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Sub-THz Vibrational Spectroscopy of Bacterial Cells and Molecular Components

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Abstract In this work, sub-terahertz (THz) spectroscopy is applied to characterize lyophilized and *in vitro* cultured bacterial cells of non-pathogenic species of *Escherichia coli (E. coli*) and *Bacillus subtilis* (BG), spores of BG and DNA from *E. coli*. One of the goals of this research is to demonstrate that Fourier Transform (FT) spectroscopy in the frequency region of 10–25 cm^{-1} is sensitive enough to reveal characteristic spectral features from bio-cells and spores in different environment, to verify the differences between species, and to show the response of spores to vacuum and response of cultured cells to heat. The experimental technique was significantly improved for sensitivity and reliability. Observed spectra taken with a spectral resolution of 0.25 cm^{-1} using FT spectrometer with a detector operating at 1.7 K are rich in well resolved features having spectral widths of ~0.5–1 cm^{-1}. The reproducibility of experimental results was verified and confirmed. Measured spectra from *E. coli* DNA and from the entire cell have many similarities, thus demonstrating that the cellular components might contribute to the vibrational spectrumof the cell. The results of this work confirm that observed spectroscopic features are caused by fundamental physical mechanism of interaction between THz radiation and biological macro-molecules. Particularly, the analysis of results indicates that the spectroscopic signatures of microorganisms originate from the combination of low frequency vibrational modes or group of modes at close frequencies (vibrational bands) within molecular components of bacterial cells/spores, with the significant contribution from the DNA. The significance of this study is justified by necessity for a fast and effective, label free and reagent free optical technology to protect against environmental and other biological threats, as well as for general medical research. The obtained results show that THz vibrational spectroscopy promises to add quantitative genetic information to the characteristic signatures of biological objects, increasing the detection accuracy and selectivity when appropriate spectral resolution is used.

Keywords FTIR, *E.Coli*, *B. Subtilis*, DNA, Signature, Reliability, Molecular Dynamics

1. Introduction

Terahertz (THz) vibrational spectroscopy is an emerging field to examine biomolecular structure and dynamics, and to characterize absorption properties of biologicalmaterials in this frequency region. THz radiation excites low-frequency internal molecular vibrations that involve intra/inter molecular domains connected by the weakest interactions: weak hydrogen bonds, van der Waals forces and/or non-bonded (hydrophobic) interactions[1, and references therein]. The very far infrared (IR) range of absorption reveals these low-energy vibrations, andtheoretical studies predicted multiple resonances in absorption (or transmission) spectra of biological molecules in the THz frequency (0.1–10 THz) or millimeter- wavelength (3–300cm − 1) range[2]. Organic solid systems and relatively small biomolecules like protein fragments have beensuccessfully characterized in this range to demonstrate sharp spectral features determined by their individual symmetries and structures[3-6].

In this study, sub-THz spectroscopy is applied to characterize lyophilised and in vitro cultured bacterial cells from two non-pathogenic species of Escherichia coli (E. coli) and B. subtilis (BG), spores of BG , and DNA from *E.coli* cells. *E. coli* and BG are diverse bacterial organisms that are most commonly used as model organisms for laboratory studies. Bacteria can readily accept, replicate, and express foreign DNA and this makes them powerful agents for studying genes of other organisms in isolation[7]. However, both of these bacterial species have strains, which can be pathogenic to humans and animals. There are tens of thousands *E. coli* contamination cases every year in the United States. Bacteria can adapt to

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extreme environments and bacterial pathogens share a common trait that is their ability to live long-term inside the host's cells[8, 9]. *B. subtilis* can form endospores in response to nutrient deprivation and to other environmental stresses. Spore can survive during adverse conditions, preserving the cell's genetic material. Endospores are resistant to heat (>100℃), radiation, many chemicals (i.e. acids, bases, alcohol, chloroform), and desiccation.

There has been an increasing need for fast, reliable, non-invasive methods for rapid recognition and characterization of bacterial cells and the critical destructive changes inside the living cell. It has already been reported that using Fourier Transform Spectroscopy identification of bacteria and other microorganisms to the level of species is possible in the near- and mid-IR[10, 11]. At the same time many benefits may result from experimental observations of spectroscopic features in the sub-THz region that was not yet widely explored. The most important advantages include low absorption by water vapors and liquid water, at least two orders of magnitude less than in the far IR. Thus, water does not mask absorption by biological materials, and characterization of molecules in solution is possible. Spectroscopic sensors in the sub-range of 10-25 cm^{-1} do not require evacuation or purging with dry nitrogen. THz spectroscopy is an optical method, it is nondestructive for living organisms and does not produce health risks in direct scanning of people. THz radiation penetrates non-metallic materials, such as skin and clothing, allowing detection of hidden bio-agents.

Bacteria are very complex biological objects. Because of their small size and relatively low absorption coefficient, the THz radiation propagates through an entire object, allowing the genetic material and proteins all contribute to the THz signature of bacteria or spores.

