Chemical Physics Letters 592 (2014) 302–306

Contents lists available at ScienceDirect

Chemical Physics Letters

journal homepage: www.elsevier.com/locate/cplett

Bacillus spores and their relevant chemicals studied by terahertz time domain spectroscopy

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ARTICLE INFO

Article history:
Received 12 November 2013
In final form 20 December 2013
Available online 28 December 2013

ABSTRACT

Terahertz time domain spectroscopy has been used to investigate 0.2–2.2 THz transmission responses of *Bacillus* spores and their related chemical components. Whilst no THz signatures could be clearly associated with either sporulated cells or their chief chemical components, differing degrees of signal attenuation and frequency-dependent light scattering were observed depending on spore composition and culture media. The observed monotonic increase in absorption by spores over this THz spectral domain is mainly from Mie scattering and also from remnant water bound to the spores.

1. Introduction

The detection of *Bacillus* spores is important in defence-science and heath technologies. Spores are dormant, tough, and temporarily non-productive structures which are formed by vegetative cells in response to adverse changes in the environment and produced by certain bacteria from the firmicute phylum. Sporing bacteria are the cause of a number of serious diseases in humans, such as Anthrax, an acute disease caused by *Bacillus anthracis*. An extensive investigation of bacterial cells of different species and strains has been conducted by three main spectroscopies and their derivatives: Raman [1,2] and Fourier transform infrared (FTIR) spectroscopy [3–11] and mass spectrometry [12–14], each of which has its own technical strengths and weaknesses.

The terahertz (THz) spectral sub-domain of the electromagnetic spectrum spans 0.1–10 THz (~3.3–333 cm^{-1}), corresponding to the long-wavelength end of the far-infrared, has successfully demonstrated capability in detecting explosives [15] and drugs [16] and is steadily gaining a diagnostic footing in biological and chemical sciences [17,18]. There are, however, few publications systematically investigating the interaction of THz energies with microbial cellular components or their related spores. In contrast to the direct power detection method of FTIR, THz time domain spectroscopy (TDS) coherently detects the complex electric field amplitude, allowing direct estimation of real and imaginary parts of the refractive index. The measurement philosophy [19] leads to a superior signal-to-noise performance compared with FTIR methods. The purpose of this study is to systematically investigate the THz spectra of two *Bacillus* spores and their related chemical cellular components. The effects of culture media on the spectra of the spores are also discussed.

2. Materials and methods

2.1. Measurement system

THz TDS [20] was applied to investigate the spectra of spores and their related chemical components. The samples were placed at the focus (spot-radius <1.25 mm at 200 GHz) of a THz spectroscopy beam and spectra were recorded in transmission against the spectra without any samples in the focus. The TDS measurements were performed under a controlled temperature of 24.0 ± 0.1 °C and with a nitrogen purge that reduced the relative humidity to ⩽0.3%.

2.2. Sample preparation

Bacillus cereus (*BC*) and *Bacillus subtilis* (*BS*) identified by matrix-assisted, laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF), were used to produce the spores. The bacteria were cultured overnight in a nutrient broth medium. A sterile loop was dipped into the medium before streaking on a plate. After overnight culture, one pure colony was placed into 20 ml of a nutrient broth medium (NB) for further overnight culture. The bacterial broth was then inoculated in a 200 ml nutrient broth to be cultured at 37 °C and spun at 230 rpm until 95% of the vegetative cells were observed under a confocal microscope to form spores. The spores were then washed with sterile water and collected using a centrifuge spun at 4000 rpm for 20 min. The collected spores were further washed three times by sterile water to remove

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http://dx.doi.org/10.1016/j.cplett.2013.12.061

all cell debris. The spore-water solution was stored at 4 °C and washed again before drying using a freeze-dryer (Virtis) to form the powder. The spore powders were mixed evenly in a 1:1 (20 mg:20 mg) mass ratio with polyethylene (PE), a compound which is near-transparent at THz frequencies, and then compressed by a vacuum-presser into pellets having a 13 mm diameter and thickness <0.5 mm (variable for different samples), which were then stored in a desiccator to prevent humidity influencing the samples. In order to investigate the influence of culture media on the THz properties of the spores, the spores were also cultured in two other media: Tryptone Soya Broth (TSB) and Leighton Doi broth (LDOI) under exactly the same procedures as described above. NB is a standard medium which serves as a reference for the culture and accumulation of microorganisms, and it consists mainly of beef-extract, peptone and water. TSB is another general medium for culturing microorganisms whose nutrient content is casein, peptone and soya peptone. LDOI broth is a sporulation medium which is based on NB with more metallic ions added.

