Published in final edited form as:

Analyst. 2008 May ; 133(5): 649–654. doi:10.1039/b715948j.

Label-free flow-enhanced specific detection of *Bacillus anthracis* using a piezoelectric microcantilever sensor†

John-Paul McGovern^a, Wan Y. Shih^b, Richard Rest^c, Mitali Purohit^c, Yognandan Pandya^c, and Wei-Heng Shih^d

^aDepartment of Materials Science and Engineering, Drexel University, Philadelphia, PA, 10104, USA

^bSchool of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, PA, 19104, USA. E-mail: shihwy@drexel.edu

^cDepartment of Microbiology and Immunology, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA, 19129, USA

^dDepartment of Materials Science and Engineering, Drexel University, Philadelphia, PA, 10104, USA. E-mail: shihwh@drexel.edu

Abstract

Differentiation between species of similar biological structure is of critical importance in biosensing applications. Here, we report specific detection of *Bacillus anthracis* (BA) spores from that of close relatives, such as *B. thuringiensis* (BT), *B. cereus* (BC), and *B. subtilis* (BS) by varying the flow speed of the sampling liquid over the surface of a piezoelectric microcantilever sensor (PEMS). Spore binding to the anti-BA spore IgG coated PEMS surface is determined by monitoring the resonance frequency change in the sensor's impedance vs. frequency spectrum. Flow increases the resonance frequency shift at lower flow rates until the impingement force from the flow overcomes the binding strength of the antigen and decreases the resonance frequency shift at higher flow rates. We showed that the change from increasing to decreasing resonance frequency shift occurred at a lower fluid flow speed for BT, BC, and BS spores than for BA spores. This trend reduces the cross reactivity ratio of BC, BS, and BT to the anti-BA spore IgG immobilized PEMS from around 0.4 at low flow velocities to less than 0.05 at 3.8 mm s⁻¹. This cross reactivity ratio of 0.05 was essentially negligible considering the experimental uncertainty. The use of the same flow that is used for detection to further distinguish the specific binding (BA to anti-BA spore antibody) from nonspecific binding (BT, BC, and BS to anti-BA spore antibody) is unique and has great potential in the detection of general biological species.

1. Introduction

Bacillus anthracis (BA) is a Gram positive spore-forming bacterium that can survive for long periods in harsh environments in its sporulated state, and is listed as a 'Category A' bioterrorism agent by the Centers for Disease Control and Prevention (CDC).¹ Current CDC-approved positive identification techniques involve staining for the vegetative cell after culturing of collected spores.² Clearly, this is a time-consuming endeavor, while in the event of a bioterrorist attack, early detection of BA is of the utmost importance when human or animal

†The HTML version of this article has been enhanced with colour images.

© The Royal Society of Chemistry 2008

Correspondence to: Wan Y. Shih.

exposure is a possibility. Among the various techniques under development, polymerase chain reaction (PCR),^{3–5} which requires amplification and takes at least four hours for detection,^{6, 7} is regarded as more specific than other methods as it looks for a genetic signature for detection. However, even the most advanced molecular techniques have difficulty consistently distinguishing between BA and its close relatives,⁸ such as *B. thuringiensis* (BT), *B. cereus* (BC), *B. subtilis* (BS) and others. There is an urgent need to develop rapid and reliable detectors that can detect BA spores specifically in real time.

Recent developments in fiber optics,⁹ silicon microcantilevers¹⁰ and piezoelectric microcantilevers¹¹ have led to direct, label-free detection of target antigens. In particular, antibody-functionalized piezoelectric microcantilevers sensors¹² have been demonstrated to be capable of rapid, label-free, and yet highly sensitive detection of BA in a short time (less than 30 min). One major issue with antibody–antigen-based detection is specificity, as nonspecific binding from spores other than the target antigen can occur if their surfaces are similar. This can give rise to false positive detection events as BA's close relatives bear a nearly identical chemical structure in the spore coat.¹³ False positive detection events can, in turn, lead to costly and unnecessary public alarm. Given the simplicity of the antibody–antigen based detection method, it is desirable that the sensor system be able to discriminate nonspecific binding to improve the specificity of the detection system.

