Abstract—Short peptide ligands were synthesized to selectively capture spores using microcantilever arrays. Our initial studies focused on the capture of Bacillus subtilis (a stimulant of Bacillus anthracis) spores in liquids. A peptide-ligand-functionalized microcantilever array was mounted on a flow cell filled with a B. subtilis suspension while cantilever deflection was monitored. Also, fifth mode resonant frequency measurements were performed before and after dipping a peptide-ligand-functionalized microcantilever array in a static B. subtilis solution. Both techniques showed selective binding to functionalized cantilevers when compared to arrays functionalized with a similar control (non-binding) peptide. Conclusive confirmation of selective binding was obtained by subsequent examination of the microcantilever arrays under a dark field microscope. The concentration sensitivity, shelf-life of the binding ligands, and their binding efficiency were studied as well.

Keywords — Nano-bio-sensors, Microcantilevers, peptides, Anthrax

I. INTRODUCTION

The use of highly pathogenic microorganisms (like Bacillus anthracis) as biological weapons places a new emphasis on the rapid detection and quantification of these agents. The standard detection methods require numerous test reagents, several assay steps, expensive chromophoric substrates and are not desirable for real time monitoring.

Cantilevers have emerged as strong candidates to be used as biosensors. Because of their flexibility and sensitivity, microcantilever arrays have been used as transducers of a binding event. Cantilevers can be batch microfabricated by standard low cost silicon technology and by the virtue of the size achievable, are extremely sensitive. They provide label-free, real-time measurements in fluids and/or air in a single step reaction without sample manipulation as required in traditional diagnostic systems like ELISA or PCR. Cantilever arrays provide the possibility of multi-analyte detection, leading towards the goal of lab-on-chip. Cantilevers have been used for protein and antigen-antibody binding, assay for prostate cancer markers and single cell detection [1-8].

We have investigated the advantages that short peptide ligands might offer over more conventional antibody-antigen binding schemes. Although antibody capture on microfabricated chips has been demonstrated, [6, 7] antibody functionalized microchips may not be ideal for use as biosensors for several reasons. First, exposure to harsh environmental conditions for significant times could lead to antibody denaturation and loss of function. Second, well-educated bioterrorists could readily mutate the prominent epitopes recognized by the diagnostic antibodies. Most importantly, detection of Bacillus strains via current antibody-based sensors suffers from limitations in accuracy and sensitivity resulting in false positive and false negative results. [8]

For these reasons, we have synthesized short peptide ligands that have been shown to selectively target B. subtilis spores. [9]

II. CANTILEVER SENSING

A. Frequency measurements

For a cantilever, with rectangular cross section having length l, width w and thickness t, resonant frequency is given

$$f_l = \frac{1}{2\pi} \sqrt{\frac{Ewt^3}{12l^3m_c}}$$  

where $E(=1.35 \times 10^{11} \text{N/m}^2$ for silicon) is Young’s modulus for the cantilever and $m_c$ is cantilever mass. Cantilevers vibrating at higher modes have shown to offer an order of magnitude improvement in sensitivity and resolution for target mass detection in comparison to the fundamental bending mode. In the present paper we monitor added mass at higher bending modes of the cantilever $(5^{th}$ Mode) rather than at fundamental bending mode. The $5^{th}$ Mode frequency can be written as

$$f_5 = 56.78 \ f_l$$

Change (magnitude) in frequency due to added mass (in this case spores) can be written as:

$$\Delta f = \frac{1}{4\pi} \sqrt{\frac{Ewt^3m_{load}}{12l^3\sqrt{m_c^3}}}$$

where $m_{load} = Nm_{spore}$, here $N$ is the number of spores bound to the cantilever and $m_{spore}$ is the mass of a spore and $m_c$ is the mass of the cantilever prior to spore attachment. In order to simplify the system, equation (2) further assumed that...

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the flexural rigidity remained constant following the binding of the spores.

B. Stress measurements

In our experiments, spore adsorption is selectively confined only to one side of the cantilever. This selective binding of spores to one surface of the cantilever generates differential surface, leading to bending of the cantilever. To observe bending due to surface stress cantilevers with small spring constant (< 0.1 N/m) are preferred. The radius of curvature and the adsorption-induced surface stress on the microcantilever can be related using Stoney’s Formula [12],

\[ z = \frac{2\Gamma^2(1-V)}{E t^2} \sigma \]

where \( z \) is the cantilever deflection at its free end, \( V \) (\( \approx 0.25 \) for silicon) is the Poisson’s ratio, \( E \) (\( \approx 1.35x10^{11} \) N/m\(^2 \) for silicon) is Young’s modulus for the cantilever, \( t \) is the length of the cantilever, \( I \) is its thickness and \( \sigma \) is the differential surface stress on the microcantilever. An increase in the tensile surface stress causes the microcantilever to bend toward the absorptive side; an increase in the compressive surface stress causes the microcantilever to bend away from the adsorptive side.

