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A Miniature Biochip System for Detection of Aerosolized *Bacillus globigii* Spores

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The feasibility of using a novel detection scheme for the analysis of biological warfare agents is demonstrated using *Bacillus globigii* spores, a surrogate species for *Bacillus anthracis*. In this paper, a sensitive and selective enzyme-linked immunosorbent assay using a novel fluorogenic alkaline phosphatase substrate (dimethylacridinone phosphate) is combined with a compact biochip detection system, which includes a miniature diode laser for excitation. Detection of aerosolized spores was achieved by coupling the miniature system to a portable bioaerosol sampler, and the performance of the antibody-based recognition and enzyme amplification method was evaluated. The bioassay performance was found to be compatible with the air sampling device, and the enzymatic amplification was found to be an attractive amplification method for detection of low spore concentrations. The combined portable bioaerosol sampler and miniature biochip system detected 100 *B. globigii* spores, corresponding to 17 aerosolized spores/L of air. Moreover, the incorporation of the miniature diode laser with the self-contained biochip design allows for a compact system that is readily adaptable to field use. In addition, these studies have included investigations into the tradeoff between assay time and sensitivity.

Recently, there has been a growing interest in rapid detection of pathogens for homeland defence.[1] Among the potential biological warfare (BW) agent candidates for use as a weapon, *Bacillus anthracis* is of particular concern for a number of reasons. First, it is a highly pathogenic organism, requiring medical attention within 24−48 h of initial exposure. Second, *B. anthracis* can easily be produced and released in large numbers in the spore form, which is highly resistant to inactivation. In addition, infective doses have been estimated to be 8000−10 000 spores inhaled.[2] Consequently, there is a current need for detection devices capable of identification and quantitation of BW agents, such as *B. anthracis* spores.[2]

In general, the two main approaches for detection and species-specific identification of BW agents are based on either nucleic acid or immunological recognition.[3] An advantage of immunological methods is that surface antigens can be targeted, thereby eliminating the need for a cell/spore lysis step required for the extraction of DNA or RNA in nucleic acid-based analyses. For the detection of bacterial spores, such as *B. anthracis*, this is particularly advantageous as it is very difficult to disrupt the strong, resistant shell of the spore.[4] In addition, antibody-based identification is not only capable of high sensitivity and specificity but is also adaptable to field use.[5−7] For example, detection of *Bacillus globigii*, a surrogate species for *B. anthracis*, has been previously demonstrated at levels of 3×10^3−1×10^5 colony forming units (cfu)/mL of air, using a sandwich immunoassay method.[5,6]

In developing portable detection devices, the tradeoff between the size of the instrumentation and detection sensitivity should be considered. However, when detecting biological agents (such as *B. anthracis*) in the field, sensitivity is essential. Furthermore, identification of low spore concentrations could provide an early warning of a release, thereby minimizing further spread of the spores and human casualties. One route to sensitive detection is to utilize a method that incorporates amplification of the signal, such as enzyme-linked immunosorbent assays (ELISA).[8] In an ELISA, an antibody specific to the target species is immobilized onto a solid support or platform. The antigen (i.e., target species) binds selectively to the capture antibody and is thus immobilized on the platform. Following immobilization, a labeled second antibody, which is used for detection, specifically recognizes another epitope or site on the target, as illustrated in Figure 1. Amplification is made possible through the modification of the detector antibody or antibodies to include the attachment or conjugation of an enzyme. The final step of the assay involves addition of a substrate upon which the enzyme (conjugated to the detector antibody) acts with a very high turnover rate to cause

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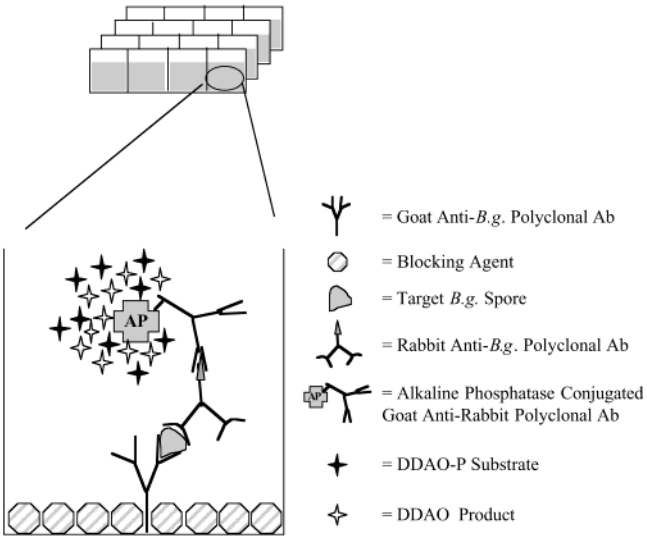

