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# Detection of *Bacillus subtilis* Spores Using Peptide-Functionalized Cantilever Arrays

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***Abstract:*** We move beyond antibody−antigen binding systems and demonstrate that short peptide ligands can be used to efficiently capture *Bacillus subtilis* (a simulant of *Bacillus anthracis*) spores in liquids. On an eight-cantilever array chip, four cantilevers were coated with binding peptide (NHFLPKV-GGGC) and the other four were coated with control peptide (LFNKHVP-GGGC) for reagentless detection of whole *B. subtilis* spores in liquids. The peptide-ligand-functionalized microcantilever chip was mounted onto a fluid cell filled with a *B. subtilis* spore suspension for ∼40 min; a 40 nm net differential deflection was observed. Fifth-mode resonant frequency measurements were also performed before and after dipping microcantilever arrays into a static *B. subtilis* solution showing a substantial decrease in frequency for binding-peptide-coated microcantilevers as compared to that for control peptide cantilevers. Further confirmation was obtained by subsequent examination of the microcantilever arrays under a dark-field microscope. Applications of this technology will serve as a platform for the detection of pathogenic organisms including biowarfare agents.

## Introduction

The use of highly pathogenic microorganisms as biological weapons places a new emphasis on the rapid detection and quantification of these agents.[1−6] Recent events in which the spore forming bacterium *Bacillus anthracis* (*B. anthracis*), the causative agent of anthrax, was intentionally released in the United States underscore the need for real-time sensors capable of the reliable detection of biological threats. Compounding the problem with *B. anthracis* infection is a mortality rate approaching 95% if medical treatment is not sought within 24−48 h post infection.[4]

Current *Bacillus* spore detection methods rely on the use of the polymerase chain reaction (PCR) to identify specific gene sequences unique to pathogenic strains,[5−11] immunoassays to detect specific spore surface antigens,[8,12,13] and standard microbial assays including colony morphology, penicillin susceptibility, and absence of hemolysis.[14] These techniques have significant limitations because they require numerous test reagents, several assay steps, expensive chromophoric substrates, well-trained personnel, and a significant time commitment to obtain reliable results. Furthermore, these methods are undesirable for use in real-time monitoring given the inherent complexity associated with multistep detection systems. Thus, any demonstration of a direct capture, identification, and quantitative assessment of pathogenic organisms could enable a rapid first-alert detection scheme.

Although antibody capture on microfabricated chips has been demonstrated,[15,16] antibody-functionalized microchips may not be ideal for use as biosensors for several reasons. First, exposure to harsh environmental conditions (e.g., remote air monitoring) for significant lengths of time could lead to antibody denaturation and loss of function. Second, a well-educated bioterrorist 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 both false positive and false negative results.[12]

A promising alternative approach for biosensor construction would be the use of small molecular weight ligands that are relatively inexpensive to synthesize and easy to chemically functionalize. Recently, short peptide sequences, which preferentially bind to *Bacillus* spores for both *B. anthracis* and *B. subtilis* (a *B. anthracis* simulant), have been identified by phage display peptide library screening[7,17−19] and demonstrate exceptional selectivity in discriminating closely related *Bacilli* species.[17−19] Moreover, these peptide ligands can easily be

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adapted for covalent attachment to gold surfaces typically used in microchip fabrication.

Assuming highly specific binding can be achieved with peptide ligands, a rapid and sensitive transduction scheme is still required before a viable sensing paradigm becomes possible. In this regard, microcantilevers have been developed as sensitive transducers in many micro-/nanomechanical sensor systems.[20−25] Cantilevers can be microfabricated by standard low-cost silicon technology and, by 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 the sample manipulation required in traditional diagnostic systems such as ELISA (enzyme-linked immunosorbent assay), oligonucleotide (DNA or RNA) hybridization capture, PCR (polymerase chain reaction), and fluorescence-based techniques. Cantilevers can also be batch fabricated, leading to a decrease in production costs and allowing the possibility of integrating multiple functional devices onto the same platform, i.e., moving toward the proverbial goal of a "lab-on-a-chip". They can also be fabricated into large arrays, permitting autonomous detection of multiple analytes in a single step and allowing a built-in passive (unfunctionalized) cantilever that can be used to provide background readouts. This capability is particularly effective in reducing false reporting of a targeted threat. With further integration of detection electronics, cantilever arrays promise to offer a sensitive yet reliable platform for use in clinical and public safety venues.