THz spectroscopy has already found applications in characterizing biological macromolecules including molecular components of biological cells/spores. Our previous results on characterization of bio-molecules using Fourier transform (FT) spectroscopy detected multiple resonances in transmission/absorption spectra of bio-molecules in the sub-THz frequency region[12-14]. Simulations can help us to interpret the experimental data by assigning spectral features to specific molecular motions, and simultaneously with experimental characterization computational modeling techniques have been developed using the energy minimization, normal mode analysis and molecular dynamics (MD) approaches to predict low frequency vibrational absorption spectra of short artificial DNA, and RNA[12, 15-18], large macromolecules of DNA[19, 20] and proteins[21, 22]. Direct comparison of experimental spectra with theoretical prediction for a short chain RNA fragment with known structure showed reasonably good correlation[12, 17] validating both experimental and theoretical results.

Some of cellular components of E. coli, DNA, transfer RNA[20], and protein thioredoxin[21, 22] were also characterized, and absorption spectra of relatively small macromolecules were simulated using MD. Vibrational frequencies from simulated spectra for components correlate rather well with the observed features. Although experimental difficulties have severely limited the direct identification of phonon modes in biological materials at submillimeter-wave frequencies inthe past, nevertheless, the spectral response of both, relatively small and macromolecules, revealed spectral features caused by interactions between radiation and the biological material. Thus, multiple resonances due tolow frequency vibrational modes within biologicalmacromolecules, components of bacterial organisms, areunambiguously demonstrated experimentally in the sub-THz frequency range in agreement with the theoretical prediction.

Several challenges however arise when trying to characterize an entire microorganism. In the past, transmission spectra of bio-particles (cells and spores) have been investigated in the sub-THz range using photomixing[23-25] and FTIR technologies[12, 26-31] with a relatively good spectral resolution, better than 0.25 cm-1 Spectra reveal detectable resonant features in the range below 25 cm-1. To enhance the sensitivity, we used a Fourier spectrometer model FTS-66v with a Si-bolometer operating at 1.7 K. Nevertheless, the intensity of spectral features in most cases was only 1-2 % for solid samples, and the reproducibility was not good. There was always a probability to mix real spectral features with artifacts of measurements.

In addition, there are still many fundamental disagreements and challenges in the field. The researches, who use pulsed time-domain spectroscopy, usually observe only smooth broad absorption bands in spectra of biological macromolecules, and there is still a wide spread skepticism caused by large density of overlapping states contributing to absorption bands that might obscure vibrational resonances and yield essentially structureless spectra. Although this last statement can be argued since vibrational bands in spectra of macromolecules are observed and very well studied in the far IR region at even higher density of states, nevertheless low intensities of spectral features and their variability still support the skepticism.

The existing controversies in relatively young sub-THz spectroscopy field are caused in most cases by a poor spectral resolution and sample preparation techniques that are not adequate to the problem. In particular, the samples used with time-domain spectroscopy are often prepared in the form of thick pellets from the mixture of biomaterials with polyethylene powder that cause multiple reflection effects on pellet surfaces. These geometrical optic effects mask characteristic spectral features and prevent application of good spectral resolution required for resolving relatively narrow lines.

In addition, there are challenges in estimates of decay time scales, an important problem concerning THz oscillations in biological molecules and species. It is clear that the decay (relaxation) time is the factor limiting the

spectral width and the intensity of vibrational modes, the required spectral resolution, and eventually the discriminative capability of sub-THz spectroscopy. At the same time, the entire mechanism that determines intra-molecular relaxation dynamics is still not completely understood. There is a number of related studies, and the relaxation time scale between 1 ps and tenths of ns is discussed in recent works[32,33] (see section 4). These uncertainties have to be eliminated to justify the choice of spectral resolution in experiments as well as for calculating absorption spectra from MD simulation. In our simulations, we used till now the relaxation time estimated from the observed width of spectral lines.

In this work, the experimental THz spectroscopy technique was significantly improved for sensitivity and reliability thus resulting in more reliable signatures from macromolecules and bacterial cells/spores. Earlier it was only possible in most cases to receive intensities of vibrational modes in transmission not higher than 1-2% when solid samples characterized. The correct choice of substrate type, material concentration in solution or suspension and material alignment in thin layer deposition procedure permitted us to significantly enlarge intensities of modes and reproducibility of frequencies of resonance features when using Bruker FT66v. Transmission /absorption spectra observed in this study, taken with a spectral resolution of 0.25 cm-1 using FT spectrometer with a detector operating at 1.7 K are rich in well resolved features having spectral widths of ~0.5–1 cm-1. The reproducibility of experimental results was verified and confirmed. Similar experimental technique was successfully applied in the last several years to enhance spectroscopic features from not only natural macromolecules and bacterial cells/spores[31], but from artificial molecules as well[18, 34-35], thus resulting in more reliable signatures.