Figure 1. Schematic diagram of the enzyme-linked immunosorbent assay, used for the detection of *B. globigii* (*B.g.*) spores. The final stage of the bioassay involves enzymatic cleavage of the substrate dimethylacridinone phosphate (DDAO-P) by the enzyme-conjugated detector antibody, converting the substrate into the fluorescent product dimethylacridinone (DDAO).

amplification of a detectable product. This measurable end point, typically the production of a colored reaction product, is usually detected spectrophotometrically, and the absorbance of the reaction product is used to quantitate the amount of antigen or target species present in the sample. By using a substrate that produces either a fluorogenic or chemiluminescent product (upon enzymatic cleavage by the conjugated enzyme), the sensitivity of the assay can be enhanced even further.[9]

Typically, the two most common enzymes that are utilized in an ELISA protocol are alkaline phosphatase and horseradish peroxidase. Both of these are ideally suited for numerous applications since they possess very high turnover rates, allowing for rapid signal amplification. Recently, a novel fluorogenic alkaline phosphatase substrate, dimethylacridinone phosphate (DDAO-P) was introduced as an alternative to fluorescein diphosphate (FDP) and 4-methylumbelliferyl phosphate (MUP), which have been in widespread use for many years. This new DDAO-P substrate has a number of advantages over other fluorogenic substrates, including avoidance of the biphasic kinetics of fluorescein- and rhodamine-based systems due to a single hydrolysis-sensitive moiety.[10] In addition, upon enzymatic cleavage, DDAO-P produces a species with an absorption maximum that is red-shifted over 200 nm relative to the unreacted substrate, allowing the two species to be easily distinguished spectroscopically. This 200-nm shift in the absorption spectrum of the DDAO product means that the excitation maximum (i.e., 633 nm) is extremely red-shifted relative to other fluorogenic substrates and therefore is easily and efficiently excited using diode lasers. Once excited, the resulting fluorescence emission (maximum at 659 nm) can then be efficiently detected, since most photodiode-based detectors exhibit a maximum sensitivity in this spectral region. Finally, DDAO-P also possesses good water solubility, a low K_m, and a high turnover rate.[10]

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Previously, we developed an integrated circuit chip, known as the multifunctional biochip (MFB), that has demonstrated great potential for field use. The MFB has a number of distinct advantages over alternate biosensing technologies.[11−14] These advantages include a fabrication process based on complementary metal oxide semiconductor (CMOS) technology and multianalyte detection. For example, the CMOS fabrication process allows for application-specific circuitry (i.e., signal amplification and filtering) to be integrated into the chip, thereby significantly reducing the size and power requirements of the system. Another important consideration is that the CMOS process is very cost-effective, which is ideal when one is mass-producing portable detection devices. Furthermore, the chip is composed of an array of individual detector elements, each of which could be devoted to the detection of a different biological agent for multiplexed detection. For example, in this work, a 4 × 4 array of photosensors was used, which could be capable of performing 16 simultaneous biowarfare agent analyses in a single, compact unit.

To provide a compact system, readily amenable to field use, it is important to consider the size and power requirements of the supporting components to the biochip system, such as the laser used for excitation of the fluorescent product. Since the red-fluorescent DDAO is conveniently excited at 635 nm, a miniature diode laser can be used. This, along with the self-contained biochip integrated circuit, allows for the development of a small yet sensitive system.