Two modes of detection are possible with cantilevers: (i) stress/deflection measurements (when differential stress is generated because of molecule adsorption or absorption onto one surface) and (ii) resonant frequency measurements (frequency shift due to uploaded mass). Recent experiments have used AFM cantilevers as versatile sensors to distinguish between DNA oligonucleotides,[20] to measure pH changes,[21] to measure surface stress associated with protein and antigen−antibody binding in the liquid phase,[24−27] to identify analyte vapors in gases,[22] and to assay for prostate cancer markers.[23] Although the detection of cells and microorganisms such as *E. coli*[28,29] and *Listeria*[30] has been demonstrated using mass detection methods employing changes in cantilever resonant frequencies, the methodology employed cannot be considered a real-time detection. Furthermore, the capture of larger entities such as cells on antibodies/peptides attached to cantilevers has not been reported using real-time stress detection.[31]

Herein, we report the nanomechanical biodetection of whole *B. subtilis* spores (a nonpathogenic *B. anthracis* simulant) with peptide-functionalized silicon cantilever arrays in two modes: stress/deflection measurements as well as resonant frequency measurements.

## Materials and Methods

**Cantilever Characterization.** Commercially available cantilever array chips (available from Veeco Metrology, Santa Barbara, CA) 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 spring constant $k$ for these cantilevers is 0.0207 $Nm^{-1}$; the resonance frequency $f_0 = 4.94$ kHz, and the fifth bending mode frequency $f_5 = 280.45$ kHz.

Experiments were performed using a Digital Instruments Scentris system (available from Veeco, Santa Barbara, CA; see Supporting Information Figure 1). Eight superluminous diodes (SLDs) 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.

**Reagents and Bacterial Strains.** All Fmoc-protected amino acids, starting resins, and coupling reagents were obtained from Novabiochem (La Jolla, CA). *R*-Phycoerythin, a pyridyl disulfide derivative, was obtained from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). *Bacillus subtilis* (*trpC2*) 1A700 (originally designated as ATCC168) was provided by Arthur Aaronson (Purdue University, West Lafayette, IN), and *Bacillus licheniformis* 5A36 (originally ATCC 14580) was purchased from American Type Culture Collection, (Manassas, VA).

**Preparation of Peptide Capture Ligands.** *Bacillus subtilis* binding peptide, Asn-His-Phe-Leu-Pro-Lys-Val (NHFLPKVGGGC), and the randomized control peptide, Leu-Phe-Asn-Lys-His-Val-Pro (LFNKHVPGGGC),[17] were synthesized by standard solid-phase peptide synthesis and characterized by high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry.

**Preparation and Characterization of *Bacillus* Spores.** *Bacillus* spores were generated, purified, and quantified as previously described.[17] Determination of peptide spore binding by fluorescent-activated cell sorting (FACS) was performed according to a previously published methodology.[17]

**Cantilever Fuctionalization.** To remove contaminants from the surfaces of the cantilevers, the silicon cantilevers were rinsed for 10−15 min sequentially with acetone, ethanol, and deionized water (DI water). For further cleaning of organic contaminants from silicon cantilevers, the whole cantilever array was placed in an oxygen plasma cleaner for 10−12 min (Harrick Scientific, model: PDC − 32G, 100 W RF). Medium-power RF settings (700 V DC, 15 mA DC, 10.5 W) were used to generate the plasma when the chamber was filled with oxygen gas. All cantilevers were then coated with 20−22 nm of gold on one side (either top or bottom) by thermal vacuum evaporation (NRC 3114 Thermal Evaporator). To ensure the adhesion of gold onto the silicon cantilevers, a thin 4−6 nm chromium layer was vacuum evaporated prior to gold deposition. Evaporation was done at a vacuum pressure of $2 \times 10^{-7}$ to $5 \times 10^{-7}$ Torr, and the deposition rate was 0.5−0.6 Å/sec. The distance between the source and sample was ∼10 in. The gold-coated cantilevers were then cleaned in the above-mentioned sequence immediately before functionalizing cantilevers with peptides. Four cantilevers from the array were coated with the binding peptide sequence NHFLPKV-GGGC (1 mg/mL in phosphate buffer solution) using a glass capillary (inner diameter of 180 $\mu$m and outer