A piezoelectric microcantilever sensor (PEMS) is a new type of sensor that has been demonstrated for repeatable, highly sensitive, real-time, label-free biological detection.^{12,14} For detection, antibody to the target antigen is immobilized on a PEMS surface. Interaction of the target antigen with the antibody on the sensor surface shifts the PEMS resonance frequency, which is monitored to achieve detection. Since a PEMS uses an antibody immobilized on its surface for detection, it would be of use to augment the natural specificity of the antibody–antigen interaction. It has previously been shown that fluid flow can impinge forces on cells adhered to a surface¹⁵ and lead to the removal of cells from the surface.¹⁶ In these studies, unbinding occurred when the flow-induced forces overcame the antibody–antigen interaction forces. If the strengths of nonspecific interactions are smaller than that of specific antibody–antigen interactions, conceivably, flow may be used to discriminate between the two, as weaker interactions will be overcome or prevented from occurring altogether by fluid flow above a certain flow speed.

The purpose of this study is to explore this use of flow as a means to minimize nonspecific binding of BT, BC, and BS spores in BA spore detection using anti-BA spore immunoglobulin G (IgG)-functionalized PEMS. We show that, as with the detection of BA, the nonspecific interactions of BT, BS, and BC with the anti-BA antibody functionalized sensor surface initially increase with an increasing flow speed as the fluid flow helps bring more spores to the sensor surface. However, as the flow rate (velocity) becomes larger than 10 ml min^{-1} (2.7 mm s^{-1}), the nonspecific binding of BT, BS, and BC diminishes, while the binding of BA remains steady, dramatically enhancing the specificity of the detection. The observed reduction of nonspecific binding at higher flow rates is similar to the result of a recent study where flow and micrometre-size labels conjugated to a secondary receptor were used to improve specificity in DNA detection.¹⁷

In addition to improving the specificity of our PEMS sensor by means of controlling the fluid flow rate, the data obtained will also allow for an estimation of the interaction forces involved in the antibody–antigen interactions that take place at the sensor surface. Such force interrogation has been previously investigated by rupture event scanning (REVS) on the QCM.^{18–20} The PEMS system differs from REVS in that, with PEMS detection, it is the binding of the antigen to the antibody that is probed rather than the unbinding of an antigen bound in a previous preparation step, as is the case with REVS. Additionally, the PEMS system uses fluid

flow for its basic detection,¹² while the experiments presented here further probe the utility of this same fluid flow for specificity enhancement and force interrogation.

II. Experimental

II.1 Piezoelectric microcantilever sensor fabrication

A lead zirconate titanate (PZT)/gold-coated glass PEMS was constructed by bonding a 2 mm wide and 150 μm thick glass wafer (Fisher Scientific, Pittsburg, PA, USA) to a 1.5 mm wide and 127 μm thick PZT wafer (T105-H4E-602, Piezo Systems Inc., Cambridge, MA, USA) with insulating epoxy (Loctite Hysol 1C, Rocky Hill, CT, USA). Prior to cantilever fabrication, a 150 nm thick gold layer (with 10 nm thick chromium bonding layer) was coated on both sides of the glass wafer by e-beam evaporation (E-gun: 3 kW, 2×10^{-6} torr).

To achieve the cantilever geometry shown in Fig. 1a, the gold-coated glass wafer was bonded to the PZT wafer such that the long edges of both wafers were parallel and coincident, and on one end the gold-coated glass protruded past the PZT by 1.7 mm to serve as the cantilever tip. After attaching wire leads to the rear end of the PZT, the rear end of this composite strip was encapsulated in a tube with the above mentioned insulating epoxy. The final PZT/gold-coated glass PEMS was 2.2 mm long; the PZT/gold-coated glass portion was 0.5 mm long and the gold-coated glass tip was 1.7 mm long (see Fig. 1a). This tipped cantilever structure allowed the gold-coated glass tip to be dipped into an aqueous medium for *in situ* biological detection, while also allowing for all-electrical actuation and detection by the PZT layer which remained free from submersion.