III. EXPERIMENTAL PROCEDURE

Commercially available cantilever array chips with eight rectangular silicon cantilevers in parallel were used as sensors. Each cantilever had typical dimensions of 500 \( \mu \)m length, 100 \( \mu \)m width, and 1 \( \mu \)m thickness. The nominal spring constant \( k \) for these cantilevers is \( \sim 0.02 \) Nm\(^{-1} \) and resonance frequency \( f_0 \) = 4.94 KHz and a nominal fifth bending mode frequency \( f_5 \) = 280.45 KHz. Spring constants for an individual cantilever on the array were calibrated using the thermal noise method. [9, 10]

Experiments were performed using a Scentsir system. Eight super-luminous diodes (SLD’s) were used to focus onto the tip of the cantilever (one SLD for each cantilever). The diode emitted infrared light at 850 nm (< 0.12 mW). The SLD beam reflected off the cantilever was directed into a position-sensitive diode (PSD) that can detect the vertical beam position.

The gold coated cantilevers were thoroughly cleaned immediately before functionalizing cantilevers with peptides. Four cantilevers from the array were coated with the binding peptide sequence NHFLPKV-GGGC (1mg/ml in phosphate buffer solution) using a glass capillary (inner diameter 180\( \mu \)m and outer diameter 240 \( \mu \)m, 3 inch long) for 45 min each. The phosphate buffer solution (PBS) had a pH=7.2.

Once all four cantilevers were coated with the binding peptide the array was rinsed with PBS to remove unbound peptide and then with DI water to remove any residual salt crystals. Then, the whole cantilever array was submerged in a solution of the control (non-binding) peptide LFNHKPHP-GGGC (1mg/ml in PBS) for \( \sim 1h \) to allow derivatization of the remaining four cantilevers with the control peptide.

The functionalized cantilever array chip was then mounted into the fluid cell to perform either stress/deflection experiments or frequency shift measurements. The fluid cell and tubing were rinsed with deionized water for \( \sim 10 \) min directly before use. The cantilever array was also rinsed a number of times with PBS followed by DI water after every peptide functionalization and spore binding experiment to minimize non-specific adhesion.

IV. RESULTS

The result of an experiment to measure the static bending of the both the binding and control peptide coated cantilevers upon exposure to a static solution containing \( \sim 7x10^7 \) spores/ml is shown in Fig. 1. A stable baseline was obtained by submerging cantilevers in buffer solution (PBS). When \( B. subtilis \) spore solution is injected in the fluid cell containing the cantilever array, binding peptide coated cantilevers show a deflection of 40 nm (Fig 1a) where as control peptide coated cantilevers show no deflection (Fig 1b). The data from all four binding peptide coated cantilevers and control peptide coated cantilevers is similar to the data shown in Fig. 1(a) and Fig. 1(b) respectively.

While the data in Fig. 1 were collected when the cantilever array was submerged in a static spore solution, similar results were also observed when the cantilever was subjected to a flowing spore solution.

Complementary experiments were also performed by measuring the resonance frequency shift of the fifth bending mode. The cantilevers coated with the binding peptide showed an average frequency decrease of 695\( \pm 95 \) Hz, whereas the cantilevers coated with control peptide showed a decrease of 130\( \pm 50 \) Hz (from non specific binding). Dark field images of the cantilever array were made following the frequency measurements, allowing an estimate of the number of spores on each cantilever. The average number of spores on the binding peptide coated cantilevers was 1025\( \pm 75 \), while on the control peptide coated cantilevers the number was only 150\( \pm 50 \). From these results, it is possible to estimate the mass of each \( B. subtilis \) spore and this mass was found to be 7.4 \( \pm 2.1 \times 10^{-13} \) grams, an estimate obtained assuming that the added mass is concentrated at the free end of the cantilever, rather than being distributed along the cantilever length. Nevertheless, this result agrees with the reported \( B. subtilis \) spore mass previously reported in the literature. [13] A more complete discussion of the results reported above can be found elsewhere. [14]

V. CONCLUSIONS

We have investigated the use of cantilever arrays for real time detection of \( B. subtilis \) spores in a liquid medium. These experiments demonstrate that it is possible to move beyond an antibody-antigen capture paradigm to detect spores.
in liquids. Using peptide ligands, we have generated a stable and selective substrate for capturing intact *Bacillus subtilis* spores on cantilever arrays in a reliable and repeatable fashion. Real-time detection was achieved by monitoring stress changes in the cantilever due to spore binding and estimates for the induced stress per binding event were obtained. We also measured frequency shifts upon spore binding by monitoring the fifth bending mode of oscillation. In this way we were able to estimate the mass of each spore binding to the cantilever. Conclusive confirmation of selective binding was obtained by subsequent examination of the microcantilever arrays under a dark field microscope. The concentration sensitivity, shelf-life of the binding ligands, and their binding efficiency were studied as well. [14]

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