EXPERIMENTAL SECTION

Biochip Detection System. The miniature biochip detection system, consisting of excitation source, excitation and collection optics, and integrated circuit photosensing array chip, is illustrated schematically in Figure 2. A 635-nm, diode laser (model VHK 4.9 mW, Edmund Scientific), having an output power of ∼5 mW, is first filtered using an excitation filter (model HQ620/60X, Chroma Technology Corp.) and then directed through a diffractive pattern generator having >95% diffraction efficiency (Rochester Photonics Corp. part SA4X4). This diffractive pattern generator is used to create an array of 16 equally intense laser beams, corresponding to the number of elements in the IC array chip. The excitation laser was chosen for its small size (∼0.6 × 1.3 in.) diffraction-limited beam quality, and ability to optimally excite the fluorescent substrate used in these studies. In addition to the abovementioned attributes, performing the analysis in the red region of the electromagnetic spectrum is advantageous as this is the region of best quantum efficiency of the photosensors comprising the biochip device. After passing through the pattern generator, the excitation laser beams are reflected off a diochroic filter (Q660LP, Chroma Technology Corp.) and are thereby incident on the sample chambers. The distance between the diffractive pattern generator and the sample chambers was ∼4 cm, to match the center-to-center spacing of the diverging pattern with the sample chambers. The resulting fluorescence was collected back through the dichroic filter that is designed to transmit wavelengths longer

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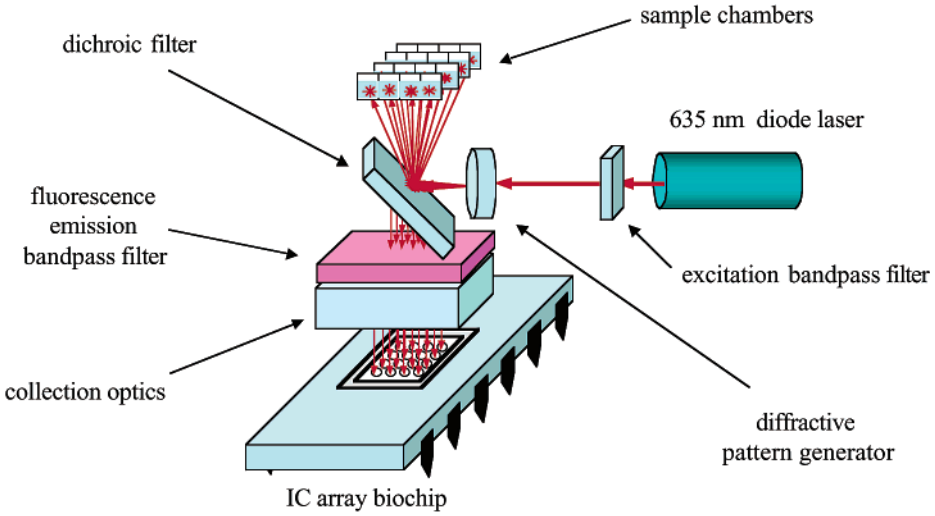


Figure 2. Schematic diagram of the miniature biochip detection system, consisting of excitation source, excitation and collection optics, and integrated circuit photosensing array.

than 660 nm, thereby providing preferential collection of the fluorescence over the scattered laser light at 635 nm. Collection was achieved using a 1-in.-diameter, *f*/2 lens, and the collected fluorescence was filtered additionally by an emission band-pass filter (HQ 700/75 nm, Chroma Technology Corp.) before being focused by another lens onto the IC photosensor array. In this way, an image of the fluorescence originating from the 16 individual sample chambers could be focused onto each of the 16 elements of the photosensing chip. The resulting output voltage from each of the individual photosensors was then recorded using a laptop computer with in-house-written Labview software. This fluorescence signal intensity, originating from the 16 locations, was then correlated to the concentration of target *B. globigii* spores.

The integrated circuit biochip prototype described above was designed and developed at Oak Ridge National Laboratory and is not yet commercially available.[11−14] The individual photosensors of the 4 × 4 array are 900 μm square in size and are arranged in a 1-mm grid array. Through a 1.2-μm n-well CMOS fabrication process, application-specific circuitry was integrated into the chip for digital control of filtering and amplification.