II.2 Bacteria, spores and antibody preparation

To determine the specificity of the anti-BA IgG functionalized cantilever, additional *Bacillus* species were chosen for study: *B. subtilis* (BS, ATCC 49760), *B. cereus* (BC, ATCC 6464), and *B. thuringiensis* (BT, kindly provided by R. Mutharasan). *Salmonella typhimurium* (ST) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) was used as an additional unrelated control species.

2XSG²¹ medium was used as the standard complex medium for the growth of *BT*, *BC* and *BS* and generation of spores. 2XSG contains (per liter) 16 g of nutrient broth (Difco), 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2 g of KCl, with the following supplements added: 1 mM $\text{Ca}(\text{NO}_3)_2$, 0.1 mM MnSO_4 , 1 mM FeSO_4 , 0.1% glucose. PA²² medium was used to make BA spores. Spore preparations were heat treated at 65 °C for 30 min to kill any remaining vegetative BA or germinating spores, purified through Percoll (GE Healthcare), 17-0891-01), $3600 \times g$ for 25 min in the cold, and extensively washed in distilled water to remove debris. Spores were then inactivated by exposing them to UV light in a bio-safety cabinet at concentration 10^9 spores ml^{-1} in distilled water in an open plastic Petri dish for 120 min. Spores were quantified microscopically with a hemocytometer (INCYTO, Seoul, Korea). UV inactivated spore preparations were tested for viability by spreading 100 μl of spore suspension on a Brain Heart Infusion (BHI) medium plate (Remel, Lenexa, KS, USA), and checking for colonies the next day.

Anti-BA spore was raised in rabbits by Lampire Biological Laboratories, Inc. (Pipersville, PA, USA) against UV-killed PA medium BA Sterne strain 7702 spores. Serum was collected 7 days after a 3rd subcutaneous injection of spores in Freund's Incomplete Adjuvant. IgG was purified from the serum by protein A column chromatography.

II.3 Surface functionalization and detection setup

For anti-BA spore IgG functionalization of the cantilever tip, the gold-coated glass was first cleaned with a diluted (1 : 40 in water) piranha solution (one part 98% sulfuric acid (Fisher, Fair Lawn, NJ, USA); one part 30% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ, USA)) at room temperature for 2 min. This cleaned tip was then dipped in 2 mM 3-mercaptopropionic acid (MPA) (Alfa Aesar, Heysham, Lancashire, UK) for 2 h to form an MPA monolayer on the gold surface.²³ The carboxyl group of the MPA was then activated in a solution of 2 mM *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (Aldrich, St. Louis, MO, USA) and 5 mM *N*-hydroxysuccinimide (Aldrich, St. Louis, MO, USA) in water to allow covalent bonding of primary amines on the anti-BA IgG to the MPA on the gold surface²⁴ of the cantilever tip.¹⁴

For continuous, real-time analyte detection, the tip of this anti-BA IgG functionalized PZT/gold-coated glass PEMS was submerged 1 mm into the center of an open flow channel 80 mm long and 8.0 mm wide, as schematically shown in Fig. 1b. The flow channel was 9 mm deep with a semi-circular bottom of 4.0 mm radius.

Since the present PZT/gold coated glass PEMS was not electrically insulated, the gold-coated glass tip was only partially dipped in the liquid to avoid electrically short circuiting the PZT through the electrolyte detection medium. The humidity was controlled at 90% relative humidity to ensure that the resonance frequency of the partially-dipped PZT-glass PEMS was stable with time. The analyte suspension was circulated using a Masterflex C/L pump (Model 77120-62, Cole-Parmer, Vernon Hills, IL, USA) with an adjustable volumetric flow rate of 0 to 14 ml min⁻¹.

