Detection of anthrax simulants with microcalorimetric spectroscopy: *Bacillus subtilis* and *Bacillus cereus* spores

Edward T. Arakawa, Nickolay V. Lavrik, and Panos G. Datskos

Recent advances in the development of ultrasensitive micromechanical thermal detectors have led to the advent of novel subfemtojoule microcalorimetric spectroscopy (CalSpec). On the basis of principles of photothermal IR spectroscopy combined with efficient thermomechanical transduction, CalSpec provides acquisition of vibrational spectra of microscopic samples and absorbates. We use CalSpec as a method of identifying nanogram quantities of biological micro-organisms. Our studies focus on *Bacillus subtilis* and *Bacillus cereus* spores as simulants for *Bacillus anthracis* spores. Using CalSpec, we measured IR spectra of *B. subtilis* and *B. cereus* spores present on surfaces in nanogram quantities (approximately 100–1000 spores). The spectra acquired in the wavelength range of 690–4000 cm^{-1} (2.5–14.5 μm) contain information-rich vibrational signatures that reflect the different ratios of biochemical makeup of the micro-organisms. The distinctive features in the spectra obtained for the two types of micro-organism can be used to distinguish between the spores of the *Bacillus* family. As compared with conventional IR and Fourier-transform IR microscopic spectroscopy techniques, the advantages of the present technique include significantly improved sensitivity (at least a full order of magnitude), absence of expensive IR detectors, and excellent potential for miniaturization. © 2003 Optical Society of America

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1. Introduction

Recent events have raised the fear of deadly pathogens in common urban environments as a result of biological warfare proliferation, terrorist activities, or accidental contamination. This points out an increasing need for rapid and reliable detection of various pathogenic micro-organisms. One of the most infamous pathogens is *Bacillus anthracis*.[1] It has been postulated since the mid-1990s that *B. anthracis* is likely to be an agent of bioterrorism.[1] *B. anthracis* is a large, aerobic spore forming rod-shaped bacillus that is 3–10 μm in length and 1–1.5 μm in width.[2] *B. anthracis* causes anthrax, which is a biological infection acquired by injection or inhalation.[1] It is interesting to note that *B. anthracis* would pose no danger to humans if it lacked the two extra gene domains, known as pXO1 and pXO2.[3] In fact, it is pXO1 that encodes the deadly anthrax toxin.[3–5]

In the development of sensors for airborne biological contaminants, *Bacillus subtilis*[6–9] and *Bacillus cereus*[5,10–13] spores have been commonly used as simulants for highly viral spores of *B. anthracis*. *B. subtilis* is a species of unicellular, aerobic, gram-positive bacteria capable of producing at the end of its growth period a new cell (spore) in its bacterium. There are seven distinct stages in the sporulation process.[9] *B. subtilis* spores are elliptical in shape, approximately 1 μm long and 0.7 μm wide.[6–9,14,15] A dormant spore is composed of the exosporium, outer and inner coats, a thick cortex, a germ cell wall, and the nucleus.[8,14] The spore coats are composed primarily of proteins that have been extensively characterized.[16] The spores can remain alive in a dormant state for many years because of their high resistance to adverse environmental conditions, such as temperature changes, drying, chemical attack, high vacuum,[14] and electromagnetic radiation.[7]

In recent years, several alternative detection strategies have been pursued to develop warning systems that could provide sensitive and selective identification of pathogenic micro-organisms, such as spores of *B. anthracis*.[11,17–21] The most common identifica-

The authors are with the Oak Ridge National Laboratory, P.O. Box 2009, Building 9102-2, Oak Ridge, Tennessee 37831-8039. E. T. Arakawa and P. G. Datskos (pgd@ornl.gov) are also with the University of Tennessee, Knoxville, Tennessee 37996-1200.

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tion systems[17] are based on biomolecular recognition technologies,[22,23] such as enzymolinked immunosorbent assay (ELISA) and polymerase chain reaction. These methods can be implemented in various formats, ranging from more traditional "labs on wheels"[17] to portable systems[20,24] carried by a small remotely piloted airplane[24] and to miniature handheld devices. An alternative to molecular recognition approaches is a combination of various extraction–separation techniques and mass spectroscopy,[25–27] which offers unprecedented sensitivity at a cost of significantly increased size, cost, and operation complexity. Although immunochemical, DNA-selective, mass spectroscopy, and chromatographic methods provided the required selectivity and sensitivity, preliminary screening of airborne particles and distinguishing between microbes and other particulate contaminants relied on simpler UV-scattering and UV-luminescence measurements.[17] It should be noted that the operational costs of mass spectroscopy based analysis as well as immunochemical and DNA-selective methods could be prohibitively high in the case of continuous monitoring; therefore preliminary screening is typically required so as to trigger the sample collection and delivery when the concentration of suspected biological particles increases.[17] The question arises as to whether highly specific and sensitive detection of airborne contaminants of biological origin can be conducted without the high operational cost or the expensive consumables.