The chief chemical components in the cell wall of spores, such as dipicolinic acid (pyridine-2,6-dicarboxylic acid, DPA) and its calcium chelates (Ca-DPA), have been intensively investigated by Raman spectroscopy [21–26], mass spectrometry [27] and FTIR spectroscopy [28–30], in an effort to seek for a potential biomarker in spore detection. Likewise, this THz spectral domain study sought to identify spores through the spectra of unique chemicals present in the cell wall of bacterial spores. In addition to DPA, Diaminopimelic acid (DAP) and D-glutamic acid (D-Glu) are also selected for analysis. DAP, a sub-unit of the peptide portion of peptidoglycan, is also a characteristic component of the cell wall of the spore, and is a component allied to bacteria and does not occur in non-bacterial cells. Poly-D-glutamic acid is one of the major virulence factors of *B. anthracis*, which causes a highly lethal infectious disease. D-Glutamic acid (D-Glu), as a sub-unit of Poly-D-glutamic, was also therefore investigated. In order to get comparison spectra, DAP, DPA and D-glutamic acid powders were prepared by two separate methods: the first was to compress the mass ratio 1:1 for the chemicals and PE (total 40 mg) into thin (approx. 0.40 mm thick) and 13 mm diameter pellets; the second was to press PE and the chemical mixtures based on their real proportion according to the dry-weight of the spores. Details are listed in Table 1. All the chemicals are from Sigma–Aldrich, and are used without further purification.

Table 1
Proportion of PE and the chemicals.

Chemical	Chemical (mg)	PE (mg)	Ratio (%)	Thickness (mm)	Refs.
DAP	0.8	39.20	2	0.417	[31]
DPA	4.00	36.00	10	0.348	[32]
D-Glu	0.15	39.85	0.3	0.417	[33]

3. Results and discussion

The absorption spectra of 1:1 mass-ratio chemical mixtures with PE and their related proportional mixtures with PE are plotted in Figure 1. The effective spectra are cut-off around 2.2 THz, which vary with the different chemicals. The spectra above 2.2 THz give a poor signal to noise ratio due to the limited system dynamic range from both high loss properties of the test samples and a falling photoconductive efficiency of the GaAs THz emitter. For 1:1 mass-ratio chemical DAP and DPA mixtures with PE, absorptions all increase with increasing frequency. The absorption features of D-glu at 1.21 and 2.01 THz are clearly observed, which have been well explained by weak skeleton torsion and torsion of the whole structure from DFT calculation [34]. The ripples at 1.12 and 1.68 THz are observed through all the measured spectra due to remnant THz water absorption, which can come from the measurement environment despite the humidity being reduced to 0.3% by nitrogen purge. Recent experimentally and theoretically work from Johnson et al. [29,30] clarifies that a key spore component is not DPA, as is often reported, but rather the trihydrate of the calcium salt of the DP anion. $CaDP{\cdot}3H_2O$ exhibits reproducible IR spectra specifically a unique 'quartet' of peaks at 22.98, 21.75, 21.03 and 19.77 THz due to Ca–O–H bends, H_2O–Ca–O torsion and O–C–O bends. The same features are seen too in different species of *Bacillus* endospores. Consequently it is reasonable to assume that additional torsion and libration modes exist or are shifted to lower THz bands as compared to those of the acid form of DPA. The work from

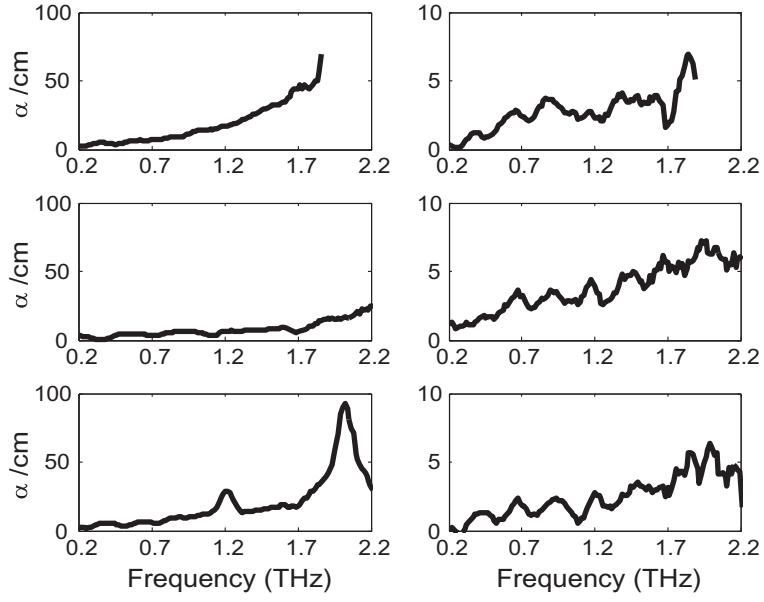

Figure 1. Absorbance of spore components. Left hand side plots are for 1:1 ratio of the chemicals with PE; right hand side plots present spectra of low percentages of chemicals mixed with PE. Spore component chemicals from top to bottom are DAP, DPA and D-Glu.