The resonance frequency of the PEMS was obtained by measuring the phase angle *versus* frequency spectra of the sensor using an impedance analyzer (4294A, Agilent), where the phase angle is the angle between the real part and the imaginary part of the electrical impedance of the PEMS. Off resonance, the PZT layer behaves like a capacitor exhibiting a phase angle close to -90 degrees. At a resonance frequency of the PEMS, the mechanical vibration induces a piezoelectric voltage in phase with the input voltage causing the phase angle to deviate from -90 degrees, towards zero. The data acquisition rate was one resonance-spectrum scan per minute. This rate can be increased or decreased depending on the detection application. In the current study, one scan per minute was chosen as to minimize noise while also resolving the PEMS-analyte interaction with sufficient detail. Prior to exposure to any suspension of spores or cells, a baseline resonance frequency of the PEMS was recorded for a period of 30 min in pure phosphate buffered saline (PBS) solution. After each detection, the PEMS was dipped in a glycine/HCl^{24,25} solution of pH = 2.5 for 7 min to release the spores¹² and allow for another round of detection.

As an example, the inset in Fig. 2 displays phase angle *versus* frequency spectra of the PEMS at different stages of a detection experiment. The resonance peak of the sensor shifted to lower frequencies with time. This resonance peak frequency position (resonance frequency, hereafter) of such spectra *versus* time is shown in Fig. 2. As can be seen, the PEMS resonance frequency remained unchanged in PBS at $t < 0$. After the introduction of the spores at $t = 0$, binding of spores to the PEMS surface lowered the resonance frequency. All the detection results described below were obtained using this peak at 313 kHz in PBS. As can be seen, during detection the resonance peak frequency decreased with time while the Q value remained stable around 33 throughout the detection period where Q is the ratio of the resonance frequency to the width of the resonance peak at half peak height.

Light microscopy, with an Olympus BX51 microscope, (Center Valley, PA, USA) and a Scion Corp CCD camera (CFW-1310C, Scion Corp, Frederick, MD, USA), was used to verify the presence of spores or bacteria cells on the surface of cantilever sensors.

II.4 Enzyme-linked immunosorbent assay determination of antibody specificity against BA spores

Experiments were performed to compare the specificity of the cantilever sensor system with that of a traditional enzyme-linked immunosorbent assay (ELISA). NUNC Maxisorp (Nunc, Rochester, NY, USA) ELISA plates were coated with serial 2-fold dilutions of spores in 100 μ l of 100 mM sodium carbonate–bicarbonate buffer (pH = 10.0) and incubated overnight at 4 °C. Plates were washed with PBS + 0.05% Tween-20 (wash buffer) and blocked with wash buffer + 2% bovine serum albumin (BSA) for 2 h at room temperature (RT). 100 μ l primary antibody (anti BA spore IgG diluted 1 : 1000 in wash buffer) was added and the plate was incubated for 1 h at RT. After washing, 100 μ l of secondary antibody (goat HRP-conjugated anti-rabbit IgG, (Jackson ImmunoResearch Labs) diluted 1 : 20 000 in wash buffer) was added and the plates were incubated for 1 h at RT. After washing, 100 μ l of freshly prepared substrate (0.075% v/v hydrogen peroxide, 50 mM Na₂HPO₄, 22 mM citric acid, and 1 OPD tablet (Sigma Chem Co, St. Louis, MO, USA)) was added to each well and the plate was incubated for 20 min at RT in the dark. Reactions were stopped with 100 μ l 5% phosphoric acid, and plates were read at 490 nm in a Spectromax Plus 384 spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA).

III. Results

In Fig. 3, we plot the resonance frequency shift of the PEMS during the detection of BA spores at various flow rates from 1.3 to 14 ml min⁻¹. Also shown in Fig. 3 is the resonance frequency of PEMS *versus* time in PBS at 14 ml min⁻¹. As one can see, the data acquisition rate of 1 scan per minute provides low levels of electrical noise (± 30 Hz) for the resonance peak monitoring in PBS, while still allowing for good resolution of the real-time analyte detection. When exposed to a BA spores suspension with no flow, a resonance frequency shift of -740 Hz was observed at 30 min. The magnitude of the resonance frequency shift was markedly increased in the presence of a flow: the resonance frequency shift at 30 min was increased from -740 Hz to -1150, -1190, -1470, and -1370 Hz at 1.3, 4.6, 10, and 14 ml min⁻¹, respectively. This result indicates that the attachment of BA spores to the sensor surface was enhanced by the presence of flow and that flow rates greater than or equal to 4.6 ml min⁻¹ all enhanced detection of BA over and above the conditions of no flow and 1.3 ml min⁻¹. The enhancement by flow in the detection of BA spores seemed to be maximized at 10 ml min⁻¹, and is understandable as the flow constantly brought spores to the sensor surface which increased the chance of BA spore detection. Unfortunately, current pump speed limitations prevented the study of higher flow rates and the determination of a speed at which the binding of BA to the sensor is reduced or prevented.

