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Detection and differentiation of biological species using microcalorimetric spectroscopy

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Abstract

We report on the application of infrared (IR) microcalorimetric spectroscopy (μ-CalSpec) to the identification and detection of trace amounts of biological species. Our approach combines principles of photothermal IR spectroscopy with ultrasensitive microcantilever (MC) thermal detectors. We have obtained photothermal IR spectra for DNA and RNA bases and for *Bacillus Cereus* (an anthrax simulant) in the wavelength range of 2.5–14.5 μm (4000–690 cm^{-1}). The measurements are accomplished by absorbing biological materials directly on a MC thermal detector. The main advantage of the developed μ-CalSpec is its unprecedented sensitivity as compared to any of the previously explored IR techniques, including FTIR and photothermal FTIR methods. Our results demonstrate that $<10^{-9}$ g of a biological sample is sufficient to obtain its characteristic μ-CalSpec spectrum that contains information-rich chemical (vibrational) signatures. This opens up a new opportunity to create inexpensive high-throughput analytical systems for biochemical detection.
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1. Introduction

Recent events have raised the fear of facing deadly pathogens and biological toxins 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 substances of biological origin, including pathogenic microorganisms and DNA or RNA fragments. In recent years several alternative detection strategies have been pursued to develop warning systems that could provide sensitive and selective identification of microorganisms [1–5]. The most common identification systems [1] are based on biomolecular recognition technologies [4,5], such as enzymolinked immunosorbent assay (ELISA) and polymerize chain reaction (PCR). These methods can be implemented in various formats, ranging from more traditional "labs on wheels" [1] to portable systems carried by a small remotely piloted airplane [6]. An alternative to molecular recognition approaches is a combination of

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various extraction-separation techniques and mass spectroscopy (MS) [7], which offers unprecedented sensitivity at a cost of increased size and expensive operation. The operational costs of MS based analysis as well as immunochemical and DNA-selective methods could be prohibitively high in the case of continuous monitoring. The question arises as to whether highly specific and sensitive detection of airborne contaminants of biological origin can be carried out without the high operational cost and/or expensive consumables.

With regard to this question, our attention has been drawn to dispersive IR and FTIR spectroscopic techniques which were traditionally used for identification of chemical entities in samples of unknown composition. In fact, studies of biological microorganisms using IR spectroscopy have been attempted since the 1950s, however remained relatively scarce until the 1990s [8–10]. The main reasons for this were insufficient sensitivity of conventional IR instruments and the lack of computerized spectra analysis algorithms. Availability of sensitive FTIR instruments [11,12] and recent advances in development of computerized chemometrics approaches [13–15] facilitated much more successful application of vibrational spectroscopy to identification and differentiation of intact microorganisms [16]. However, the amount of biological material required for even the most sensitive microspectroscopic FTIR analysis [17,18] still substantially exceeds infective doses of many pathogens. In order for vibrational spectroscopy to become a viable approach to biological threat warning systems, acquisition of vibrational signatures of as few as 1–1000 microorganisms is needed. This translates into the required mass sensitivity of a few picograms to a few nanograms. Vibrational spectroscopy with a similar mass sensitivity would also become competitive with DNA and immuno-selective sensing methods.

In order to address the issue of sensitivity, the present work explores an innovative approach to vibrational spectroscopy that is based on a photothermal mode of transduction and highly sensitive MC thermal detectors [19–22]. In our previous studies, we used this novel microcalorimetric spectroscopy (μ-CalSpec) for chemical analysis and identification of some organic absorbates based on their photothermal IR spectra [20]. Here we demonstrate the potential of μ-CalSpec for measuring vibrational signatures of DNA and RNA bases, and bacterial spores in the wavelength range of 2.5–14.5 μm. Less than 10^{-9} g of biomolecules adsorbed on a MC thermal detector was sufficient to obtain information-rich photothermal spectra. We thus extend the use of μ-CalSpec to highly sensitive and selective differentiation of biologically relevant samples. In the development of sensors for airborne biological contaminants, *Bacillus Cereus* spores have been commonly used as simulants of highly viral spores of *Bacillus Anthracis,* which is one of the most infamous pathogens [23]. *B. Cereus* 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. A dormant *B. Cereus* spore is approximately 1 μm in diameter and composed of the exosporium, outer and inner coats, a thick cortex, a germ cell wall, and the nucleus [24,25]. The spore coats are composed primarily of proteins which have been extensively characterized [24].

