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An Anthrax "Smoke" Detector

Online Monitoring of Aerosolized Bacterial Spores

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The anthrax attacks in 2001 have highlighted the need for new methods of detecting bioaerosols including airborne *Bacillus anthracis* spores. Current methods of bacterial spore (endospore) detection, such as colony counting and PCR, require trained personnel for sampling and analysis. The labor requirement obviates these methods for continuous, online endospore monitoring; it would be like firemen inspecting your home periodically to ensure that there is no fire. Here we report a method for automated monitoring of airborne endospores, which combines an aerosol capture technique with endospore detection based on terbium luminescence turn-on.

Endospores can be formed by certain bacteria during times of stress or lack of nutrients. This dormant bacterial form can survive harsh conditions such as boiling, freezing, and desiccation that readily kill vegetative bacteria [1]. Indeed, *Bacillus stearothermophilus* and *Bacillus subtilis* spores are used to check the performance of autoclaves. Two spore-forming genera of medical importance, *Bacillus* and *Clostridium*, are the causative agents of anthrax, tetanus, botulism, and gas gangrene. The fact that endospores are so durable is the primary reason for time-consuming and expensive sterilization procedures employed in hospitals, canneries, and other food preparation facilities. Although the vegetative cells of endospore-forming bacteria are most commonly found in the soil, endospores exist almost everywhere, including the atmosphere, where they are frequently carried on dust particles [2].

Online monitoring of aerosolized bacterial spores is essential in locations such as mail sorting, food preparation, and healthcare facilities. Among the desired characteristics for bacterial spore alarm systems are online operation, minimal maintenance, few false alarms, and no false negatives. Methods for bioaerosol sampling [3], [4] such as filtering, suspending, or impacting, coupled to methods of monitoring such as PCR analysis of gene segments [5], culturing and analysis of the colonies using standard microbiolocal assays (e.g., shape, staining), pyrolysis-GC-IMS [6], or fluores-

cence flow cytometry [7] have been reported. The PCR method requires extensive sample preparation prior to running the PCR reaction, and the procedure for culturing requires an incubation period of ~ 2 days; both methods require the active participation of a technician. The cost of labor, technical complexity of PCR, and slow response time of colony counting have prevented the widespread application of these methods for monitoring of bacterial spores in the air.

We and others [8]-[11] have investigated methods for the rapid detection of bacterial spores based on dipicolinic acid (DPA) triggered terbium (Tb) luminescence. The core of bacterial spores contains up to 1 M DPA [1], which can be released into bulk solution by microwaving the sample [12]. The released DPA binds Tb ions with high affinity and triggers intense green luminescence under UV excitation. The luminescence intensity can then be correlated to a DPA concentration and subsequently to bacterial spore concentration. Here we report the implementation of the terbium luminescence assay in conjunction with an aerosol capture device, a microwave, and a miniature lifetime-gated luminescence spectrometer to enable unattended, online monitoring of aerosolized bacterial spores.

Materials and Methods

Stock solutions of purified *Bacillis subtilis* spores were purchased from Raven Biological Laboratory (Omaha, Nebraska) and purified with three water wash cycles after which no vegetative cell fragments were seen with phase contrast microscopy. Terbium(III) chloride hexahydrate, 99.999%, and glycerol, 99.5% spectrophotometric grade, were purchased from Aldrich (Milwaukee, Wisconsin).

B. subtilis spores were aerosolized with a Lovelace nebulizer system (InTox Products, Albuquerque, New Mexico) to simulate an anthrax attack. The instrument for monitoring the concentration of aerosolized bacterial spores consisted of a bioaerosol sampler (SKC, BioSampler, Eighty Four, Pennsylvania), a microwave with temperature control (CEM Corporation, Discover Microwave System, Matthews, North Carolina), and a miniature lifetime-gated fluorescence spectrometer with fiber-optic probe (Ocean Optics, USB2000-FL-2048-element fluorescence spectrometer and PX-2 Pulsed Xenon

While background indoor spore concentrations do not yield a signal throughout a workday, a spore event like the one simulated here will be recorded within 15 minutes.
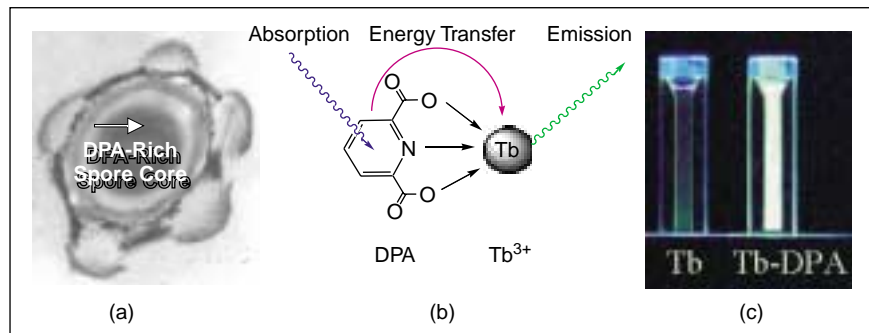