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diameter 240 of $\mu$m, 3 in. long; available from Veeco Metrology, Santa Barbara, CA) for 45 min each. The phosphate buffer solution (PBS) had a pH of 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 submersed in a solution of the control peptide LFNKHVP-GGGC (1 mg/mL in PBS) for ~1 h to allow derivatization of the remaining four cantilevers with the nonbinding (control) peptide. The functionalized cantilever array chip was then mounted onto the fluid cell to perform stress/deflection experiments or frequency measurement. The fluid cell and tubing were rinsed with deionized water for ~10 min directly before use. The spring constant for an individual cantilever on the array was calibrated using the thermal noise method.[32,33] The cantilever array was rinsed off a couple of times with PBS followed by DI water after every peptide functionalization and spore binding to minimize nonspecifice adhesion.

## Results and Discussion

To explore the specificity of peptide ligands to selectively capture *B. subtilis* spores on a solid surface, a number of preliminary experiments were first performed using silicon chips (~1 × 1 cm) cut from a commercially available silicon wafer. This strategy proved to be effective because the binding chemistry and substrate cleaning protocols could be rapidly established *before* handling the delicate cantilever arrays. After cleaning, a patterned gold−silicon (Au−Si) substrate was prepared by thermal evaporation through TEM mesh grids. These TEM grids are comprised of a mesh of 100 squares, with each square size being 200 × 200 $\mu$m, with 30 $\mu$m spacing between squares. First, a 3−5 nm adhesion layer of chromium was evaporated onto the silicon, followed by evaporation of a thin layer of gold, ~20−30 nm, as already described in the cantilever fictionalization section.

After evaporation, the gold-coated chips were suspended halfway in the binding peptide solution (1 mg/mL in PBS for 1 h). The peptide sequence Asn-His-Phe-Leu-Pro-Lys-Val (NHFLPKV) is used as the binding peptide for *Bacillus subtilis*, and a randomized sequence of the same composition, Leu-Phe-Asn-Lys-His-Val-Pro (LFNKHVP), is used as the control peptide[17] (for preparation, see Materials and Methods). Both peptides are tethered to the desired gold-derivatized surface using the spacer Gly-Gly-Gly-Cys (GGGC) attached to the COOH terminal amino acid. Attachment of the peptide to the patterned Au−Si chip is facilitated by a cysteine residue placed at the COOH terminal end of the peptide. After functionalization, the whole chip is then dipped into control nonbinding peptide (1 mg/mL in PBS, 1 h). We find that the control peptide does not displace the binding peptide already bound to Au on the bottom half of the chip, giving a Au−Si chip that is coated on the bottom half with binding peptide and on the top half with the nonbinding control. After completing these steps, the chip was then submersed in a solution of *B. subtilis* spores (~5 × $10^7$ spores/mL in PBS) for 1−1.5 h. Any selective binding of spores to the Au−Si chip was then quantified using a dark-field microscope (see Figure 1). The number of spores attached to the chip was counted using commercially available image processing software. The average number of spores that selectively adhered to the binding peptide on the Au−Si chip was 210 ± 20, as measured over an area of 50 × 50 $\mu$m (averaged over ten different areas chosen at random). For the control nonbinding peptide on the upper half of the Si chip, only 30 ± 10 spores were counted over an equivalent area. The *B. subtilis* spores showed very little nonspecific binding to the bare silicon, yielding an average retention of only ~1 or 2 spores per 50 square microns.