1.1. Microcalorimetric spectroscopy-based detection

The approach to detection of DNA/RNA bases and biological microorganisms used in the present work is based on CalSpec [20] developed in our laboratory previously and successfully used for detection and identification of medium molecular weight environmental contaminants in our prior work. CalSpec involves two main steps. In the first step, the sample is allowed to interact with the surface of a microcantilever thermal detector. In the second step, a photothermal spectrum is obtained for the molecules adsorbed on the detector surface, by scanning a broadband wavelength region with the aid of a monochromator [20]. The temperature change in the active detector region is proportional to the number of photons absorbed which, in turn, is proportional to the amount of sample species adsorbed on the detector surface. As a rule, photothermal spectra are similar to conventional IR infrared spectra and contain a superposition of characteristic

vibrational signatures of the target species chemical which can be used to uniquely identify the adsorbed species.

1.2. Microcantilever thermal detector

The selection of the thermal detector plays an important role in the ultimate sensitivity of the μ-CalSpec technique. Presently, there are a number of families of commercially available IR detectors, including thermopiles, bolometers, pyroelectrics in addition to various cooled solid state detectors [26]. In this work, we employed inexpensive bimaterial microcantilever thermal detectors which operate based on the bending of the microcantilever as a result of absorption of IR energy [20,21,27,28]. When a microcantilever is exposed to IR radiation, its temperature rises due to absorption of this energy. Bimaterial microcantilevers are constructed from materials exhibiting dissimilar thermal expansion properties; for example, microcantilevers made from silicon nitride (or silicon) coated with a thin film of gold or platinum can be used. The bimetallic effect causes the microcantilever to bend in response to this temperature variation. Previous work [27] has shown that microcantilever bending can be detected with extremely high sensitivity. Earlier studies have reported [22] that a microcantilever thermal detector had an observed sensitivity of 100 pW corresponding to an energy of 150 fJ. However, using an optimally designed microcantilever, the sensitivity may be improved even further. An estimate of the minimum detectable power level was on the order of 10 pW, corresponding to a detectable energy of 10 fJ and a temperature sensitivity 10^{-5} K. For chemical detection based on microcalorimetric spectroscopy, microcantilevers can be coated with an appropriate layer providing the bimetallic effect, and a moderate chemical selectivity. Theoretical evaluation of cantilever deflections due to the absorbed energy has been reported previously [29–31].

Our previous studies have shown that there is a good correlation between CalSpec and IR spectra for polymers as well as medium molecular weight organic compounds, such as chloroaromatic and nitroaromatic [20] species. In general, it is a well established fact that both conventional and photothermal IR spectra can be used for "chemical fingerprinting" of analytes due to the predominant contribution of vibrational modes of chemical bonds into optical properties of materials in the middle infrared. Vibrational signatures of nucleic acids are known to be sensitive to their chemical compositions and structure [32] which, in turn, can be related to thermodynamic and kinetic properties.

2. Experimental

2.1. Biological material

B. Cereus (ATCC-7064) was purchased from American Type Culture Collection (10801 University Blvd., Manassas, VA 20110–2209, USA) 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 $CaCl_2$, 0.25 ml of 0.01 M $FeCl_3$, 0.5 ml of 0.01 M $MgSO_4$, and 0.5 ml of 50% glucose per liter. 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 4 days. Spores were harvested by foam flotation and the richness of the spores were confirmed by optical microscopy. The harvested spores were washed with sterile deionized water several times and lyophilized for the further use in our experiments.

2.2. CalSpec measurements

Commercially available gold coated silicon nitride cantilevers obtained from thermomicroscopes, Inc. were used as thermal detectors for the CalSpec measurements without any modification. The A-shaped cantilevers selected for our experiments were 0.6 μm thick, 250 μm long and had 60 nm thick gold coatings. Typical thermal responsivities of these cantilevers were on the order of hundreds nanometers per Kelvin. The reference photothermal spectra of the cantilevers as well as their mechanical resonances were measured prior

to the deposition of the sample adsorbates. In order to obtain ng quantities of adsorbates on microcantilevers, we carried out sublimation of pure DNA/RNA bases and spores of *Bacillus* family from resistively heated 0.1 mm thick stainless steel boats in air. The cantilever resonance frequency shifts were analyzed in order to estimate a total mass of the analyte deposited. The temperature of the boat with a powdered biological material was in the range 180–230°C during sublimation of nucleotides. Sublimation of *B. Cereus* spores was carried out by heating the boat with approximately 5 mg of the powdered spores to 210°C. Sublimation of *B. Cereus* 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 and the photothermal spectra were acquired in the 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 [20]. All measurements were conducted at the ambient temperature and pressure. The samples were deposited on the silicon nitride side, which was also the side facing the IR radiation from the monochromator (Foxboro Miran-80 IR). The experimental setup is depicted schematically in Fig. 1. An Al aspherical mirror with f/#1 was used to focus IR radiation onto the cantilever. The readout of microcantilever deflections was provided 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. The details of these types of measurements were described previously [20,29].