One of the goals of this research is to demonstrate that FT spectroscopy in the range of 10–25 cm-1 is sensitive enough to reveal characteristic spectral features from bio-cells and spores and their molecular components, to verify the differences between species, and to show the response of spores to vacuum and response of cultured cells to heat. Analysis of the sub-THz absorption spectra revealed some differences between species of B. subtilis and E. coli. High temperature treatment (~100℃) was used as a method of destruction of living cells and it was found to have a detectable effect on transmission/ absorption spectra by changing certain resonance frequencies. Effects that desiccation has on the transmission spectra of spores were studied as well. The FT IR spectroscopy appears to be sensitive enough to detect the differences between cultured (grown in the laboratory) and thermally treated cells. It has also been demonstrated that different substrates can modify the orientation of bacterial cells in the sample. The orientation effects are more pronounced in DNA spectra since molecules can be significant in length (compared to width) and therefore can be preferentially oriented during sample preparation. In addition, absorption spectra have been studied for quantitative characterization of samples with different amount of biomaterial.

The important motivation of our research is however to demonstrate the physics behind observed spectroscopic features. The method we used is based on comparison of experimental absorption spectra of relatively small macro-molecules, components of bacterial cells, with molecular dynamics (MD) simulation. The work revealed several important results. Simulated spectra for components correlate rather well with experimental data. These results confirm that observed spectroscopic features are caused by fundamental physical mechanism of interaction between THz radiation and biological macro -molecules. At last, the decisive confirmation is obtained in this work from direct comparison between E. coli DNA and E. coli cell absorption spectra that show many similarities. This is one of our the most important experimental results demonstrating that DNA indeed contributes significantly to the absorption spectra of an entire cell. Therefore, we conclude that the combination of sub-terahertz vibrational modes from molecular components of bacterial cells/spores contribute to the spectroscopic signatures of microorganisms. Particularly, the analysis of results indicates that the spectroscopic signatures ofmicroorganisms originate from the combination of low frequency vibrational modes or group of modes at close frequencies (vibrational bands) within molecular components of bacterial cells/spores, with the significant contribution from the DNA

The significance of this study is justified by necessity for a fast and effective, label free and reagent free optical, reliable, non-invasive methods for detection and identification of biological and chemical materials and organisms to protect against environmental and other biological threats, as well as for general medical research. The obtained results suggest that THz vibrational spectroscopy promises to add quantitative genetic information to the characteristic signatures of biological objects, increasing the detection accuracy and selectivity when appropriate spectral resolution is used.

The benefits from this study results are based on broad potential dual applications of vibrational spectroscopy of microorganisms together with generated data base, which include rapid detecting and identification of bio threat and environmental agents, food quality and water contaminations control, disease diagnostic and therapy. New THz molecular recognition signatures that are complimentary to those present in IR and UV pave the road for development of sensitive optical biosensors with increased discrimination of biological threats and in more varied environments. Simulated and experimental results from this project permit us to find the optimal sub-range with the maximum number of the most intense absorption lines to build spectroscopic sensors with the best detection and discriminative capability.

Although significant progress in experimental THz spectroscopy was demonstrated and reliable information

was received for transmission/absorption spectra from different species, the spectral resolution of Bruker spectrometer (0.25 cm–1) still does not provide good discriminative capability. Very recently, sub-THz characterization with better spectral resolution became possible, and we plan to continue this study and present the new, highly resolved spectroscopic results in the nearest future.

2. Materials and Methods

2.1. Materials

Lyophilized *B.subtilis* cells from Sigma were used in this work. BG spores from Dugway Proving Grounds (DPG)[26] were used in a form of a milled powder. *E.coli* DNA, Deoxyribonucleic acid, sodium salt, from *E. coli* strain B, type VII, was purchased from Sigma (USA) and re-hydrated to a concentration of ~60 mg/ml.

BL21 (DE3) strain of *E. coli* cells was purchased from Novagen (USA). Cultured cells were grown aerobically in the Luria Bertani (LB) broth at 37℃. Obtained cell culture was centrifuged and wet pellet was suspended in the LB broth to a concentration of ~50 mg/ml (recalculated from the dry material). *B. subtilis subsp. spizizenii* 6633^{TM} strain was purchased from ATCC (Manassas, VA). *B. subtilis* culture was grown aerobically at 30℃ for 48 hours. Obtained cell culture was centrifuged, and wet pellet was suspended in Nutrition Broth Medium (NBM) to a concentration ~ 120 mg/ml (recalculated from dry material). Half of the prepared *E. coli* and *B.subtlis* cultured cell suspensions was subjected to the heat treatment. During thermal treatment the cells were heated in the oven at 100℃ for 1 hour. Heat treatment was used as a method of cell destruction since high temperatures irreversibly damage the cells by altering the cell walls and membranes, damaging proteins, and nucleic acids.

2.2. Sample Preparation

BG spores and lyophilized BG cells were mixed with distilled water in 1:5 ratio and set aside for 24 hours. Half of the mixture with cells was heated at 100℃.

Liquid and air-dried samples for the spectra measurements were prepared from the three suspensions (spores, cells, and thermally treated cells) by pipetting 5–30 µl aliquots onto a surface of polytetrafluoroethylene (PTFE) disposable IR cards (International Crystal Laboratory) or other substrates. The PTFE cards are characterized by a high transmission level (~ 98%) and by an absence of strong peaks in the frequency range of 10–25 cm^{-1} (See Figure 1).