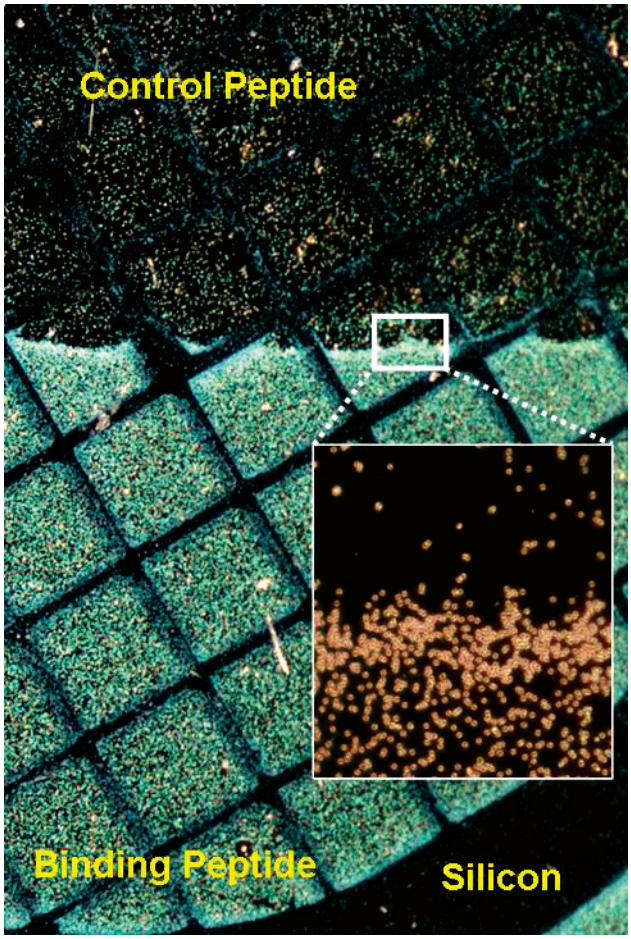


***Figure 1.*** Dark-field image of *B. subtilis* spores. A silicon chip was patterned with gold evaporated onto an array of squares with an area of 250 × 250 $\mu$m. The bottom half was then derivatized with a binding peptide, and the top half was derivatized with a nonbinding or control peptide (of the same amino acid composition but randomized sequence), as described in the text. The chip was then dipped in *B. subtilis* spore solution. The inset shows the magnified dark-field image. Each bright spot represents a *B. subtilis* spore (~1.2 $\mu$m in diameter, as observed in the microscope).

Control experiments were performed using ~5 × $10^7$ *B. licheniformis* spores/mL (another spore from the *Bacillus* genus)[17] to check the specificity of *B. subtilis* for the peptide. Minor nonspecific binding of the *B. licheniformis* spores to the peptide Au−Si chips was observed (20 ± 5 spores per 50 × 50 $\mu$m area), but no preference for binding peptide over control peptide was detected (data not shown).

After optimizing the chip fabrication procedure demonstrated in Figure 1, the functionalization chemistry was transferred to the microcantilevers. A cantilever array chip with eight rectangular silicon cantilevers (500 $\mu$m long, 100 $\mu$m wide, and 1 $\mu$m thick) was used for these studies. A 4−6 nm chromium layer followed by 20−22 nm of gold was thermally evaporated onto the top side of each cantilever. Four of these cantilevers were then coated with the binding peptide, and the other four were coated with control peptide (each 1 mg/mL, see Experi-

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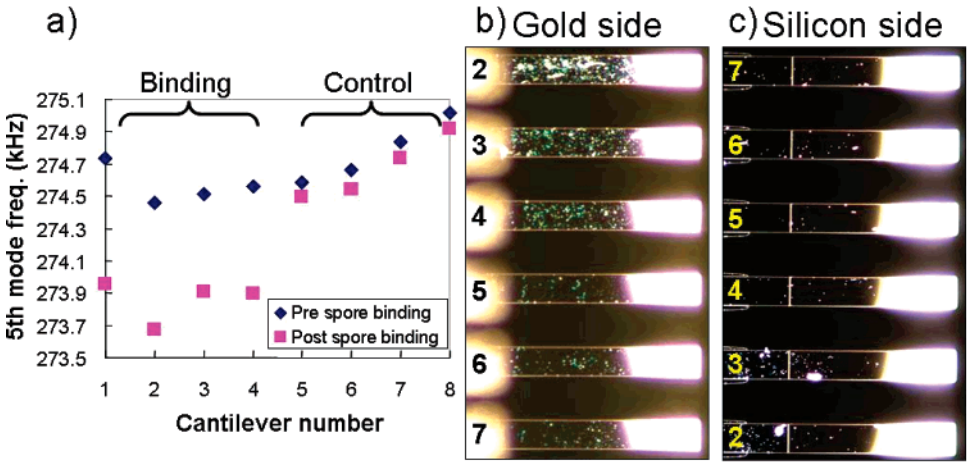