In fact, detection of micro-organisms by use of IR spectroscopy has been attempted since the 1950s. Although IR spectroscopy and microspectroscopy[28] have been extensively used for chemical analysis of organic and inorganic samples based on characteristic vibrational signatures of their constituents, IR studies of biological micro-organisms (bacteria, spores, and viruses) remained relatively scarce until the 1990s. The main reasons for this were insufficient sensitivity of conventional IR instruments and the lack of computerized spectra analysis algorithms. Starting from the early 1990s, wide availability of sensitive Fourier-transform infrared (FTIR) instruments and computerized chemometric approaches facilitated much more successful applications of vibrational spectroscopy for identification and differentiation of intact micro-organisms.[29–32] More recently, Mariey *et al*.[33] have reported a detailed review of use of FTIR as a characterization tool for micro-organisms. Although a number of recent studies confirm the potential of vibrational spectroscopy for differentiation of micro-organisms,[12,34–37] the amount of biological material required for even the most sensitive microspectroscopic FTIR analysis substantially exceeds the infective doses of pathogens effective as biological warfare agents. Hence acquisition of vibrational signatures from as few as 1–100 micro-organisms is required for vibrational spectroscopy to become suitable for biological threat warning systems.

In this paper we demonstrate the potential of a novel microcalorimetric spectroscopy (CalSpec) technique[38] for detection of *B. subtilis* and *B. cereus* spores by measuring their vibrational signatures in the range 690–4000 cm^{-1} (2.5–14.5 μm). Our CalSpec instrument utilizes a photothermal mode of transduction and takes advantage of highly sensitive micromachined bimaterial cantilevers to IR radiation.[39–44] In our previous studies, we used CalSpec for chemical analysis and identification of organic absorbates based on their vibrational photothermal spectra.[38] In the present paper we extend our use of CalSpec to detection and identification of micro-organisms and report photothermal spectra for nanogram quantities of *B. subtilis* and *B. cereus* spores.

2. Microcalorimetric Spectroscopy-Based Detection

The approach to detection of biological micro-organisms used in the present study is based on CalSpec,[38] which was previously developed in our laboratory and used successfully for detection and identification of medium molecular weight environmental contaminants in our prior research. The detection of any molecular species with CalSpec consists of two main stages. At the first stage, a sample is allowed to interact with the surface of a sensitive microcantilever thermal detector.[38–44] An appropriate chemically selective layer can be used to enhance the adsorption and retention of the sample species on the microcantilever surface. The chemical specificity of the method, however, stems from the capability of acquiring a vibrational spectrum of the absorbed sample. Therefore moderately selective surfaces, such as chromatographic stationary phases, can be successfully used. At the second stage, the microcantilever thermal detector with the adsorbate is exposed to IR radiation from a scanning monochromator. The signal obtained by use of an optical readout of the microcantilever thermal detector is proportional to the heat produced as a result of IR absorption in the sample. Therefore photothermal spectra recorded while the monochromator is scanning are similar to conventional IR spectra and contain a superposition of characteristic vibrational signatures of the chemical entities in the sample.

3. Materials and Methods

A. Biological Material

B. cereus (ATCC-7064) was purchased from American Type Culture Collection (10801 University Blvd., Manassas, Va. 20110-2209) and was grown in a medium consisting of 24.00 g of Nutrient Broth (Difco), 7.5 g of KCl, 0.5 ml of 1-M CaCl2, 0.25 ml of 0.01-M FeCl3, 0.5 ml of 0.01-M MgSO4, and 0.5 ml of 50% glucose/l. All the ingredients except the Nutrient Broth and KCl were filter sterilized and added after autoclaving. A half milliliter of overnight preculture was inoculated into 250 ml of the medium in a 1000-ml flask and incubated at 37 °C in a shaker for four days. Spores were harvested by foam flotation, and the richness of the spores was confirmed by optical mi-