2.3. Reference IR measurements

A Buck Scientific model 500 single beam IR spectrometer 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. A small portion of *B. Cereus* 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 based on the mass of the spores added and the cross-sectional area of the pellet.

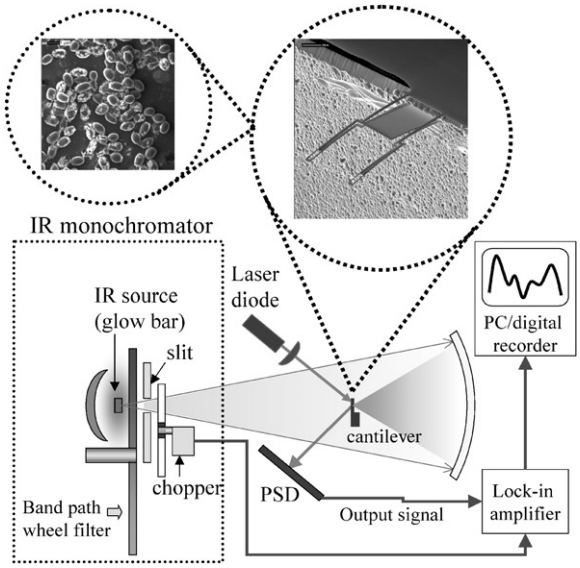

Fig. 1. Schematic of the experimental setup. Photons of different wavelength irradiate the thermal detector and the adsorbed species absorb photons producing a photothermal response.

3. Results and discussion

We conducted CalSpec measurements of a series of biological samples deposited on the cantilever transducers using thermal evaporation. In the case of nucleic acid bases and spores that are represented by rather polar chemical entities, this method produced very stable and well-adhering amorphous coatings on the cantilever. The cantilevers with thus adsorbed analytes retained their photothermal signatures (both the magnitude and the position of the peaks) over periods of at least several months. The coatings with total mass in the range of 0.5–1.5 ng were used in our experiments. These amounts correspond to approximately 1000 individual spores of *B. Cereus*.

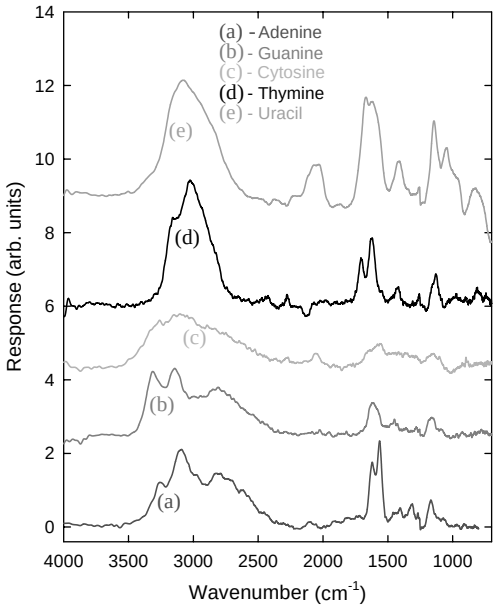

Fig. 2. CalSpec spectra of DNA bases. The photothermal spectra of adenine, guanine cytosine, thymine and uracil were obtained using gold-coated silicon nitride microcantilevers.

Fig. 2 shows representative photothermal responses of the nucleic acid bases adenine, guanine, cytocine, thymine and uracil as a function of wave number. As can be seen in Fig. 2, the photothermal spectra of the DNA/RNA bases contain a number of overlapping bands in the region 1100–1600 cm^{-1}, which are attributed to in-plane vibrations [32]. The carbonyl group stretching vibrations of thymine, guanine, and cytosine are expected to give vibrational bands in the region between 1600 and 1750 cm^{-1} [32]. The data shown in Fig. 2 clearly indicate that differentiation of the different DNA/RNA bases can be based on their photothermal signatures. However, an important question arises as to whether it is possible to discriminate samples of genomic DNA with different base sequences based on their vibrational spectra.