Aerosolized *B. globigii* Spore Experiments. A six-jet modified MRE collision nebulizer, (BGI Inc., Waltham, MA) using 20 psig of compressed air was used to create an aerosol of *B. globigii* spores from concentrated stock solutions. A fraction of the aerosolized spores was collected over a 2-min time period at an air sampling rate of 150 L/min into a 5-mL volume of phosphate-buffered saline (PBS) using a portable bioaerosol collection system (Biocapture BT-550, MesoSystems Technology, Inc., Kennewick, WA), The number of spores collected by the system was determined through visual inspection under a microscope.

ELISA Procedure for Detection of *B. globigii* Spores. An ELISA for antibody-based capture and identification of *B. globigii* spores, was used in conjunction with the biochip detection instrumentation. As illustrated in Figure 1, antibodies specific to a surface antigen on the *B. globigii* spore (goat anti-*B. globigii*) were immobilized onto a Nunc Maxisorp protein binding platform (Nunc Maxisorp 96-well plate surface) overnight at 4 °C. For all other steps, incubation times can range to up to 5 min. The goat antibody was diluted to 10 μg/mL in 0.1 M carbonate buffer, pH 9.6. The remaining binding sites were blocked for 1 h at room temperature using a bovine serum albumin (BSA) diluent/blocking solution concentrate, diluted 1:10 in distilled water (KPL, Gaithersburg, MD). Following blocking, the immobilized antibodies were then incubated with the *B. globigii* spores at 37 °C **(***B. globiggi* Var. Niger, Baker Labs Dugway Proving Grounds, UT), diluted to various concentrations in PBS, and then washed thoroughly in PBS + 0.5% Tween 20 to remove any unbound target species. Subsequently, a detector antibody (rabbit anti-*B. globigii*) recognizing another epitope on the *B. globigii* spore surface was diluted in BSA diluent/blocking solution concentrate (1:15 dilution in distilled water) to a final concentration of 5 μg/mL, incubated at 37 °C with the captured spores, and finally washed several times (PBS + 0.5% Tween 20). The final antibody, goat anti-rabbit IgG (H + L) conjugated with alkaline phosphatase (Jackson Immunoresearch Laboratories, Avondale, PA), was diluted 1:3000 in AP stabilizer (KPL) and was incubated at 37 °C with the sample complex. The unbound enzyme−antibody conjugate was then removed through several washes as described above. This antibody concentration was found to be ideal for production of the maximum fluorescence signal of the final product, while minimizing the fluorescence originating from the negative control. Finally, the fluorogenic substrate DDAO-P 20 μM in 0.1 M Tris, pH 9.9, plus 1 mM $MgCl_2$ was incubated with the immunocomplex to yield a detectable fluorescence product. Upon enzymatic cleavage, DDAO-P produces a product (DDAO) with a shift in absorption maximum of over 200 nm relative to the unreacted substrate, allowing the two species to be easily distinguished. For all experiments here, the reactions were carried out under "saturated substrate" conditions, since the heterogeneous assay will be diffusion limited. The results from three separate bioassays were recorded three times each per chamber and averaged for each of the data points described below. The error bars shown on the graphical results represent plus or minus one standard deviation calculated from these replicate assay experiments and measurements.