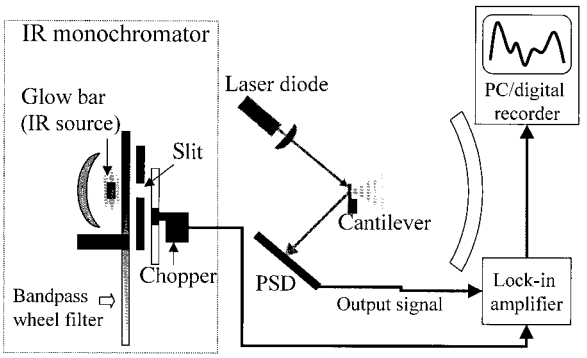

Fig. 1. Experimental setup used in the present studies for CalSpec measurements. PSD, position-sensitive photodetector.

croscopy. The harvested spores were washed with sterile deionized distilled water several times and lyophilized for use in our experiments. The *B. subtilis* spores were obtained from the Genetic Stocks Center, Department of Biochemistry, Ohio State University, and multiplied for use in our studies by J. A. Lindsay of the University of Florida.[6]

B. Microcalorimetric Spectroscopy Measurements

Commercially available gold-coated SiN_x cantilevers obtained from ThermoMicroscopes, Inc. were used as bimaterial thermal detectors for the CalSpec measurements without any further modification. The reference photothermal spectra of the cantilevers as well as their mechanical resonances were measured prior to the deposition of the sample adsorbates. To obtain nanogram quantities of adsorbates on microcantilevers, we carried out sublimation of *B. subtilis* and *B. cereus* spores from resistively heated 0.1-mm-thick stainless-steel boats in air. We carried out sublimation of *B. cereus* spores by heating the boat with approximately 5 mg of the powdered spores to 210 °C. Sublimation of *B. subtilis* spores taken in the amount of 4 mg was carried out in the same manner except that the boat temperature was 230 °C. The cantilevers with the adsorbates were then transferred into the CalSpec setup (Fig. 1), and the photothermal spectra were acquired in the range of 690–4000 cm^{-1} (wavelength range of 2.5–14.5 μm). In addition, the change in the microcantilever mechanical resonance frequency as a result of the mass loading was measured and recorded. The mass of each of the adsorbed samples was determined from this change in the microcantilevers resonance frequency as described elsewhere.[38] All measurements were conducted at ambient temperature and pressure. The samples were deposited on the silicon nitride side of the cantilever, which was also the side facing the IR radiation from the monochromator (Foxboro Miran-80 IR). An aluminum aspherical mirror with a numerical aperture of 1 was used to focus the IR radiation onto the cantilever. We carried out the readout of microcantilever deflections according to an optical lever scheme by focusing a beam from a 650-nm diode laser onto the gold-coated side of the microcantilever and detecting displacements of the light spot reflected onto a position-sensitive photodetector.

It is important to note that spontaneous cantilever oscillations, that is, thermomechanical noise, that are due to continuous dynamic exchange between cantilever thermal and mechanical energy could be observed in a wide frequency range. The root-mean-square magnitude of such oscillations is comparable to the cantilever deflections in response to the radiation from a monochromator. To improve the signal-to-noise ratio of the CalSpec method, the preamplified signal from the position-sensitive photodetector was fed into a lock-in amplifier synchronized with a mechanical chopper modulating the IR beam. Tuning of the chopper modulation frequency in the range of 25–150 Hz was based on the conditions of minimal interference with ambient cantilever noise and cantilever time thermal constant (approximately 10 ms). The details of these measurements were described previously.[38,44]

C. Reference Infrared Measurements

A single-beam spectrometer (Buck Scientific M500) was used in our studies to obtain reference transmission-mode IR spectra of the spores in the wavelength range of 2.5–14.5 μm (690–4000 cm^{-1}). A small portion of *B. subtilis* spores was powdered and mixed with KBr to form a pellet for the transmission-mode IR measurements. The effective surface density of spores in the sample was calculated on the basis of the mass of the spores added and the cross-sectional area of the pellet.