***Figure 2.*** (a) Frequency shifts for all eight cantilevers present in the cantilever array. The cantilevers coated with the binding peptide showed an average frequency decrease of 695 ± 95 Hz, whereas the cantilevers coated with control peptide showed a decrease of 130 ± 50 Hz (from nonspecific binding). (b) Dark-field images of the gold side of the cantilever array showing six cantilevers of the eight cantilevers, three of which are coated with binding peptide and the other three with control peptide. The average number of spores on the binding-peptide-coated cantilevers was 1025 ± 75, and on the control-peptide-coated cantilevers, the number was only 150 ± 50. (c) Almost no spores are bound to the bottom silicon surface. (Note that the cantilevers in the image are out of focus at the free end because they are bent due to postprocessing (involving bimetallic effects) on gold-coated cantilevers.)

mental Protocol). This functionalized cantilever array chip was then dipped into a *B. subtilis* spore suspension in PBS (~5 × $10^7$ spores/mL) for 1−1.5 h. After exposure, the fifth bending mode frequencies (see Supporting Information eq 6) were measured (in air) for each cantilever using an optical beam deflection system. Cantilevers coated with binding peptide showed a subsequent decrease of 695 ± 95 Hz, whereas the cantilevers coated with control peptide showed a decrease of 130 ± 50 Hz. Dark-field microscope images revealed the average number of spores on the cantilevers coated with binding peptide to be 1025 ± 75, whereas the cantilevers coated with the control peptide showed only 150 ± 50 when counted (see Figure 2).

From these results, it is possible to estimate the mass of each *B. subtilis* spore using Supporting Information eq 11, and this mass was found to be 7.4 ± 2.1 × $10^{-13}$ g. Equation 11 is an approximation because it assumes 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.[34]

For real-time detection of spores, we chose to monitor the change in surface stress of the cantilevers as spore binding occurs. The functionalized cantilever array chip (four cantilevers coated with binding peptide and the other four coated with control peptide) was mounted onto a fluid flow cell and equilibrated in PBS solution until a stable baseline was obtained. *B. subtilis* spore suspension (7 × $10^7$ spores/mL in PBS) was next injected into the fluid cell, and the cantilever deflection was monitored in real-time using LabView software to record the deflection signal from the photodetector. All experiments were carried out at room temperature inside an enclosed isolation box to facilitate thermal stability; however, no active temperature control was required to implement the experiments. Although there is some thermal drift (~1−3 nm bimetallic effect due to gold on silicon), this did not affect our results, as all cantilevers were coated with gold of the same thickness.

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Two different sets of experiments were performed:

(1) Static mode. The flow cell was filled with a suspension of *B. subtilis* spores (for ~35−45 min), and cantilever deflection was monitored in situ. Because there was no flow through the fluid chamber, the binding proceeded in a static environment. Figure 3 shows the deflection of a typical cantilever in the array as a function of time derivatized with either a binding peptide or a nonbinding (control) peptide. All four cantilevers coated with binding peptide showed a differential deflection of ~40 nm with respect to the four cantilevers coated with the control (nonbinding) peptide. The bending was due to stress caused by preferential binding to one surface and can be estimated by Supporting Information eq 2. After completing the experiment, the number of spores observed on each binding-peptide-coated cantilever was 462 ± 30 and on each control-peptide-coated cantilever was 60 ± 11. No spores were observed on the underivatized silicon surface of the cantilevers, in agreement with the results presented in Figure 1. From the known cantilever dimensions and the measured deflection, it was possible to estimate the total surface stress that each cantilever experiences. Calculations using Supporting Information eq 2 give a stress value of ~9.6 × $10^{-3}$ N/m. From the number of spores counted on each cantilever, it is possible to estimate the average increment in surface stress produced by an individual binding event of a *B. subtilis* spore. This quantity, which was important for the further optimization of cantilever arrays, was found to be ~0.02 × $10^{-3}$ N/m. Because of the controls exercised during implementation of the experiments, it is possible to also estimate the binding efficiency of the spores to the microcantilevers in our setup. Because the number of spores in the 65 $\mu$l flow cell was 4.5 × $10^6$ and because ~462 × 4 = 1850 spores were detected on the cantilever array, one can estimate that approximately 1 spore for every 2400 spores in the suspension was captured and detected on the cantilever surface. Although the approach to surface area saturation could have limited this capture efficiency, a better presentation of the peptide might permit improvement of this parameter.