The transmission spectra of elongated particles are sensitive to the direction of the electric field of radiation. Bacterial cells are rod-shaped and some orientation effects are expected. Orientation effects are even stronger for DNA samples, since DNA strands are very long. Although sample material could not be completely aligned on the substrate, we applied the mixture only in one direction, to form a 5 mm x 7 mm oval in the center of the card to ensure a constant sample preparation protocol. Additional advantage of this procedure is that the thickness of the sample material inside the aperture is not uniform. This approach results in eliminating or significant reducing of interference effects, which might occur when thin samples are characterized. This very simple procedure for the sample preparation results in a rather high intensity of spectral features, much more pronounced compared to the spectroscopic features from materials in a very dilute solutions (0.1–0.01 mg/ml)[14].

Measurements on the liquid samples started about 5 minutes after preparation and continued until all excess water evaporated and the sample material dried. Dry samples were measured again after 1 hour and after 24 hours. Additional drying occurred while taking measurements under vacuum.

2.3. Measurements of transmission spectra

Transmission spectra were measured in the spectral range of 10 – 25 cm^{-1} using commercial FTIR infrared spectrometer, Bruker IFS66v equipped with a mercury lamp and with a Si-bolometer that is used for signal detection (operating at 1.7 K). The parameter settings for measurements were: aperture 12 mm, beam-splitters Mylar 75 and 125 µm, scanner velocity 80 KHz. A cooled filter with a cut-off frequency of 35 cm^{-1} was used inside the bolometer cryostat (when using beam-splitter 125um). Each data point is an average of 32 scans. The experimental technique is described in more details in earlier publications[13, 18]. Spectral resolution for all experiments was 0.25 cm^{-1}, which is the smallest allowable setting in the hardware. The choice of the spectral resolution is important. With poor resolution, resonance features have diminished intensity and spectra look flat. Spectral resolution of 0.25–0.3 cm^{-1} allows us to observe spectra rich in resonance features with the spectral lines width of 0.5–1 cm^{-1}. These spectral line widths are of the same order as those observed in the experiments with high resolution photomixing spectroscopy at higher frequencies (~1THz)[4-6]. Test measurements indicate about 80% polarization of the radiation with electric field vector oriented in the vertical direction in Bruker spectrometer. Some materials show sensitivity to the direction of the electric field of radiation. In our notation, angle of 90° corresponds to the vector of electric field of radiation parallel to the direction of material alignment. Rotating the sample by 90° clockwise corresponds to 0° orientation.

A prepared sample was mounted on the standard Bruker sample holder and placed inside a sample compartment in the focus of the beam. The optics inside Bruker (except sample compartment) was under vacuum of 10 mbar. Because there is almost no disturbance from water vapors in the 10 – 25 cm^{-1} range, all samples were measured

without evacuating the sample chamber. However, some measurements were also done with the sample chamber under vacuum. Measurements that were done without evacuating the sample chamber show the disturbance from water vapors at the absorption band around 18.6 cm^{-1}. Therefore, the region from 18 – 19 cm^{-1} is not included in the plots in most cases. Each sample was measured multiple times and the results for the dry samples were later averaged.

2.4. Modeling Absorption Spectra

Our MD simulations were described in previous works[12,16,17] with the most detail analysis of procedures, protocols and parameters in the latest publication[22]. The obtained phonon modes can be convoluted to derive the far IR absorption spectrum of a molecule.

$$\alpha(\nu) \sim \nu^2 \sum_k S_k \gamma_k / ((\nu^2 - \nu_k^2)^2 + \gamma_k^2 \nu^2). \quad (1)$$

Normal mode analysis enables calculations of eigenfrequencies k. Oscillator strengths Sk can be found from calculations of vibration trajectory along each normal mode. However, oscillator dissipations k cannot be found from harmonic approximation of the potential energy and, hence, remain to be determined from the comparison of theory and experiment. In the first approximation, however, the decay can be considered frequency independent in our relatively narrow sub-THz range. In this simplified form the absorption will depend on the frequency as:

$$\alpha(\nu) \sim \gamma \nu^2 \sum_k S_k / ((\nu^2 - \nu_k^2)^2 + \gamma^2 \nu^2). \quad (2)$$

At one particular vibrational frequency, the specific absorption coefficient is inversely proportional to the oscillator decay:

$$\alpha(\nu) \sim S_k / \gamma_k . \quad (3)$$

2.5. Transmission and Absorption Calculations

Where m is the mass of material in mg, T is transmission. The amount of material in the sample was calculated from

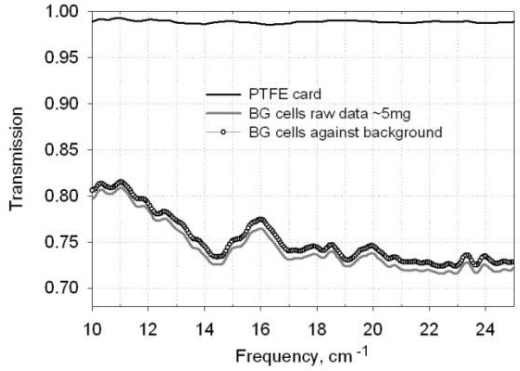

Figure 1. Transmission of PTFE substrate, BG cells raw data, and BG cells relative to the substrate background

All transmission spectra presented are recalculated to remove the contribution from the substrate. Fig. 1 shows the comparison between the raw data, substrate spectrum and a recalculated result for the sample material computed by taking ratio of the sample's raw data to the substrate spectrum. It can be seen that our substrate only slightly reduces the level of transmission and does not modify spectral features.