4. Results and Discussion

Our first series of experiments focused on the comparison of photothermal CalSpec spectra and conventional IR spectra (Fig. 2). Our previous studies showed that there is a close correlation between CalSpec and IR spectra in the case of medium molecular weight organic compounds, such as nitroaromatic[38] and chloroaromatic species[45] as well as in the case of polymers. In general, it has been demonstrated that both conventional and photothermal IR spectra can be used for chemical fingerprinting of analytes because of the predominant contribution of vibrational modes of chemical bonds into optical absorption of materials in the middle IR. As can be seen in Fig. 2, the photothermal and transmission spectra obtained for *B. subtilis* have common major bands located around 3000 cm^{-1} and 1500–1000 cm^{-1}. Naumann *et al.*[32,46] have pointed out that five important regions of interest (windows) in IR spectra of microorganisms should be analyzed. Each of these windows is associated with specific chemical groups of the different bacterial constituents. For example, it is assumed that the spectral region 3000–2800 cm^{-1} has a major contribution from fatty acids; the region from 1700 to 1500 cm^{-1} is characteristic of proteins; the region from 1450 to 1200 cm^{-1} contains mostly carboxylic bands of proteins and amino acids; and the region from 1250 to 1200 cm^{-1} is strongly affected by

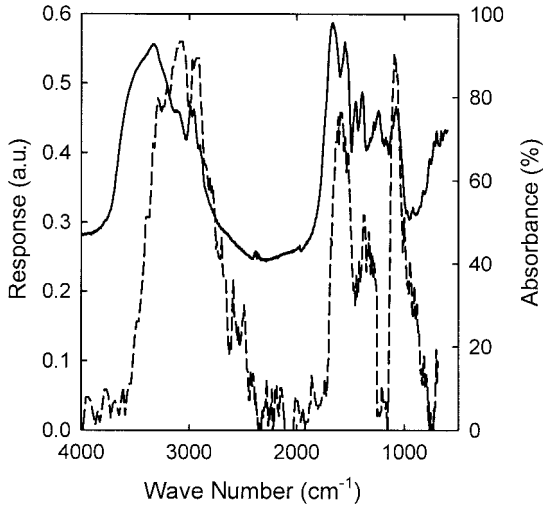

Fig. 2. Comparison of the CalSpec photothermal and conventional transmission-mode IR spectra of *B. subtilis*. The IR spectrum was measured on a pressed KBr pellet with the addition of powdered *B. subtilis* spores. The resulting surface concentration of the spores in the pellet was 1 mg cm^{-2}. The CalSpec spectrum was obtained by use of a gold-coated silicon nitride microcantilever with 1.12 ng of *B. subtilis* that was sublimed in air from a stainless-steel boat at 230 °C.

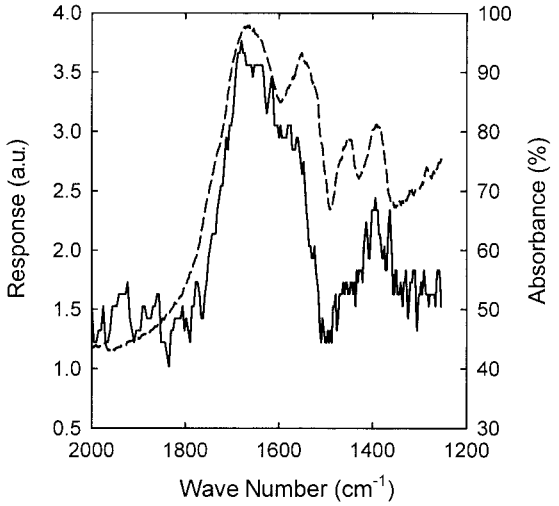

Fig. 3. Slow-scan spectra in the region of 4.5–8.0 μm (2222–1250 cm^{-1}) show remarkable correlation between the respective features in the two spectra of *B. subtilis* obtained with CalSpec (solid curve) and conventional transmission IR spectroscopy (dashed curve).

RNA and DNA components. In addition, the region below 900 cm^{-1} has been recognized to be of significance for the discrimination of micro-organisms at the strain level.[35] Because micro-organisms contain a wide variety of chemical entities, subtle differences within each of the mentioned regions as well as intensity redistribution among the windows should be taken into account. Although this can be accomplished with computerized algorithms as described above, it is important to emphasize that, except for the low-energy region below 800 cm^{-1}, the obtained CalSpec spectrum of *B. subtilis* contains sufficient amount of information-rich signatures and is consistent with the previously reported FTIR data.[35]

Figure 3 shows slow-scan spectra of *B. subtilis* obtained with CalSpec (solid curve) and conventional transmission IR spectroscopy (dashed curve) for the region of 2000–1200 cm^{-1}. As can be seen from the data in Fig. 3, there is an excellent correlation between the spectra of *B. subtilis* obtained with CalSpec and conventional transmission IR spectroscopy. The subtle differences between the CalSpec and the IR spectra in Fig. 3 are not unexpected because different contributions of certain vibration states into photothermal and absorbance IR spectra are commonly observed.[47] We therefore assume that detection and differentiation of micro-organisms with CalSpec would rely on the libraries of photothermal rather than absorbance IR spectra.