(2) Flow mode. In this experiment, the suspension of *B. subtilis* spores was flowed through the cantilever chamber at a

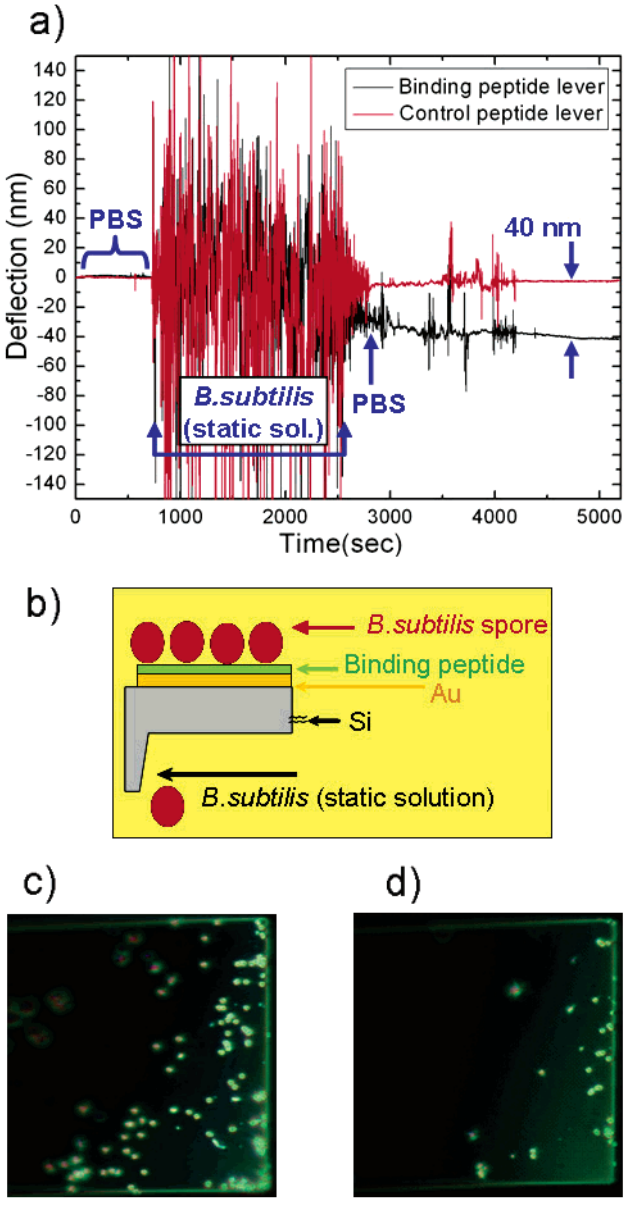


***Figure 3.*** (a) Cantilever deflection vs time for *B. subtilis* spore solution ($7 \times 10^7$ spores/mL). The graph demonstrates a differential deflection of 40 nm between a binding-peptide-coated cantilever and a control-peptide-coated cantilever, when the chip was submersed into a static solution of *B. subtilis* spores for ~40 min. (b) Block diagram of functionalized cantilever. (c) Dark-field image (with focus on the cantilever's free end) of the binding-peptide-coated cantilever. (d) Dark-field images (with a focus on the cantilever's free end) of the control-peptide-coated cantilever.