To characterize biomaterial rather than individual samples, transmission data was recalculated into spectra of specific absorption for 1 mg of material using the following formula:

$$\alpha = \frac{-2.3 \cdot \log(T)}{m}, \quad (4)$$

the amount of suspension deposited and the concentration of biomaterial in suspension. Beer's law was not used in this case since sample thickness was not uniform.

Some samples were rather thick (~20-50 m) and uniform in thickness, and the interference phenomena (multiple reflections on two surfaces) were observed in their transmission spectra. This was especially clear in the case of DNA samples that were characterized in the extended frequency range of 8-35 cm-1. To calculate absorption spectra for these samples we used an approach described in[36]. Results from two approaches were close in the more narrow frequency range below 25 cm-1 with significant differences in the overall slope of . vs. frequency at higher energies. Resulted resonance modes, however, were the same in both methods, since their widths are more narrow compare to the interference fringes.

2.6. Substrate Selection

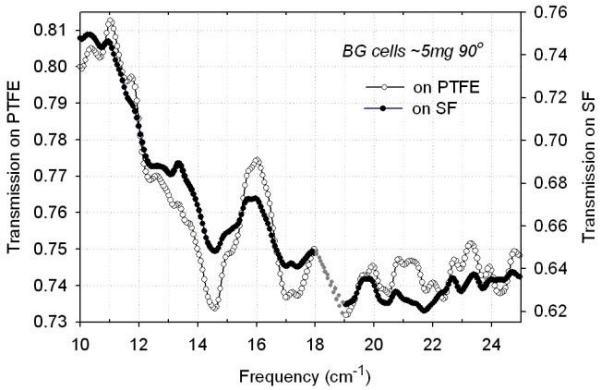

Figure 2. Transmission spectra of lyophilized BG cells on PTFE and SF substrates

The interface between biomaterial and the substrate is important. Particularly significant is the first monolayer of biomaterial, as it can affect the orientation of cells and molecules and contributes greatly to the vibrational peak intensities[13]. For example, cells deposited on a porous substrate can wedge themselves into the pores. Substrate with a wrinkled texture can force cells to line up along the faults and allow more material in the certain portions on its

surface. Finally substrate can create unwanted optical effects such as multiple reflections at the surfaces. Our substrates are very thin (~12 μm), thus minimizing these effects.

Fig. 2 shows the spectra of lyophilized BG cells (~5 mg) at the same orientation of 90 °on two different substrates: PTFE and polyvinylidene chloride polymer film, Saran film by S.C. Johnson (SF), 12 μm thick. The total absorption is higher in the case of SF. At the same time, the peak positions (transmission minima) are very close. At the other orientation (0 °) the intensity of peaks on SF is much smaller. We can conclude that using certain substrates over others can allow a clearer and easier interpretation of the data.

3. Results

3.1. Reproducibility and Reliability

The reproducibility of our experimental characteriza -tion is demonstrated in Fig. 3. The figure depicts the transmission curves of the same sample taken 24 hours apart. The difference in the absolute value of transmission reflects sample drying with time. We attribute higher transmission on the second day (solid circles) to lower moisture content. he initial measurements (open circles) were taken only 30 minutes after sample preparation, and as a result it is highly likely the sample was still not completely dry, whereas after 24 hours all excess water had a chance to escape, resulting in a higher transmission level. The positions of the transmission minima (vibrational frequencies), however, correlate very well between the two spectra. There is not a single feature that was not reproduced after 24 hours from initial measurements.

To obtain the repeatability, one sample containing approximately 5 mg of thermally treated BG cells was

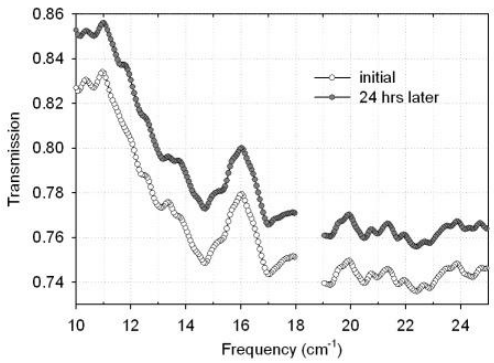

Figure 3. Reproducibility of spectra measured over 24 hrs period (thermally treated BG cells, 2.5 mg, 90 °)

measured 11 times. Standard deviation for the repeatability that includes the differences between mounting the sample onto the sample holder and collecting the background is between 0.1 and 0.3% at frequencies below 24 cm^{-1}. Seven samples containing 5.2–5.6 mg of lyophilized BG cells were measured to determine the reliability of our experiment. Small variations in the sample mass caused slightly different transmission levels between seven samples. Therefore before computing standard deviation, transmission spectra were converted into absorption per 1 mg of material (Fig. 4). Standard deviation was found from the normalized absorption spectra and it includes the differences between the individual samples, mounting samples on the holder and mismatch with the background. The standard deviation is shown in Fig. 5. The reliability is within 0.5 % over the entire range.

