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Label-Free Optical Detection of Anthrax-Causing Spores

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Due to the emergence of pathogenic bacteria as bioterrorism agents and the increase in bacterial infections in general, continuous monitoring of the environment for infectious agents is important and requires the integration of rapid, label-free, and inexpensive methodologies into compact devices that are highly sensitive and specific.[1] *Bacillus anthracis* is a spore-forming bacteria causing the disease anthrax in humans and a potential bioterrorism agent requiring medical attention within a few hours of initial inhalation.[2] Therefore, the rapid detection of *B. anthracis* spores in the environment prior to infection is extremely important for human health and safety. In this direction, we developed a rapid and label-free biosensor integrated with robust peptide ligands for detection of as few as 34 *B. anthracis* spores. This biosensor, in conjunction with an aerosol capture unit, can test anthrax spores circulating in air.[3]

Rapid microbial screening methods currently use a series of assays that often respond to multiple organisms. However, standard laboratory testing, though more precise, is often time-consuming.[4] High-throughput and accurate identification of pathogens is a vital task to facilitate timely and appropriate actions in the event of an outbreak.[5] Some of the presently available detection methods for *B. anthracis* are based on matrix-assisted laser desorption−ionization (MALDI) mass spectrometry,[6] flow-through polymerase chain reaction (PCR),[7] multiplexed immunoassay flow cytometry,[8] enzyme-linked immunosorbent assay (ELISA),[9] and surface-enhanced Raman spectroscopy (SERS).[10] Although these methods possess a high degree of specificity since they precisely match and measure the genes or proteins of the organism, they involve sophisticated and expensive detection equipment, extraction of DNA followed by PCR amplification, and isolation and/or labeling of biomarkers. Hence, a simple and inexpensive alternative approach would be of great significance for the rapid and accurate detection of pathogens and bioterrorism agents. Toward accomplishing this goal, we developed an inexpensive and label-free approach for the specific capture and detection of *B. anthracis* spores.

Our method comprises laser light transmission measurement integrated with immobilized short peptide ligands.[11] Using short peptides as specific spore recognition elements in the biosensor offers several advantages. Short peptides are robust, capable of withstanding denaturing conditions compared to proteins and antibodies, can be easily and covalently coupled to the sensor surface, and can be synthesized by simple experimental procedures.[12] Short peptides having amino acid sequences ATYPLPIRGGGC and NHFLPKVGGGC that can specifically bind to spores of *B. anthracis* and *B. subtilis*, respectively, were identified from a phage display library and used as ligands in our study.[13,14]

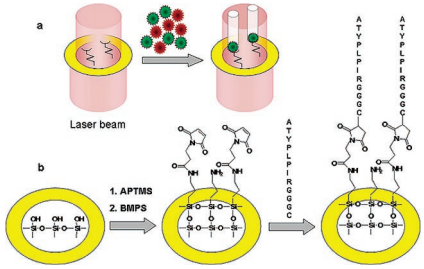

Figure 1. (a) Schematic of the detection strategy: laser light blocked by the presence of captured *B. anthracis* spores and (b) coupling of *B. anthracis*-specific peptide to the sensor surface.

Since these peptides bind directly to the spore surface, the whole spore is detected, thus eliminating the need for extraction of its nuclear and protein components and allowing the archival of captured spores for future reference.

The sensor array, microfabricated on a glass wafer, consists of two columns of gold-coated rings of 4.8 mm outer diameter and 200 μm inner diameter. The transparent inner circle functions as the sensor well. The gold-coated ring completely blocks the laser light transmission. The laser light can pass through the inner transparent well, and upon spore binding, a small part of the laser beam is blocked by the captured spores, thus decreasing the overall transmission intensity that is measured (Figure 1a).[11]

In our experiment, short-peptide molecules were coupled to the sensor well surface using simple attachment chemistry (Figure 1b).[15] A piranha-cleaned transparent sensor well was coupled with aminopropyl trimethoxysilane (APTMS, 10% solution in methanol, 2 h) to obtain an amine-terminated monolayer. To prevent the binding of the thiol group of the peptide to the gold surface around the transparent well, it was blocked with a short PEG-SH by incubating it in a solution of $HS-(CH_2)_{11}-EG_3$ (1 mg/mL EtOH, 1 h, not shown in Figure 1b). The terminal amine groups of the monolayer were reacted with a heterobifunctional cross-linker [*N*-(β-maleimidopropyloxy)succinimide ester, BMPS, 3 mg/mL PBS, pH 7.4, 30 min]. The succinimide ester end of the cross-linker reacts with the amine group on the glass well surface and leaves the maleimide group at the other end of BMPS. This step provides a surface monolayer with densely packed maleimide groups, which react with the thiol group of cysteine in the short peptide, forming a thioether linkage.