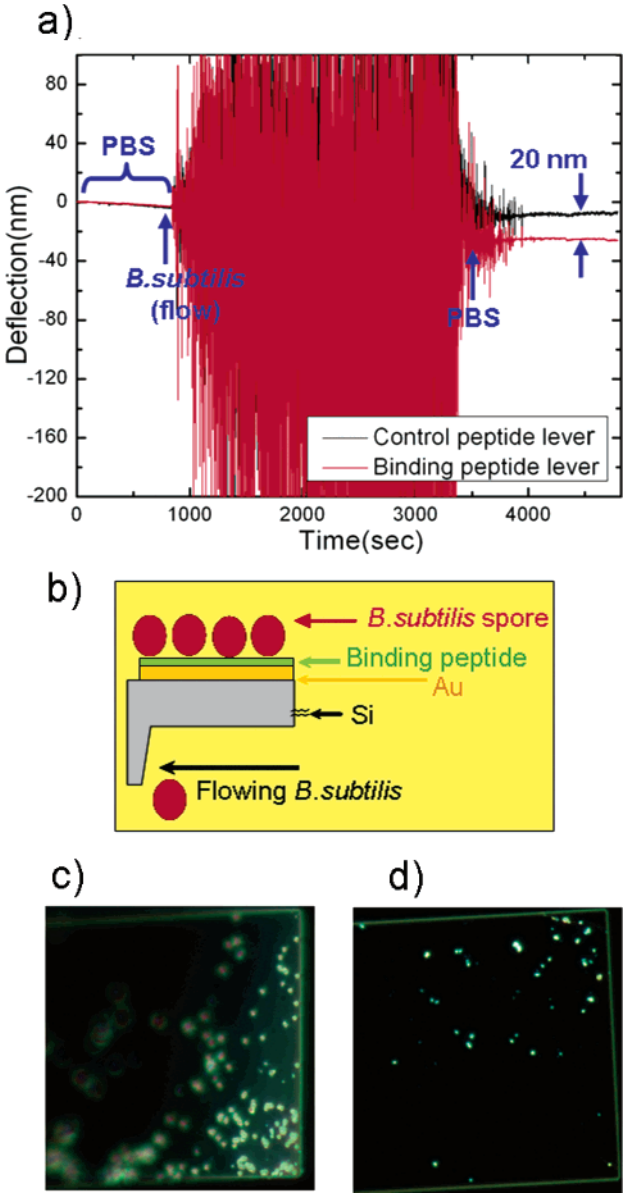


***Figure 4.*** (a) Cantilever deflection vs time for *B. subtilis* spore solution ($7 \times 10^7$ spores/mL) is made to flow through the flow cell at a rate of 0.5 mL/h for 30 min. The graph shows a differential deflection of 20 nm between a binding-peptide-coated cantilever and a control-peptide-coated cantilever. (b) Block diagram of functionalized cantilever. (c) Dark-field images (with a focus on the cantilever's free end) of the binding-peptide-coated cantilever. (d) Dark-field images (with a focus on the cantilever's free end) of the control-peptide-coated cantilever.

speed of 0.5 mL/h for about 40 min. A differential deflection of ~20 nm was observed as shown in Figure 4. The number of spores was again counted using dark-field microscopy, and the number observed on the binding-peptide-coated cantilevers was $282 \pm 20$; on control-peptide-coated cantilevers, only $26 \pm 9$ were observed. Because a total volume of 0.3 mL of suspension containing a total of $2.1 \times 10^7$ spores passed through the flow cell and because $\sim 282 \times 4 = 1130$ spores were captured, a capture efficiency of 1 spore in every $1.8 \times 10^4$ can be estimated. Thus, detection efficiency in the flow mode is ~7 times worse than that in the static mode.

The noise observed in Figures 3 and 4 could be interpreted as cantilever deflection/vibration. However, the spore size and wavelength of the SLD are comparable, so light scattering by the spores in suspension could produce an alternative explanation for the noise.

The difference in capture efficiency between flow and static mode experiments allows us to speculate that attachment of a spore to a peptide-coated substrate takes place in at least two stages. The first phase may occur as the spore approaches the substrate and may be controlled by nonspecific physiochemical forces. The second phase, the attachment of the spore to the peptide, is due to specific interactions based on molecular recognition and minimization of surface energy. By adjusting the rate of flow of the spores past the cantilevers, it is possible to control the first phase. The implication is that by further controlling the flow speeds, an optimal value for flow speed might emerge.