Subsequently, non-specific binding to the anti-BA functionalized PEMS at various flow rates was examined in BS, BC, BT and ST suspensions at the same 20,000 spores(cells) ml⁻¹ concentration. As an example, Fig. 4 shows the resonance frequency shift *versus* time of the anti-BA functionalized PEMS in a 20,000 spores ml⁻¹ BT suspension at various flow rates, from 0 to 14 ml min⁻¹. Also shown, is the background run in PBS at 10 ml min⁻¹ which exhibited no discernable resonance frequency shift with a standard deviation of ± 25 Hz. We can see that, initially, the magnitude of the resonance frequency shift was increased by the introduction of flow. The resonance frequency shift at 30 min was enhanced from -120 Hz with no flow to -350 Hz at 1.3 ml min⁻¹. However, as the flow rate was further increased, the magnitude of the resonance frequency shift was reduced. At 30 min, the resonance frequency

shift was changed from -350 Hz at 1.3 ml min^{-1} to -210 , -100 , and 60 Hz at 4.6 , 10 , and 14 ml min^{-1} , respectively. Interestingly, the magnitude of the resonance frequency shift at 14 ml min^{-1} was even smaller than that with no flow.

In Fig. 5, we plot the resonance frequency shift after 30 min of detection *versus* flow rate over the anti-BA spore IgG coated PEMS, of $20\,000\text{ spores ml}^{-1}$ BA, BT, BC, BS, and ST suspensions. This comparison of shift values at 30 min allows for a ‘snapshot’ comparison of the performance of the PEMS under the various experimental conditions. As discussed above, we see that the magnitude of the resonance frequency shift of the anti-BA functionalized PEMS increased with a flow rate in a BA suspension due to flow-enhanced spore attachment. Meanwhile, the anti-BA IgG functionalized PEMS exhibited no obvious resonance frequency shift in an ST suspension at all flow rates. This indicated that there was no nonspecific binding between an unrelated species, such as ST to the anti-BA IgG functionalized PEMS. Unlike ST, the genetically closely related BT, BC, and BS spore suspensions all caused the anti-BA functionalized PEMS to exhibit a resonance frequency shift, indicating weak binding to the sensor. Similar to BT, the magnitude of the resonance frequency shift of the anti-BA functionalized PEMS also exhibited a maximum shift of -460 Hz at 1.3 ml min^{-1} for BS and -390 at 4.6 ml min^{-1} for BC, both of which decreased to very low values at higher flow rates: -40 Hz at 14 ml min^{-1} for BS and -20 Hz at 14 ml min^{-1} for BC. The -40 Hz for BS and -20 Hz shift for BC at 14 ml min^{-1} was very close to or within the noise level ($\pm 30\text{ Hz}$) of the PEMS in pure PBS and is thus, insignificant. Clearly, there was a specificity enhancement by the increased flow, which increased the specific binding of BA spores while suppressing the weak binding of closely related BT, BS, and BC spores.

IV. Discussion

From the data presented in Fig. 2–Fig. 4, we see that continuous, real-time monitoring of BA and its close relatives has been achieved using the PEMS. While detection experiments were run for 30 min in all cases, Fig. 3 clearly shows that positive detection of BA at all flow rates was achieved in just 2–5 min.