It has been recognized that IR spectra of intact microorganisms contain major contribution from DNA-RNA bases in the region from 1250 to 1200 cm^{-1} [14]. In the case of purified oligo and polynucleic acids a number of intense, partially overlapped Raman bands were typically observed in the range of 1600–600 cm^{-1} [32]. Although exact assignment of each of these bands is rather difficult, different weights of the different bases can clearly be concluded from comparative analysis of various DNA samples [32]. Similar studies were conducted using FTIR spectroscopy. For instance, purified samples of DNA extracted from normal and malignant fibroblast cells were shown to exhibit very distinct FTIR spectra in the range of 1600–1000 cm^{-1} [33]. It was also established that certain features in the FTIR spectra of complete cells provide a measure of cellular RNA/DNA ratio [33]. More importantly, comparative analysis of vibrational spectra of the DNA fragments with different base sequences revealed sequence-dependent spectral features [32]. The observed deviation of the measured DNA spectra from the weighted contributions of the DNA components may provide a new approach to sequence-specific DNA analysis. Explanation of the sequence-dependent intensity distribution in vibrational spectra of various DNAs can be based on the models of backbone-base pair interactions that involve the deoxyribose-phosphate backbone geometry and base-pair step parameters.

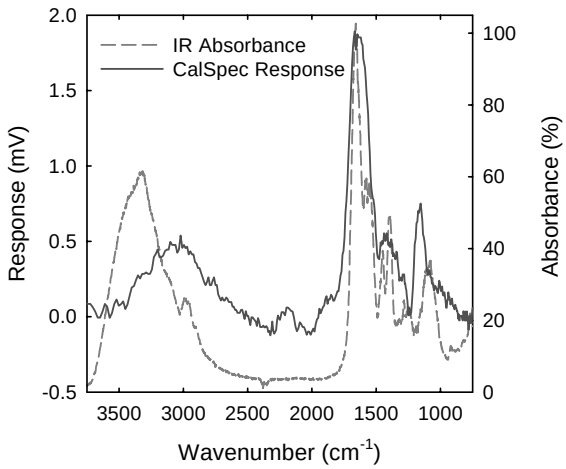

Fig. 3. CalSpec and conventional absorption mode IR spectra of *B. Cereus*. The photothermal spectrum *B. Cereus* was obtained using a gold coated silicon nitride microcantilever with 1.18 ng of *B. Cereus* that was sublimed onto the cantilever from a stainless steel boat at 210°C in air.

Fig. 3 shows the photothermal and transmission IR spectra obtained for *B. Cereus*. They have common major bands located around 3000 cm^{-1}, and within 1500–1000 cm^{-1}. In the case of regular IR absorption spectra, a large part of the intense broad band around at 3000–3500 cm^{-1} , especially closer to 3500 cm^{-1}, is attributable to the stretching mode of hydrogen-bonded OH groups of water molecules, for instance, in protein hydration shells. We have found that the majority of the studied biological samples exhibit this feature. As a result, vibrational bands attributable to organic molecules are often seen as relatively small peaks or shoulders on the background of this strong band [8,14,16]. For instance, only a small shoulder and a peak close to 3000 cm^{-1} in the IR spectrum of *B. Cereus* shown in Fig. 3 can be attributed to organic components of the sample. By contrast to regular IR absorption spectra, the band attributable to OH stretching mode is always strongly suppressed in photothermal responses obtained using CalSpec. The exact mechanism of this phenomenon is yet to be elucidated.

Naumann et al. [8,16] 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 instance, 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 RNA/DNA components. In addition, the region below 900 cm^{-1} has recognized significance in discriminating microorganisms at the strain level [14]. Because microorganisms contain a great 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. While this can be accomplished using computerized algorithms as described previously, it is important to emphasize that, except for the low energy region below 800 cm^{-1}, the obtained CalSpec spectrum of *B. Cereus* contains sufficient amount of information-rich signatures and generally is consistent with the previously reported FTIR data [14].

4. Conclusions

The present studies demonstrate the applicability of the innovative photothermal spectroscopic technique, CalSpec, to sensitive detection and identification of biological species. The CalSpec approach offers an inexpensive alternative to FTIR methods which have already been recognized as a powerful means in discriminating different types of microorganisms. Using commercially available gold-coated silicon nitride microcantilevers as thermal IR detectors, we were able to acquire vibrational signatures of subnanogram quantities of biological samples for DNA/RNA bases and *B. Cereus*. The amounts of *B. Cereus* required for CalSpec measurements correspond to less than 1000 individual spores. The limits of detection can be further improved by using optimized IR sensitive microcantilever detectors, more efficient IR sources, and better sampling techniques.

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