1. (a) An electron microscope image of a spore (~1 μm diameter) highlighting DPA-rich endopore core. (b) Unbound Tb^{3+} ion (shaded ball) by itself has a low absorption cross section (<1 $M^{-1}cm^{-1}$) and consequently has low luminescence intensity. Binding of the light-harvesting DPA (absorption cross section >$10^4 M^{-1}cm^{-1}$), originating from endospores, gives rise to intense Tb luminescence due to an absorption, energy transfer, emission mechanism. (c) Two cuvets on a UV lamp filled with 1 mM $TbCl_3$ (Tb cuvet), and 1 mM $TbCl_3$ + 1 μM DPA (Tb-DPA cuvet), respectively. The amount of DPA in the Tb-DPA cuvet corresponds to 10^6 spores/ml.
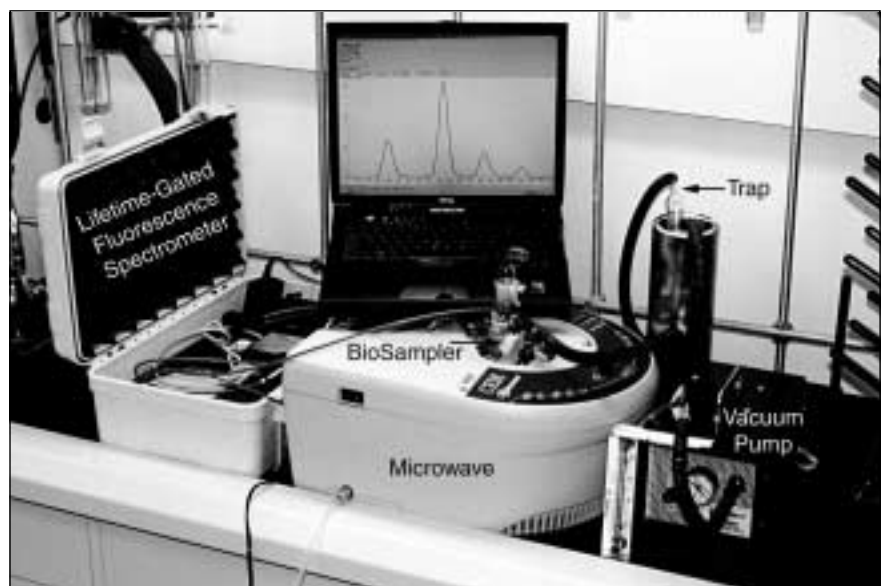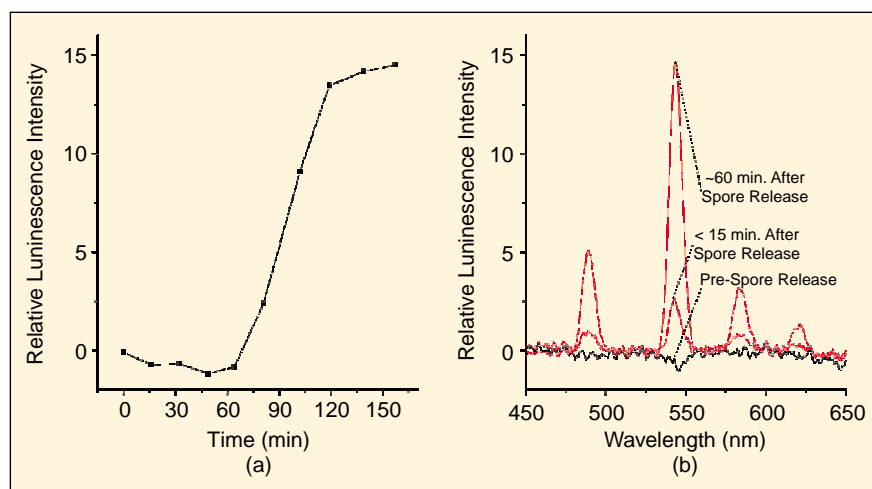