To more easily compare the PEMS specificity enhancement achieved by flow, a cross reactivity ratio of BT, BS, or BC is defined as the ratio of the resonance frequency shift at 30 min obtained in the BT, BS, or BC suspension at a given flow rate to that obtained in the BA suspension at the same flow rate. Fig. 6 shows the obtained cross reactivity ratio *versus* flow rate for BT, BS and BC. Note that the cross reactivity of ST was essentially zero and was omitted in Fig. 6. For all the cross reactive species, the cross reactivity ratios were less than 0.4 at flow rates less than 10 ml min^{-1} and decreased to less than 0.1 at 10 ml min^{-1} and to less than 0.05 at 14 ml min^{-1} . The reduction of cross reactivity ratio at 10 and 14 ml min^{-1} was attributed to the increased force impinging on spores near or attached to the sensor surface with increasing flow velocity that eventually overcame the binding strength of the spores. The sequence of cross reactivity among BT, BC, and BS depends on the flow rate as shown in Fig. 6. Note that as mentioned above, the noise level of the PEMS in pure PBS was $\pm 30\text{ Hz}$ at 14 ml min^{-1} . With this noise level one can define a cutoff cross reactivity ratio, which is depicted as the horizontal dashed line in Fig. 6. Spores with cross reactivity ratios less than or close to the cutoff would be deemed undetectable. Clearly BT, BS, and BC at 14 ml min^{-1} were such cases.

Note that the flow rates of 1.3 , 4.6 , 10 , and 14 ml min^{-1} corresponded to average linear flow velocities, u , of 0.35 , 1.2 , 2.7 and 3.8 mm s^{-1} , respectively. With the Reynolds number²⁶ $Re = \rho uw/\eta$ of just 34 at 14 ml min^{-1} , where $\rho \cong 1000\text{ kg m}^{-3}$ is the density of the fluid, $\eta \cong 1\text{ cP}$ is the viscosity of the fluid and w is the flow channel width, the flow was laminar and the flow velocity profile in the width direction was parabolic. That is, u was zero at the channel wall and a maximum at the center of the flow. Thus, unlike other sensor systems where the

sensor surface is actually part of a wall of the flow channel,¹⁶ at which point the fluid velocity diminishes to zero, the present system situates the sensor at the center of the flow where the flow velocity is at a maximum (as shown schematically in Fig. 1b).

Applying the Goldman *et al.* concept²⁷ to a sphere parallel to a plane wall in a uniform flow, the condition of the middle of the flow channel where the sensor is situated, one can obtain an

analytical expression for the impingent force on a bound spore as $F = (1.7)6\pi\eta a \left(\frac{3}{2}u\right)$ where u is the average flow velocity, $\left(\frac{3}{2}u\right)$ the flow velocity at the channel center, and $a = 0.6 \mu\text{m}$ the spore radius. The deduced impingent force on the spores by the flow was about 100 pN with $u = 3.8 \text{ mm s}^{-1}$ at 14 ml min^{-1} . Although it is unclear exactly how many antibody–antigen bonds were involved in the present spore-sensor surface binding, it suffices to say that the deduced force was consistent with the 1–200 pN range of antibody–antigen interactions.^{18, 20, 28} While it is desirable to have more detailed information about the spore-sensor surface binding strength, this is not within the scope of this study, but rather will be the focus of a future publication.

In addition, we performed direct ELISA as a semi-quantitative tool to examine the cross-reactivity or relatedness of the various spore preparations, as related to BA spores. By definition, ELISAs are static assays, with no flow of reagents, aside from the washing steps. Anti-BA spore-specific IgG, prepared from rabbit serum as described above, was used as primary antibody, and various spore preparations were serially diluted 1 : 2 as targets in the assay. The results, shown in Fig. 7, are the absorbance due to the binding of the spores with the anti-BA spore IgG *versus* the dilution of the spores. As expected, the antibody reacted most strongly with BA spores, but it also reacted to various, lesser degrees with spores from BC and BT, the two most closely related bacterial species to BA. BS spores reacted negligibly with the anti-BA spore IgG. This may be explainable by the fact that BS is more distantly related to BA, than is either BC or BT.