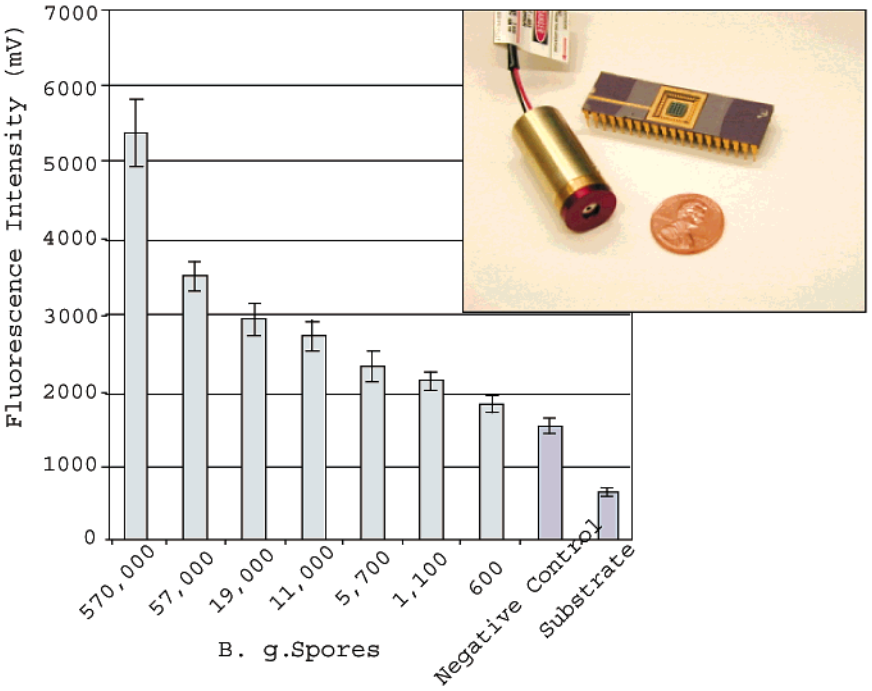


Figure 3. Detection of *B. globigii* spores using the ELISA method and miniature detection system. The fluorescence intensity of the enzymatic product, DDAO, is shown for different *B. globigii* spore concentrations. The negative control, was obtained using identical conditions, except there was no introduction of the spore samples. Also shown is the signal from the substrate alone, which has not been hydrolyzed to product. The miniature diode laser used for excitation of the fluorescent product is shown along with the integrated circuit biochip (Inset).

RESULTS AND DISCUSSION

To assess the ability of the miniature MFB system to detect anthrax spores, known concentrations of *B. globigii* spores, a surrogate species for *B. anthracis*, were analyzed using an ELISA method. For example, Figure 3 shows the intensity of the fluorescent product of the enzymatic reaction, DDAO, obtained after a 30-min incubation with the substrate, for different concentrations of *B. globigii* spores. In general, the fluorescence intensity was found to increase with increasing spore concentrations. For comparison, the last bar on the graph depicts the fluorescence intensity of the substrate, which has not been hydrolyzed to the DDAO product. Also shown are the results obtained from a negative control, using all assay steps except the introduction of *B. globigii* spores. For this work, the nonspecific binding was minimized through the introduction of a blocking agent containing BSA, which prevents protein binding sites on the substrate that do not contain the capture antibody from being available to bind nonspecifically with either subsequent assay components (i.e., detector antibodies) or species present in the sample. Although the negative control exhibited some nonspecific binding, as evidenced by the slightly higher fluorescence intensity relative to that observed for the substrate, the fluorescence intensity for the 600-spore sample is significantly greater than the negative control. Perhaps the nonspecific binding could be reduced further through optimization studies of all assay steps, in particular the addition of the third enzyme-conjugated antibody, as each enzyme-conjugated species will act on many substrate molecules to produce the fluorescent product. None the less, these results show that enzyme-based amplification offers a potentially attractive alternative to nucleic acid-based amplification methods (e.g., polymerase chain reaction) since a sensitive analysis can be performed without requiring a cell-lysing step that is particularly difficult to achieve with the hard, resistant shells of spores. In addition, the red-shifted DDAO product allows the use a small diode laser for excitation of the product; this along with the self-contained ORNL biochip integrated circuit (shown in Figure 3 inset), provided a compact detection system.

In addition to a need for sensitivity, there is often a desire for rapid analysis times. For this reason, we have investigated the effect of assay time on the sensitivity of the analysis. Figure 4 shows the fluorescence intensity of the DDAO product versus concentration of *B. globigii* spores, using different substrate incubation times. For reference, the fluorescence intensities of the negative controls (not shown) were 150 ± 19, 320 ± 20, and 630 ± 50 mV for 15-, 30-, and 45-min substrate incubation times, respectively. These results show that the substrate incubation time could be cut in half in most cases from the 30-min incubation used when the data in Figure 3 were obtained. For example, the fluorescence intensity of the 500-spore sample (230 ± 12 mV), is significantly greater than the fluorescence observed for the negative control 150 ± 19 mV using only a 15-min substrate incubation time. It is also important to note that the longer incubation time (i.e., 45 min) provided the best results, measured by comparing the fluorescence intensity for the 500-spore sample (1030 ± 30 mV) to the corresponding fluorescence background of the negative control (630 ± 50 mV). As expected, these results suggest that a tradeoff will exist between detection limit and analysis time, where a longer analysis time could allow for a more sensitive analysis.