Each column of the sensor wells was coupled with *B. anthracis*-specific peptide of amino acid sequence ATYPLPIRGGGC (10 mg/mL PBS, pH 7.4, 30 min). The first column of sensor wells was incubated with 2 μL of *B. subtilis* spores (non-infectious simulant for *B. anthracis*) using a series of concentrations (2.5×10^5 to 5×10^4 spores/mL) in PBS and functioned as the reference, while the second column of sensor wells was spotted with 2 μL of attenuated *B. anthracis* spores (non-infectious Sterne) using the

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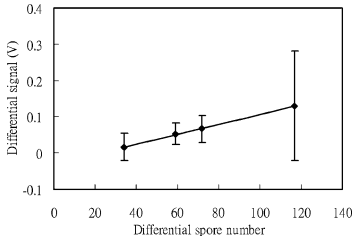

Figure 2. Relationship between differential laser light transmission intensity (ΔI) and the differential number of spores (probe − reference) captured (error bars indicate 95% confidence interval).

same concentrations as the reference column and functioned as the probe sensor. The sensor array was incubated with spores for 30 min, followed by rinsing with PBS and nanopure water to remove any unbound spores, and dried with nitrogen gas. The spores were stained with a fluorescent dye prior to their transfer onto the peptide-coupled sensor wells for independent verification with fluorescence microscopy and to count them upon capture. The spores of *B. anthracis* and *B. subtilis* were dyed with red rhodamine B isothiocyanate (RITC) and green fluorescein isothiocyanate (FITC) (2.5 μL of 1 mg/mL dye solution in DMSO, 30 min), respectively.

Laser scanning confocal fluorescence microscopic (LSCM) examination revealed the capture of 140, 73, 59, and 34 spores of *B. anthracis* on four consecutive probe sensor wells containing its complementary peptide compared to 23, 1, 0, and 0 spores of *B. subtilis* on their corresponding reference sensor wells, respectively (LSCM images, Supporting Information Figure S1).

The assembly of the optical biosensor, in brief, consisted of a laser diode (635 nm, 5 mW) and a silicon photodiode (12 V reverse bias) in conjunction with a band-pass filter arranged on an optical bench. The photodiode was connected to a variable resistor (1 kΩ), which in turn was connected to a low-pass filter/amplifier and readout by a computer.[11]

The sensor array, with its captured spores, was affixed onto a translational stage and aligned between the laser diode and the silicon photodetector to obtain a maximum transmittance signal for each well. The average transmittance values and standard deviations were calculated for each well, and a graph of transmittance intensity against the differential spore number between the probe and reference wells was plotted (Figure 2).

The laser transmission intensity differential (ΔI) between the reference and the probe sensor represents the approximate number of captured spores. The differential laser light intensity, ΔI, increased proportionally with the differential number of spores (probe-reference) captured on the well surfaces (Figure 2). The entire detection process required 35 min. With this approach, as few as 34 *B. anthracis* spores present in a 2 μL suspension (containing 100 spores) could be detected. In comparison, QCM could detect a minimum of 450 spores of *B. subtilis*, while a SPR device requires 1.7×10^3 CFUs of *Salmonella* per 10 μL test portion to obtain a significant signal response.[16,17]

To test the species specificity further, we performed a competition binding experiment in which 2 μL of an equal mixture (100 spores/μL each) of fluorescently stained spores of *B. anthracis* (RITC) and *B. subtilis* (FITC) were spotted onto two adjacent sensor wells, one of which was coupled with *B. anthracis*-binding peptide and the other one with *B. subtilis*-binding peptide, and incubated for 30 min, followed by rinsing with PBS and nanopure water. The LSCM images revealed that the sensor coupled with *B. subtilis*-specific peptide captured 46 spores of *B. subtilis* in comparison to one spore of *B. anthracis*, while the sensor coupled with *B. anthracis*-specific peptide captured 43 spores of *B. anthracis* compared to a single spore of *B. subtilis* (Figure 3). These results

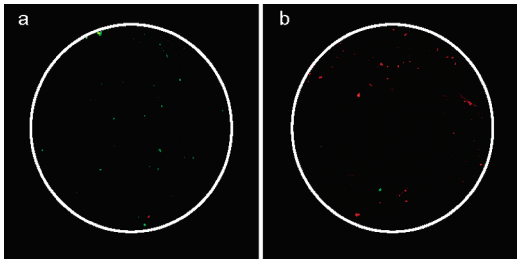

Figure 3. LSCM images from the competition binding experiment, demonstrating the high selectivity of the peptides: (a) sensor well (200 μm diameter) coupled with *B. subtilis*-specific peptide captured 46 spores of *B. subtilis* (green) and one spore of *B. anthracis* (red); (b) sensor well coupled with *B. anthracis*-specific peptide captured 43 spores of *B. anthracis* (red) and one spore of *B. subtilis* (green).

provide evidence for the high selectivity of the peptides and demonstrate their applicability in the development of a specific biosensor for *B. anthracis*.

The described optical biosensor, integrated with robust peptide ligands, demonstrates a specific detection system for *B. anthracis* spores. The differential nature of measurement reduces the possibility for false positives. This approach, with simple instrumentation and ease of use, can facilitate the development of a compact device for rapid, label-free, and accurate detection of anthrax-causing spores.

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Supporting Information Available: Experimental procedures and LSCM images. This material is available free of charge via the Internet at http://pubs.acs.org.

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