Figure 4 shows a comparison of the CalSpec spectra obtained for the two different micro-organisms we studied, *B. subtilis* and *B. cereus*. On the basis of the measured shifts in the microcantilever resonance frequency, the amount of adsorbates deposited on the cantilevers to obtain these spectra is equivalent to approximately 2000 spores. The two spectra shown in Fig. 3 exhibit a substantial difference in the intensity of the bands at 1700–1400 cm^{-1}, indicating the differences in the protein and carbohydrate content of the two types of spore. There is also a less pronounced difference in the region from 1200 to 900 cm^{-1}. The degree of these differences agrees qualitatively with previous observations.[29,31,32,35,36,46]

The improvement in the sensitivity offered by the CalSpec method was estimated on the basis of the assumption of the smallest biological sample that can be examined by use of conventional IR and FTIR

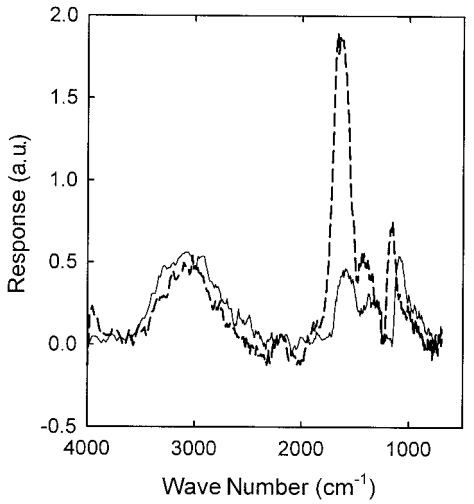

Fig. 4. Comparison of the CalSpec spectra of *B. subtilis* (solid curve) and *B. cereus* (dashed curve) reveals a clear difference between the two types of *Bacillus* spore. The spectrum *B. cereus* was obtained by use of a gold-coated silicon nitride microcantilever with 1.18 ng of *B. cereus* vapor that was sublimed in air from a stainless-steel boat at 210 °C. The spectrum of *B. subtilis* was obtained as described in Fig. 2.

spectroscopy, including use of microscopic attachments. According to recent reports, the smallest samples of biofilms that could be examined were 50 $\mu m \times 50\ \mu m \times 5\ \mu m$. This volume of organic samples corresponds to a mass of approximately 10–15 ng. Therefore the CalSpec method offers at least a full order of magnitude in sensitivity as compared with conventional IR and FTIR spectroscopic instruments.

Unlike many other types of sensor that rely on partitioning between the medium and the active sensor area as a principal mechanism of selectivity, the proposed method is capable of distinguishing various analytes and between targeted analytes and background species that are due to the differences in their vibrational signatures. Under normal atmospheric conditions, spontaneous adsorption of environmental particulate or partitioning of molecular species from air did not result in any measurable CalSpec responses. Therefore, instead of spontaneous adsorption and partitioning of analytes on sensor surfaces, forced analyte delivery that involves analyte preconcentration and condensation will be used in environmental applications of the CalSpec method. Design of the analyte delivery system optimized for use in conjunction with CalSpec micromechanical detectors is a subject of our ongoing research.

5. Conclusion

The present study demonstrates the applicability of the innovative photothermal spectroscopic technique, CalSpec, for sensitive detection and identification of pathogens. The CalSpec approach offers an inexpensive alternative to FTIR methods that have already been recognized as a powerful means in discriminating different types of micro-organism. As applied to biological warfare detection, the most important features of the proposed method is its high sensitivity and a simple design that does not require expensive components. Using commercially available gold-coated silicon nitride microcantilevers as thermal IR detectors, we were able to acquire vibrational signatures of spores sampled in nanogram quantities, which correspond to approximately 1000 individual spores. This is well below of the lethal dose of *B. anthracis* spores for humans, which, according to tests on monkeys, is approximately 10,000 spores. The implemented method therefore provides adequate sensitivity for creating an anthrax contamination warning system. Further improvements in limits of detection are anticipated from use of optimized IR-sensitive microcantilever detectors.

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