2. Picture of the instrumental setup consisting of the Ocean Optics spectrometer with fiber-optic probe attached to an SKC BioSampler that is mounted into the cavity of the CEM Discover microwave.
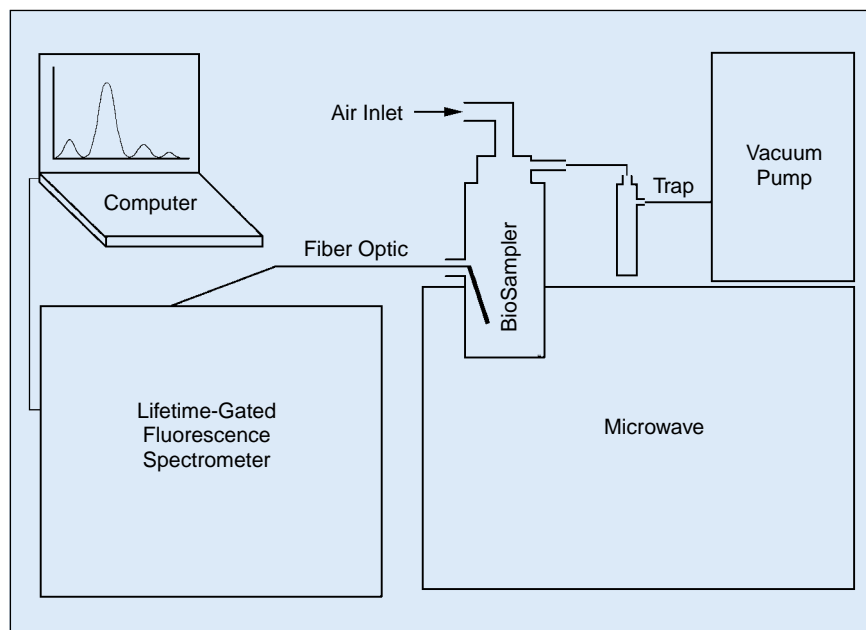
3. Diagram of the instrumental setup.

4. (a) Timecourse of bacterial spore monitoring. (b) Luminescence spectra recorded (i) just prior to spore release, (ii) less than 15 min, and (iii) about 60 min after spore release.

Flash Lamp, Dunedin, Florida). The fiber-optic probe was made of PEEK polymer to be compatible with probing the glycerol solution within the microwave cavity. The BioSampler was modified with a sidearm so that the fiber-optic probe could be immersed into the glycerol solution containing $TbCl_3$. Glycerol was used as the solvent because of its low volatility and the high solubility of $TbCl_3$ and DPA. Tubing with a T-connector to equilibrate the pressure to 1 atm was used to connect the output of the Lovelace nebulizer to the BioSampler inlet.

The Lovelace nebulizer was operated at 25 psi, which gave rise to an aerosol with an average droplet diameter of 5.5 μm. The nebulizer was filled with a 10^8 spores/ml *B. subtilis* suspension. The BioSampler was operated at a flow rate of 12.5 liters/minute and was filled with 10 μM $TbCl_3$ glycerol solution. While the BioSampler continually sampled the air, a cycle consisting of an 8-min microwaving step at 140 °C, a 6 min cooling period, and a 30 s luminescence measurement was performed repeatedly, yielding a sampling rate of ~4 measurements per hour. Lifetime-gated luminescence spectra were obtained with the following instrument settings: delay time = 100 μs, integration time = 5 ms, number of averages = 100. For the timecourse experiment (Figure 4), the luminescence spectrum of 10 μM $TbCl_3$ in glycerol solution prior to air sampling was used as the background and was subtracted from subsequent luminescence spectra.

We chose the SKC Biosampler because of its high transfer efficiency (>95%) for microbe-containing aerosols and its all-glass construction, which makes it compatible with microwave radiation and allowed side-arm modification for the fiber-optic probe. The CEM Discover microwave was chosen because it has temperature control, an open microwave cavity construction, and low power consumption (~50 W to maintain 140 °C).

Results and Discussion

Dipicolinic acid (DPA, 2,6-pyridinedicarboxylic acid) is present in high concentrations (up to 1 molar, ~15% of dry weight) in the core of bacterial spores [1]. For all known life forms, DPA is unique to bacterial spores and can thus be used as an indicator molecule for the presence of bacterial spores. Microwave radiation efficiently releases DPA from

the spore into bulk solution , which then binds to terbium ions with high affinity, triggering intense green luminescence under UV excitation [8]-[12] (Figure 1). Thus, the green luminescence turn-on signals the presence of bacterial spores, and the intensity of the luminescence can be correlated to the concentration of bacterial spores.