These results indicate that the miniature biochip system has a great potential for the analysis of spores originating from liquid samples using the ELISA-based detection method. However, an analysis method that is capable of detecting not only liquid

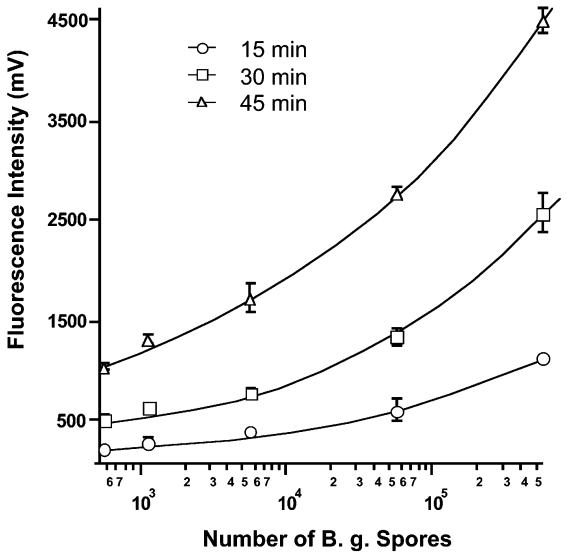
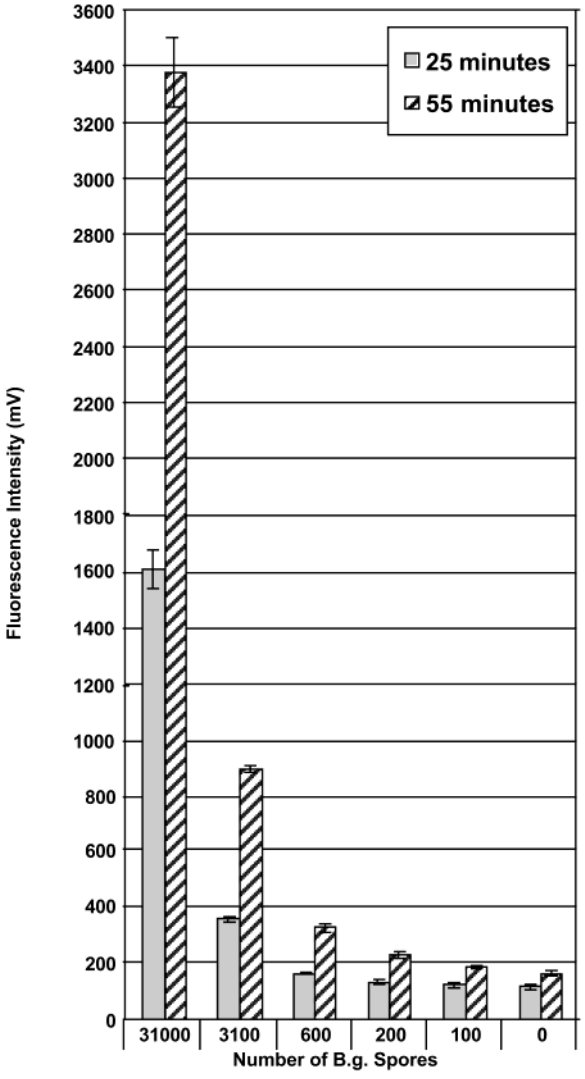


Figure 4. Fluorescence intensity of the enzymatic product for different *B. globigii* spore concentrations using a 15- (open circles), 30- (open squares), and 45-min (open triangle) incubation time with the substrate. For comparison, the fluorescence of the negative controls (all assay steps except the antigen introduction) were 150 ± 19, 320 ± 20, and 630 ± 50 mV for the 15-, 30-, and 45-min substrate incubation times, respectively.