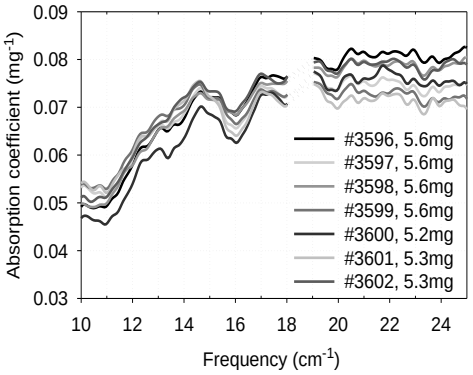

Figure 4. Absorption coefficient

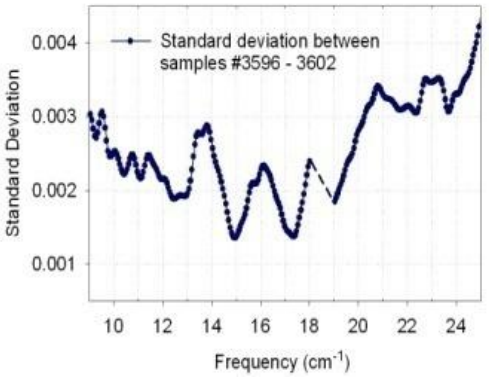

Figure 5. Standard deviation

3.2. Transmission Level Of a drying Sample

Transmission level can vary depending on the amount of the biomaterial, water content, and substrate. Fig. 6 depicts transmission level scaling with varying amount of water. In the figure, a partially wet sample containing ~15 μl of suspension was measured continuously while the sample was drying. Initially, with high water content in the sample, the transmission level is lowest (0.72–0.78). As excess water evaporates the transmission level rises (0.88–0.95). The positions of the transmission minima (absorption peaks) on the frequency scale are fairly reproducible; for example, minima at 14.5 cm-1 and at 17 cm-1 do not change during sample drying. In addition, from Fig. 6, it can be seen that the peak intensity is higher when the sample is still wet. As sample dries, the peak intensity decreases; this was demonstrated in our earlier work[13].

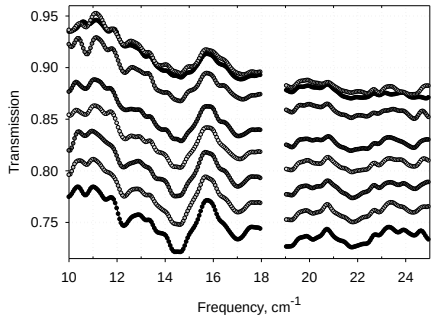

Figure 6. Transmission spectra of BG spores, wet through dry, orientation 90 º

3.3. Orientation

We did expect that BG cells should be more prone to the orientation effects because they are much longer than spores. This is indeed the case as shown in Fig. **7** that shows spectra of lyophilized BG cells with ~5 mg of material at 0° (open circles) and 90° (solid circles) Cell spectra are very similar to one another in both orientations, and yet at 90° we consistently observed a unique transmission minimum occurring at 13 cm^{-1} and two smaller peaks at 13.6 cm^{-1} and 21cm^{-1}.

Fig. 8 shows spectra of *E. coli* DNA for several measurement angles. Orientation effects can be clearly seen. Transmission minima change intensity and positions on the frequency scale while the sample is measured at different angles relative to the polarization of the light.

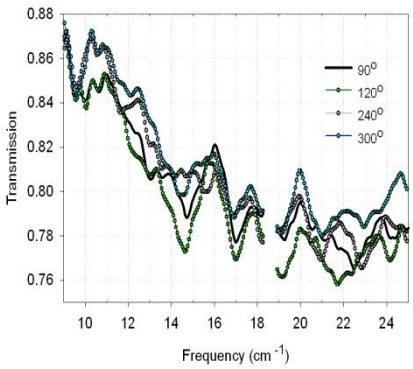

Figure 7. Transmission spectra of BG cells at two orientations

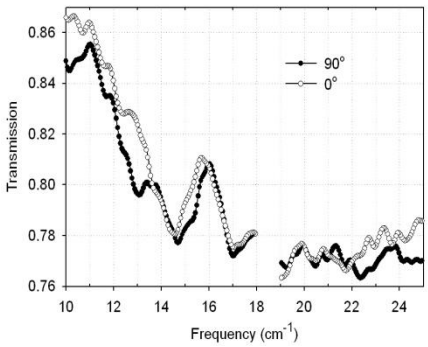

Figure 8. Transmission spectra of *E. coli* DNA at 4 orientations

3.4. Scaling of Absorption Coefficient with the Amount of Material

Fig. 9 shows the transmission spectra of four dry samples with varying amounts of BG spores. Qualitative scaling with mass is observed: more material leads to a lower transmission. At the same time, the positions of transmission minima do not change significantly from sample to sample. The overall pattern of the transmission spectra is well preserved even though transmission levels change. Specific absorption spectra (for 1 mg of biomaterial) were recalculated from the transmission and the results are shown in Fig. 10. Not only is the position of most peaks reproducible in all spectra, but absorption features become much stronger and more resolved in the samples with less biomaterial content, revealing more features than the spectra with higher amounts of biomaterial. For example, a rather broad single absorption peak at 14.5 cm^{-1}, which is present in the spectrum of 4.6 mg sample, splits into two peaks at 14.2 cm^{-1} and 14.8 cm^{-1} as the amount of biomaterial decreases to 0.6 mg. All samples, except the one with lowest biomaterial content, give the same absorption coefficient value in the low frequency sub-range, while at the high frequency end, specific absorption coefficient seems to grow with the decreasing amount of material. The different absorption coefficient level in a sample with 0.6 mg of spores can be due to a nonuniform distribution of biomaterial in a suspension and possibly some loss of biomaterial during sample preparation.