The Anthrax "Smoke" Detector instrument (Figures 2 and 3) consists of three components: 1) an SKC Bio-sampler for aerosol capture, 2) a CEM Discover microwave for releasing the DPA from the spores, and 3) an Ocean Optics miniature lifetime-gated luminescence spectrometer with a fiber-optic probe immersed in the BioSampler $TbCl_3$ glycerol solution. The fiber-optic probe contains both fibers for excitation and luminescence detection.

Lifetime gating drastically reduces the chance of false negatives, which could arise if the terbium luminescence is masked by background fluorescence from impurities. Lifetime gating takes advantage of the fact that terbium luminescence lifetimes are on the order of milliseconds, while fluorescence lifetimes from impurities generally are on the order of nanoseconds. When the Ocean Optics spectrometer is operated in lifetime gating mode, the sample is excited with a short Xe-lamp flash (FWHM ~5 μs and tailing out to ~50 μs) and luminescence is detected several microseconds after the Xe-lamp flash, thus eliminating the background fluorescence.

Figure 4(a) shows the timecourse of our online monitoring for aerosolized bacterial spores with luminescence intensity at 543.5 nm plotted against time. Each data point was collected after a cycle of microwaving and cooling. Microwaving at 140 °C is required to release the DPA, and cooling to 25 °C is necessary because the luminescence intensity is drastically reduced at high temperatures (Figure 5). First, five data points of baseline were collected (time = 0-63 min) while sampling the air without a spore event. Then the nebulizer was activated for 5 min to generate aerosolized bacterial spores, which were directed to the inlet of the BioSampler. The sixth data point (time = 81 min) clearly shows the presence of Tb-DPA luminescence, thus signaling the presence of bacterial spores. The luminescence increases for two more heating/cooling cycles and then plateaus ~45 minutes after the initiation of the spore

event. Figure 4(b) shows the luminescence spectra before (black line) and after (red lines) the generation of aerosolized bacterial spores. Clearly, the signal-to-noise ratio of 10, one cycle after spore introduction, shows that we can detect aerosolized spores with a response time of 15 min.

Figure 6 shows the spore concentration dependence of the terbium luminescence intensity at 543.5 nm. The samples for each data point were microwaved at 140 °C for 8 min in a closed vessel to release ~100% of DPA

into bulk solution [12]. In this control experiment, a closed vessel ensured that no solvent was lost due to evaporation and increased the efficiency of DPA release. The data were fit to a sigmoidal function with a near unity correlation coefficient of $R=0.99998$. From this data we determined that 1) the simulated spore event shown in Figure 4 resulted in a spore concentration of $\sim5.5\times10^5$ spores/ml in the collection vessel, and 2) the sensitivity and dynamic range are $\sim10^4$ spores/ml and four orders of magnitude, respectively.
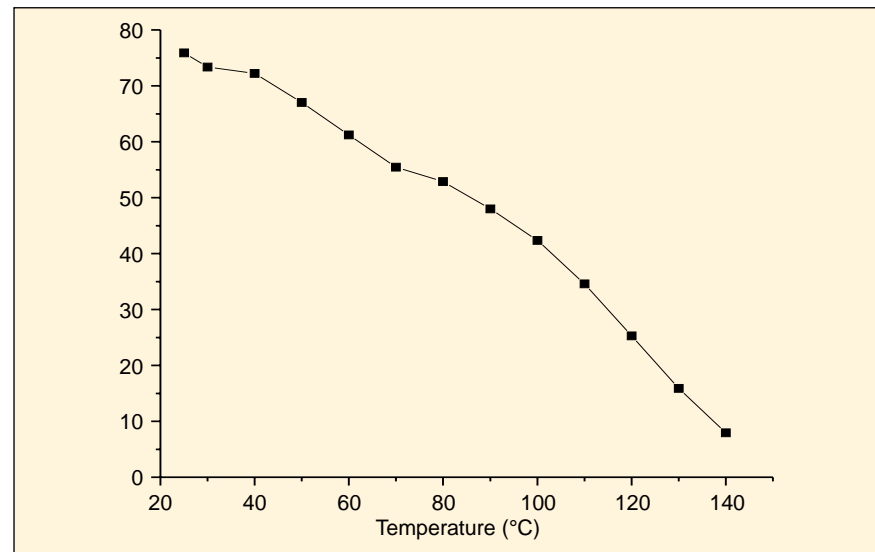
5. Temperature dependence of the luminescence intensity at 543.5 μm.
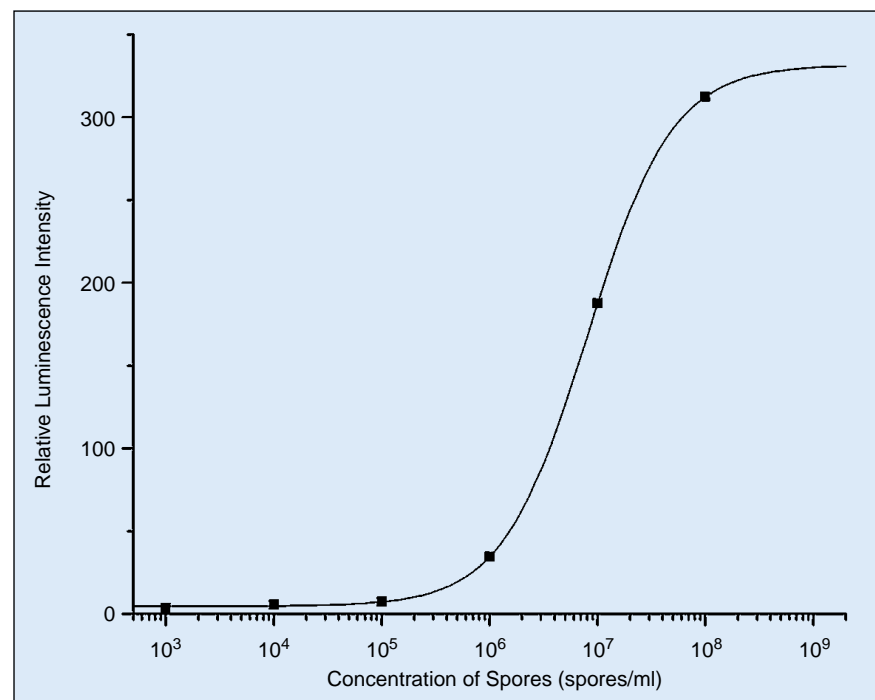
6. Spore concentration dependence of terbium luminescence intensity at 543.5 μm.