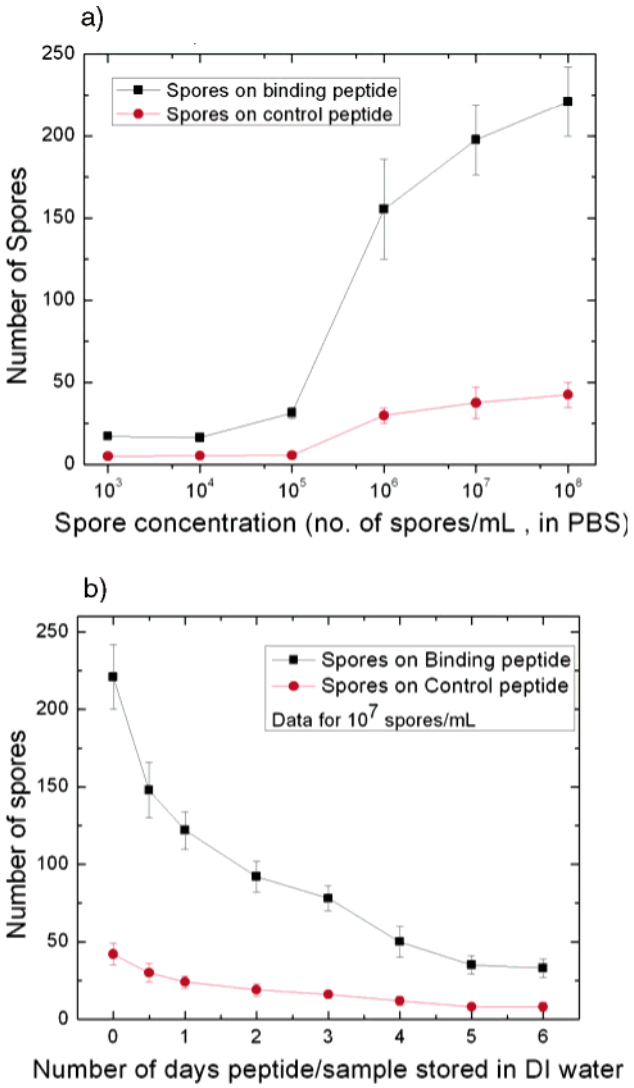


***Figure 5.*** (a) Concentration test: Number of spores bound to peptides as a function of *B. subtilis* spore concentration, when the chip is made to rest in a steady-state spore solution. Every data point is an average over seven different experiments done. (b) Peptide stability test: Peptide-coated Au/Si chips were stored in DI water for a desired time, after which each sample was dipped in a steady solution of *B. subtilis* spores ($10^7$ spores/mL) for an hour. Spores were then counted with a dark-field microscope. Each data point is an average over seven different samples. The ratio of spores bound to the binding peptide and the control peptide immediately (with no storage of peptide) is ~5.5 and after 6 days of storage in DI water is ~4.2.

These initial studies described above were followed up by a number of additional studies to better characterize the reliability of the spore-sensing strategy described above. As an example, Figure 5a shows the steady-state cantilever deflections measured as a function of *B. subtilis* spore concentration. The data indicate that spore concentrations of $\sim 1 \times 10^5$ spores/mL can be sensed with the cantilevers currently available. With further optimization of cantilever geometry (see Supporting Information eq 2), the ability to detect even lower concentrations of spores will be possible. One possible approach is to use high Q cantilevers that are thin, narrow, and long.[35]

Peptide stability studies were performed for both binding and control peptides to learn more about the durability of the peptide functionalization in liquids. For these studies, gold-coated silicon chips were derivatized on one half with binding peptide and on the other half with control peptide, in a manner similar to that shown in Figure 1. Individual chips were then stored in deionized water (DI) for different times. Figure 5b plots the number of spores bound to the peptide-coated surfaces as a function of time of storage in DI water. It was observed that even after the peptides on gold chips were stored in DI water for 6 days, the ratio of spores on the binding peptide to the control nonbinding peptide was ~4.2. This result suggests that these peptides are more stable than antibodies or proteins which have a more complex structure and are therefore more prone to denaturation.

A further study was conducted to investigate the stability of spore binding. Spores were captured on the peptide-functionalized sensors and stored in deionized (DI) water for 7 days. Statistically, we did not observe any significant change in the number of spores bound to the substrates after one week of storage (data not shown). This result reinforces our conclusion that small peptide binding provides a stable platform for selective capture of dangerous pathogens. In addition, this result indicates the possibility of achieving archival capture amenable to further analysis by other techniques at a later time.

## Conclusions

We have investigated the use of cantilever arrays for real-time detection of *B. subtilis* spores in 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* 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. Estimates for the induced stress per binding event were obtained. Using the same cantilevers, we also demonstrated a higher sensitivity to resonant frequency shifts by monitoring higher modes of vibration. In this study, we have reported frequency shifts in the fifth mode of vibration that change in response to mass uptake. The added spore mass can be estimated from these frequency shifts, and the results are found to be in good agreement with spore count performed using a dark-field microscope. The stability of the peptide ligands was also investigated, as well as the concentration dependence of spore binding. Taken together, these results suggest that real-time detection of multiple pathogenic organisms can be realized using peptide-funtionalized microcantilever arrays.

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**Supporting Information Available:** General experimental details, functionalization details, and cantilever physics equations included. This material is available free of charge via the Internet at http://pubs.acs.org.

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