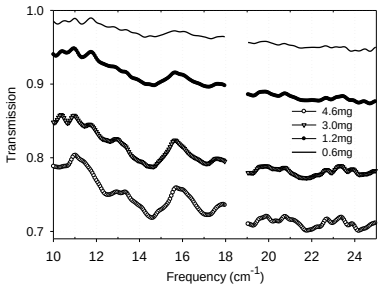

Figure 9. Transmission spectra of dry BG spores, scaling with mass

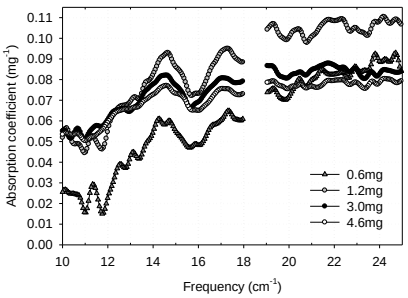

Figure 10. Absorption coefficient spectra of dry BG spores at orientation 0 º

3.5. Spores in Air and Vacuum

We also wished to ascertain what effect desiccation has on the transmission spectra. Additional measurements were performed in the vacuum at the level of ~5 mbar. The absorption coefficient spectra of BG spores dried in air (solid circles) and the vacuum (open circles) calculated

from the transmission are presented in Fig. 11. Three observations can be made from comparison. First, water contribution may influence the absorption of the samples dried in the air. When samples are placed in the vacuum, water molecules that are stuck to the substrate and spore surface are removed and absorption can change. This will result in a reduced absorption coefficient. Second, the intensity of absorption peaks in the vacuum is slightly smaller compared to air. This effect is supported by our previous work[13] where we observed that vibrational modes intensity of a dry biomaterial is much weaker than the same modes from material in a liquid environment. Third, drying in vacuum results in new absorption features. Specifically, additional absorption peaks are revealed at 13.7, 15.3, 21.2 and 22 cm^{-1} for the sample in the vacuum, while the peak at 11.5 cm^{-1} (in the air) disappeared in the vacuum. Also the maximum at 17.1 cm^{-1} observed for the sample in air shifts to 16.7 cm^{-1} in vacuum.

It has been documented that the spores can survive extreme desiccation for extended periods of time without any serious side effects[37, 38]. In our experiments, spores were exposed to a low vacuum for less than 20 minutes. Although this was not enough to cause permanent damage, perhaps our technique is able to detect small changes within the spores as a response to dehydration. These changes may or may not be harmful to the spore. More experiments are necessary to find the answer.

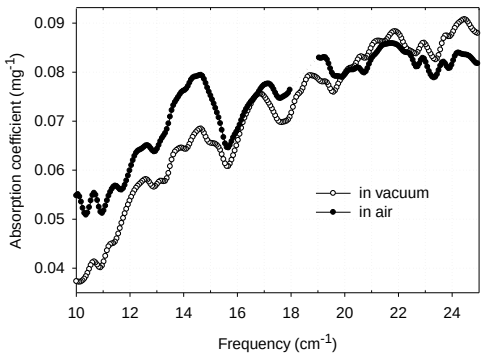

Figure 11. Absorption coefficient spectra of BG spores (3 mg, orientation 0 °) in air and in vacuum

3.6. Absorption Spectra: BG Spores vs. Lyophilized cells

BG cells transform into spores intra-cellularly during harsh environmental conditions, which would normally kill cells. Sporulation allows cells to preserve their genetic material; spores are also denser and have smaller water content than cells. The center of the spores, the core, consists of the cytoplasm, tightly bound and condensed DNA, ribosomes, enzymes and everything that is needed to function once returned to the vegetative state. The core is dehydrated, which is essential for the heat resistance, long-term dormancy and full chemical resistance. The core has large amounts of dipicolinic acid (DPA), which is not present in vegetative cells. DPA protects spore from heat and UV radiation. Outside the spore is protected by the coat containing several protein layers that are impermeable to most chemicals[39,40]. There are many common features between cell and a spore. However, cells have higher water content and have a more complicated structure, thus we expect that the differences might be revealed in the THz transmission and absorption spectra and allow us to distinguish cells from spores. Although we did not observe significant differences between cells and spores at 0° orientation, the 90° spectra have more distinguishing features as highlighted in Fig. 12. There are several new peaks in the cell absorption spectra at 11.7 cm^{-1}, 13 cm^{-1}, 19.7 cm^{-1} and 21 cm^{-1}. Besides that, cells have a shoulder at 15.2 cm^{-1} and the peak at 17 cm^{-1} in cell spectra is shifted to 17.2 cm^{-1} in spore spectra. Spores also have a peak at 20.1cm^{-1} that the cell spectra do not possess.