samples but also aerosolized spores is of great value, as this is the most probable mode of release. To demonstrate the feasibility of using the miniature MFB system and the ELISA method and for the analysis of aerosolized spores, the system was coupled to a portable bioaerosol sampler. Using this portable device, air is collected at a rate of 150 L/min, and the spores are efficiently transferred directly into a liquid reservoir, prior to analysis with the MFB system. To evaluate the possibility that the air sampler might alter the surface of the spores and thus degrade the bioassay performance, the compatibility of the method and the aerosol sampler system was investigated. The results of this study are displayed in Figure 5. For these data, the fluorescence intensity was obtained after both 25 and 55 min of incubation with the substrate for various *B. globigii* spore samples collected with the portable air sampler. The number of spores in each collected sample was determined through counting via visual inspection under a microscope. Similar to Figure 4, the last bar on the graph depicts the fluorescence intensity of the substrate, which has not been hydrolyzed to the DDAO product. For these data, however, the negative control sample was acquired by collecting the air prior to the presence of aerosolized *B. globigii* spores. After comparison of the results in Figure 5, using a 25-min substrate incubation time, to those observed in Figure 3, with liquid spore suspensions, it is clear that the bioassay performance was not significantly affected by the air sampling device. In both sets of data, the fluorescence of the 600-spore sample is significantly higher than that observed for the negative control. In other words, the cyclone-based bioaerosol collection device did not compromise the ELISA method, which relies on the antibody-based recognition of specific epitopes on the spore surface.

To see whether the detection of an even lower number of aerosolized spores was possible, the same set of samples were incubated further with the substrate for a total of 55 min and the recorded fluorescence intensity is also shown in Figure 5. Indeed,



	31000	3100	600	200	100	0
25 minutes	1604 ± 69	356 ± 8	159 ± 3	130 ± 13	121 ± 9	112 ± 9
55 minutes	3378 ± 125	892 ± 11	325 ± 13	229 ± 12	184 ± 5	160 ± 7

Figure 5. Detection of aerosolized *B. globigii* spores using the ELISA method, and biochip detection system coupled to a portable bioaerosol collector. Incubation with the substrate for 25 (solid bars) and 55 min (striped bars) is shown for various spore concentrations, with the 0-spore sample representing the negative control. The sample used for the negative control was obtained by collecting the prior to the introduction of *B. globigii* spores. Also shown are the values of the fluorescence intensity for each sample investigated, to illustrate the improved detection capability when the longer substrate incubation time was used.

a longer substrate incubation time allowed for an even more sensitive analysis as evidenced by the fluorescence intensity of the 100-spore sample, which was significantly higher than the negative control. Thus, as suggested in the discussion of Figure 4, it is possible to vary the assay development time based upon the desired mode of analysis, short for rapid screening of high concentrations, and longer for extremely sensitive detection.

CONCLUSIONS

Few reports to date have demonstrated devices capable of the identification and quantification of biological warfare agents such

as aerosolized spores. Moreover, the studies that have demonstrated these capabilities report the detection of very high (aerosolized) spore concentrations. Based on the parameters used in this study (e.g., air sampling rate, total collection time, volume of sample taken from the collection reservoir to perform the analysis), the concentration of the spores in the air, detected using the integrated air sampling and detection system can be estimated. For instance, the 100 spores detected in Figure 5, were obtained from a 100-μL aliquot of the reservoir, which corresponds to a liquid concentration of 1×10^6 spores/L. Since the portable air sampler transferred the spores to a total volume of 5 mL in the liquid reservoir, the total number of spores collected by the device was ~5000. Assuming a collection efficiency of 50% for the air sampler,[15] this corresponds to 10 000 spores available in the aerosol suspension. Finally, the air sampler collected the aerosol at a rate of 150 L/min and operated for a total of 2 min, thus collecting a total of 300 L of air. The aerosol concentration corresponding to the 100-spore signal is therefore, ~17 spores/L of air. It is interesting to note, however, that the analysis was conducted using only 1/50th or 2% of the total volume of solution of the reservoir into which the spores were deposited. Therefore, if full advantage of the entire collected sample were taken through concentrating the entire 5-mL sample onto the antibody capture surface prior to analysis, it is theoretically possible that the detection could be further enhanced, from the 17 spores/L of air reported here, to up to 0.34 spores/L of air. These results represent a significant advance in biological warfare agent detection capabilities of aerosolized spores. Furthermore, the ELISA method utilizing DDAO-P as the fluorogenic substrate, and miniature biochip system utilizing a miniature diode laser, has the potential to detect multiple biowarfare agents within a single, compact device, as each biosensing element could be devoted to the detection of a different BW agent.

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