By and large, the ELISA results were similar to those of the PEMS studies in that it also showed that some cross reactivity of the anti-BA antibody occurred with BC and BT and that BA spores bound to anti-BA antibody more strongly than BT and BC. However, the detailed order of the cross reactivity was different. For example, the ELISA results suggest that the antibody recognized $\text{BA} > \text{BC} > \text{BT}$, and did not recognize BS, the PEMS studies (with flow) suggest that the antibody recognized $\text{BA} > \text{BC} > \text{BS} > \text{BT}$ at intermediate flow rates (4.6 ml min^{-1}). Furthermore, the order of reactivity varied with flow rate. Thus, the addition of flow may reveal not only qualitative differences between bindings of spores to antibody, but also quantitative differences of the binding strength of the spores to sensor surface. While it is intriguing that the order of cross-reactivity was different in ELISA and in PEMS at different flow rates, it is not within the scope of this study to determine exactly how these differences came about. Such explanations may come from a more quantitative analysis of the binding strength of each spore at different flow rates, which, again, may be the subject of a future publication.

V. Conclusion

In this study, we have examined the specific detection of BA against BT, BS, and BC using anti-BA functionalized PZT/gold-coated glass PEMS in flow with the surface of the PEMS placed parallel to the flow at the center of the flow channel. We showed that the binding of BT, BS, and BC was greatly reduced when the flow rate was 10 and 14 ml min^{-1} , or average flow velocities of 2.7 and 3.8 mm s^{-1} , respectively, while the binding of BA at these flow rates remained more than thrice that at a flow rate of zero. As a result, the cross reactivity ratios BT, BS, and BC against anti-BA coated PEMS were reduced from around 0.4 at low flow rates to

less than 0.05 at 14 ml min⁻¹, which was essentially negligible considering the experimental uncertainty.

Acknowledgements

This document is disseminated under the partial sponsorship of the United States Department of Transportation, Federal Transit Administration, in the interest of information exchange. The United States Government assumes no liability for the contents or use thereof. The United States Government does not endorse products or manufacturers. Trade or manufacturers' names appear herein solely because they are considered essential to the contents of the report. This work was also supported in part by National Institutes of Health (NIH) grant R01 EB00720 (WYS and WHS), and Drexel University College of Medicine.

References

1. Department of Health and Human Services. Bioterrorism Agents/Diseases, CDC. <http://www.bt.cdc.gov/agent/agentlist-category.asp>
2. De BK, Bragg SL, Sanden GN, Wilson KE, Diem LA, Marston CK, Hoffmaster AR, Barnett GA, Weyant RS, Abshire TG, Ezell JW, Popoviv T. *Emerging Infect. Dis* 2002;8:1060–1065. [PubMed: 12396916]
3. Bej AK, Mahbubani MH, Boyce MJ, Atlas RM. *Appl. Environ. Microbiol* 1994;60:368–373. [PubMed: 8117091]
4. Aback BA, O'day SJ, Hoon DSB. *Ann. N. Y. Acad. Sci* 2004;1022:17. [PubMed: 15251934]
5. Oshita F, Sekiyama A, Suzuki R, Ikehara M, Yamada K, Saito H, Noda K, Miyagi Y. *Oncol. Rep* 2003;10:105. [PubMed: 12469153]
6. Sunnotel I, Lowery CJ, Moore JE, Dooley JSG, Xiao L, Millar BC, Rooney PJ, Snelling WS. *Lett. Appl. Microbiol* 2006;43:7–16. [PubMed: 16834714]
7. Xiao L, Singh A, Limor J, Graczyk TK, Gradus S, Lal A. *Appl. Environ. Microbiol* 2001;67:1097–1101. [PubMed: 11229897]
8. Rasko DA, Altherr MR, Han CS, Ravel J. *FEMS Microbiol. Rev* 2005;29:303–329. [PubMed: 15808746]
9. Krivacic RT, Ladanyi A, Curry DN, Hsieh HB, Kuhn P, Bergsrud DE, Kepros JF, Barbera T, Ho MY, Chen LB, Lerner RA, Bruce RH. *Proc. Natl. Acad. Sci. U. S. A* 2004;101:10501–10504. [PubMed: 15249663]
10. Fritz J, Baller MK, Lang HP, Rothuizen H, Vettiger P, Meyer E, Guntherodt H-J, Ch. Gerber, Gimzewski JK. *Science* 2000;288:316–318. [PubMed: 10764640]
11. Capobianco J, Shih WY, Shih W-H. *Rev. Sci. Instrum* 2006;77:125105.
12. McGovern J-P, Shih WY, Shih W-H. *Analyst* 2007;132:777–783. [PubMed: 17646877]
13. Fox A, Stewart GC, Waller LN, Fox KR, Harley WM, Price RL. *J. Microbiol. Methods* 2003;54:143–152. [PubMed: 12782370]
14. Zhu Q, Shih WY, Shih W-H. *Biosens. Bioelectron* 2007;22:3132–3138. [PubMed: 17387007]
15. Gallant ND, Michael KE, Garcia AJ. *Mol. Biol. Cell* 2005;16:4329–4340. [PubMed: 16000373]
16. Hammer DA, Tempelman LA, Apte SM. *Blood Cells* 1993;19:261–277. [PubMed: 8312563]
17. *Internat. Pat. App. Num.*, WO 2005/005951 A2.
18. Dultsev FN, Ostanin VP, Klenerman D. *Langmuir* 2000;16:5036–5040.
19. von der Werff J, Yuan YJ, Hirst ER, Xu WL, Chen H, Bronlund JE. *IEEE Sens. J* 2007;7:762–769.
20. Cooper A. *Meas. Sci. Technol* 2003;14:1888–1893.
21. Leighton TJ, Doi RY. *J. Biol. Chem* 1971;246:3189–3195. [PubMed: 4995746]
22. Thorne CB. *J. Virol* 2007;81:657–662. [PubMed: 17278096]
23. Jung SK, Wilson GS. *Anal. Chem* 1996;68:591–596. [PubMed: 8999737]
24. Fung YS, Wong YY. *Anal. Chem* 2001;73:5302–5309. [PubMed: 11721933]
25. Kandimalla VB, Neeta NS, Karanth NG, Thakur MS, Roshini KR, Rani BEA, Pasha A, Karanth NGK. *Biosens. Bioelectron* 2004;20:903–906. [PubMed: 15522608]
26. Smits, AJ. *A Physical Introduction to Fluid Mechanics*. New York: Wiley; 2000.

