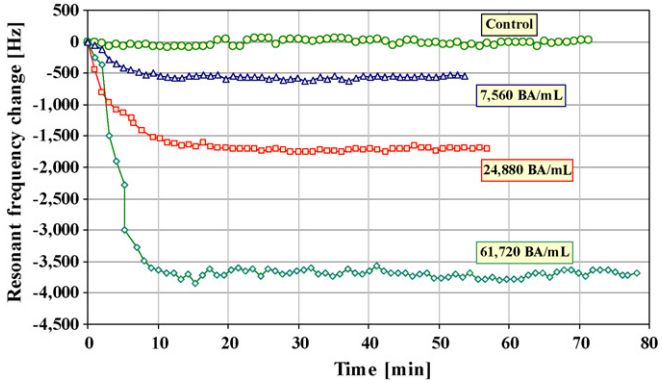

Fig. 5. By recirculating the sample, the spore attachment one can follow the transient response of PEMC sensor to the binding of *Bacillus anthracis* spores from experiments 4–6 (7560, 24,880 and 61,720 BA/mL, respectively) to the final steady-state value. The control response shown is that of the anti-BA functionalized PEMC sensor exposed to filtered PBS buffer, flowing at 1 mL/min, so as to establish the baseline frequency change of the sensor.

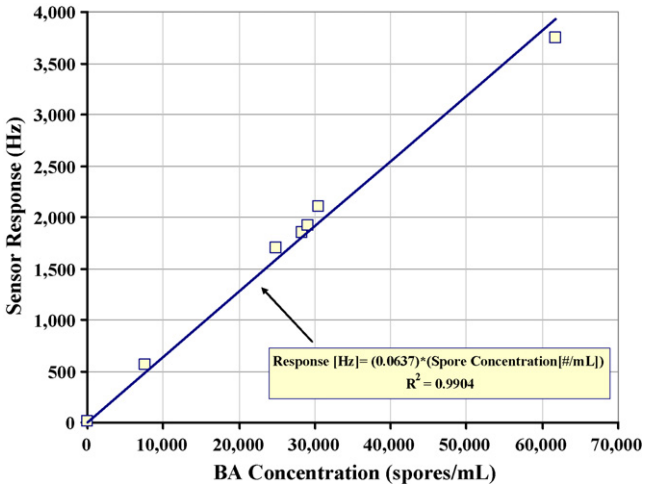

Fig. 6. The PEMC sensor shows a linear response across all six experiments. Each measurement started with a freshly prepared surface (cleaned, aminated and then immobilized with antibody). In the narrow range of spore concentration (7000–60,000) sensor response was linear.

is to be noted that this agreement results from detections where the sensor surface was re-immobilized with antibody between each measurement. It is also worth noting that there was no response to negative control, which indicates that the presence of non-BA particulates in the buffer solution did not affect the sensor response. This insensitivity to the presence of particulate matter is consistent with the findings reported by the authors for the direct detection of *Escherichia coli* O157:H7 in ground beef samples [15].

4.5. Detection confirmation

Spores bound to the antibody on the sensor surface can be released by increasing the ionic strength of the medium or by lowering the pH. The rabbit polyclonal antibody used in this study showed a very high avidity and thus a pH of 1.85 was found necessary to release the antigen [8]. Thus to confirm that the resonance frequency decrease in detection experiments (Figs. 4 and 5) is indeed due to BA spore attachment, a pH 1.85 solution was flowed through the SFC after each detection. An example response is given in Fig. 7 for experiment 6 sample. After the response frequency stabilized, PBS was pumped to re-establish the liquid environment present prior to the detection sequence. The release was signified by a rapid increase of the resonant frequency returning approximately to the original starting resonant frequency present prior to the detection. The minor overshoot of 261 ± 34 Hz is indicative of complete BA release and probably a minor antibody detachment.

4.6. Air sampler–PEMC sensor response

The results obtained in this study suggest that the combined system of an air sampler and a PEMC sensor has the potential to measure airborne *Bacillus anthracis* spores in less than 30 min; 10 min for particulate capture by the air sampler and less than 20 min for total PEMC sensor response. The time for positive

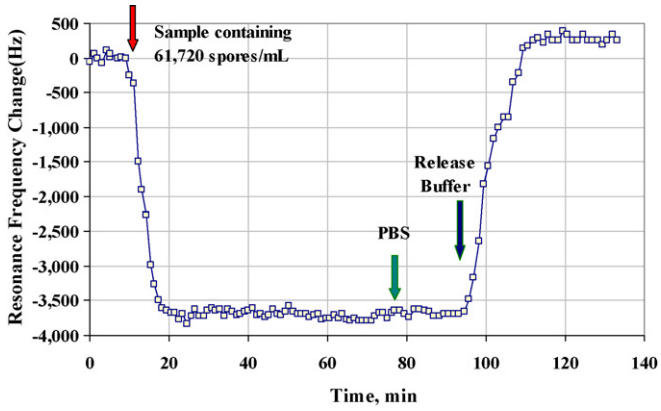

Fig. 7. Confirmation of detection of BA spores by releasing the spores using a pH 1.85 buffer. *Note*: the sensor response returned within 261 ± 34 Hz of the starting resonant frequency indicating that all of the spores were removed plus a minor amount of immobilized antibody.

indication of the presence of BA is less than 12 min. The signal-to-noise ratio in these experiments is high (>18; see Table 1). If a value of 3 is acceptable for positive detection, then the lower limit of detection is a factor of 8 below this minimum, or ~5 spores/L of air for a 10 min air sample in the configuration tested here. If the required threshold of detection is 50 spores/L of air, the air sampling time can be reduced by an order of magnitude resulting in a system response time of less than 5 min for the positive detection of BA in air.

5. Conclusion

Each of the performance indices identified in Section 1 (response time, LOD, and repeatability) were characterized for the combined air sampler–PEMC sensor system. We have shown that piezoelectric-excited millimeter-sized cantilever sensor is a sensitive and reliable method for the rapid detection of airborne *Bacillus anthracis* spores in conjunction with a commercial air sampler. We conclude that the sensor response is proportional to BA concentration, and detection/measurement of BA at 38 spores/L of air per minute was experimentally shown. Minimum signal-to-noise observed was 18, and at an acceptable signal-noise-ratio of 3, we estimate lower limit of detection as 5 spores/liter. We also showed that for the same spore concentration, PEMC sensor response gave repeatable response within ±7%. Finally, the presence of naturally found inert particle in 1–2 μm range at 1/10th of spore concentration did not affect sensor performance. Further work to quantify PEMC response in the presence of inorganic, organic and biological contaminates is currently under study.

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