Indoor airborne bacterial concentrations have been reported [13] to range from ~1 viable particle/liter in homes to 0.1 viable particles/liter in offices. At a sampling rate of 12.5 liters/minute and a sensitivity of ~10^4 spores/ml, it would take more than 12 hours before these background concentrations give rise to a signal. In contrast, the Lovelace nebulizer generated a total of ~10^6 spores at a density of ~1.8×10^4 spores/liter (sampling 12.5 liters for 5 min) that were collected in the 20 ml glycerol solution of the BioSampler. If an average person breathes in 0.5 liters of air per breath, then a breath of this aerosol, had it contained *Bacillus anthracis* spores, would have delivered approximately the LD_{50} (8,000-10,000 endospores) for an average person [14]. Thus, while background indoor spore concentrations do not yield a signal throughout a workday, a spore event like the one simulated here will be recorded within 15 min.

The overall instrument sensitivity was not optimized in this initial investigation; however, optimization of aerosol collection, spore lysing methods, and spectrometer performance is envisioned. Methods of spore lysing that do not require high temperature, such as sonication [15], [16] and chemical extraction [8], are being aimed to significantly increase the sampling rate. While the measurements detailed above do not distinguish between harmful and innocuous bacterial spores, they do demonstrate a novel alarm capability reminiscent of smoke detectors, which detect smoke to warn us of a potential fire. This alarm capability will enable rapid distinction of suspicious white powders that contain bacterial spores from those that do not without the need for a human operator.

Conclusions

We have demonstrated quantification of aerosolized bacterial spores with a response time of ~15 min, a sensitivity of 10^4 spores/ml, and a dynamic range of 4 orders of magnitude using a bioaerosol sampler, a microwave, and a lifetime-gated fluorimeter. Ultimately, the most attractive feature we have demonstrated is the unattended monitoring of aerosolized bacterial spores for the duration of a workday (~8 hours).

Acknowledgments

Elizabeth D. Lester will graduate from Baylor University with a B.S. in biology with an emphasis in prehealth care in the spring of 2003. Her interest in biological research was sparked during a semester at the University of Dallas with Dr. Geoff Ganter. Her current research focuses on spectroscopic methods for bacterial spore detection in Dr. Adrian Ponce's lab at JPL through a MURF fellowship from Caltech. She plans to continue researching new biological detection methods in graduate school.

Adrian Ponce received his B.S. in chemistry at Michigan State University in 1993 and his Ph.D. in inorganic chemistry at Caltech in 2000. He is currently employed as a senior member of the technical staff at NASA's Jet Propulsion Laboratory in Pasadena, California. His research interests include chemical/biological sensors, molecular self-assembly, electron transfer, and laser spectroscopy.

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