27. Goldman J, Cox RC, Brenner H. Chem. Eng. Sci 1967;22:653.
28. Guttenberg Z, Bausch AR, Bruinsma BH, Moroder L, Sackman E. Langmuir 2000;16:8984–8993.

Fig. 1.
(a) Photograph of PEMS and (b) Schematic of PEMS situated in center of flow channel.

Fig. 2. Continuous, real-time PEMS monitoring of analyte solution. BA is injected into PBS at $t = 0$. Example resonance peaks (numbered 1–3) monitored during this detection are shown in inset, with their peak frequencies *versus* time indicated by the corresponding encircled numbers.

Fig. 3. PEMS frequency shift responses of anti-BA functionalized PEMS to BA spores at flow rates of 0 (circles), 1.3 ml min⁻¹ (up triangles), 4.6 ml min⁻¹ (down triangles), 10 ml min⁻¹ (diamonds) and 14 ml min⁻¹ (open squares). A control of PBS with no spores at 14 ml min⁻¹ is shown for reference (closed squares).

Fig. 4. PEMS frequency shift responses of anti-BA functionalized PEMS to BT spores at flow rates of 0 (circles), 1.3 ml min⁻¹ (up triangles), 4.6 ml min⁻¹ (down triangles), 10 ml min⁻¹ (diamonds) and 14 ml min⁻¹ (open squares). A control of PBS with no spores at 10 ml min⁻¹ is shown for reference (closed squares).

Fig. 5. PEMS resonant frequency shift as a function of fluid flow rate for BA (squares), BS (up triangles), BC (down triangles), BT (circles) spores, and ST (diamonds).

Fig. 6. Cross reactivity ratio *versus* flow rate of BT (circles), BS (up triangles), and BC (down triangles) obtained by the anti-BA antibody functionalized PEMS. The cross reactivity ratio is the ratio of the resonance frequency shift after 30 min in cross reactive species relative to that in BA. The dash line indicates the detection limit of the PEMS.

Fig. 7.
Comparative ELISA of anti-BA IgG against BA (squares), BS (up triangles), BC (down triangles), BT (circles) spores, and background (diamonds).