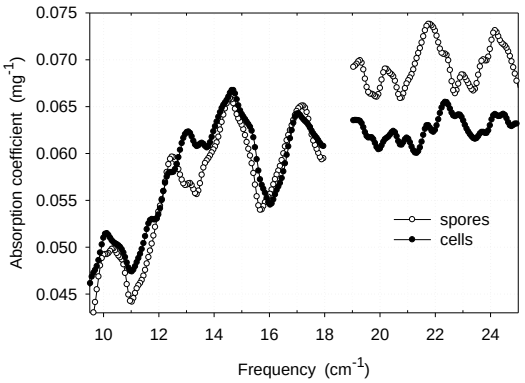

Figure 12. Absorption coefficient spectra of BG lyophilized cells and spores, ~ 5mg, orientation 90 °

3.7. Thermal Treatment of Cultured Cells.

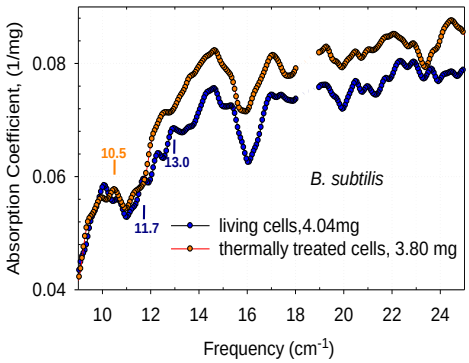

Figure 13. Absorption spectra of thermally treated and untreated *B subtilis* cells (90 °, PTFE substrate, dry)

Fig. 13 gives the results of the measurements performed on the thermally treated and untreated samples of cultured *B. subtilis* cells on the PTFE cards. Both spectra of *B. subtilis* are characterized by many similar features but at the same time have specific differences. The peaks at 11.7 and 13 cm^{-1} are observed only in the spectrum of the untreated cells, while the spectrum of the thermally treated cells only has a peak at 10.5 cm^{-1}. The differences in the spectra of living and thermally treated *E. coli* on PTFE substrate are shown in Fig. 14. The differences between the

thermally treated and living cells spectra can be explained in terms of the changes of the frequency modes that occurred under the irreversible transformation of the protein structure, and the structural changes of the nucleic acids.

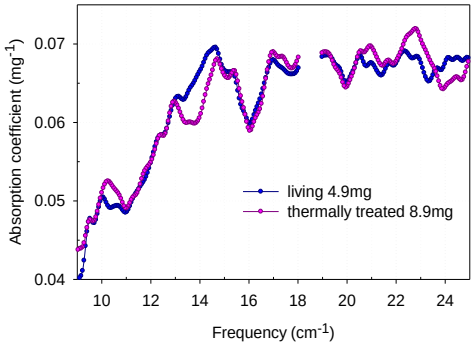

Figure 14. Absorption spectra of thermally treated and untreated *E. coli* cells (90°, PTFE, dry)

3.8. Absorption Spectra from different Biological Species

The ability to discriminate between the different bacterial species quickly and reliably is important. For example in a medical field it would enable a faster and more tailored treatment once bacterial organism is identified. Spectroscopic signatures of air-dried living cells E. coli and B. subtilis are depicted in Fig. 15. The resemblance of the spectra is obvious. The main shape of the curves, peak positions and their intensities are rather close. In the averaged absorption spectrum of E.coli, several bands are apparently slightly shifted to lower frequencies compare to the spectra of BG. Although some specificity could be found, particularly in the 9 – 13 cm-1 and 21 – 24 cm-1 ranges, better spectral resolution is required for more certain discrimination result.

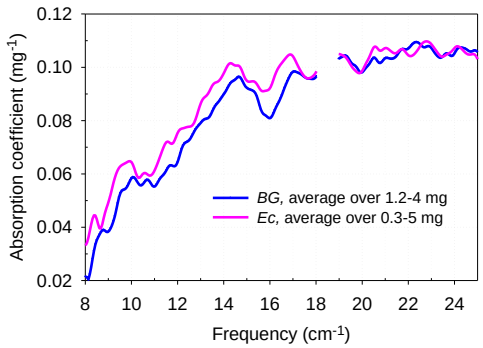

Figure 15. Sub-THz absorption spectra of *E. coli* and *B. subtilis* living cells (PTFE substrate, dry)

3.9. Absorption Spectrum of E. coli DNA

Bacterial cells do not contain nucleus, but rather DNA is contained in the cytoplasm. It is typically a single, closed circle concentrated in the region called nucleoid. There has been extensive research for decades on the characterization of different cell components such as DNA, proteins, etc. We chose to characterize the *E.coli* DNA, which constitutes 5 – 15% of an entire cell weight[41]. Fig. 16 shows the absorption coefficient spectrum of *E.coli* DNA in air using interference technique, and the average results over 7 samples with the mass from 1.2 mg to 5.2 mg demonstrating reproducibility.

The measurements were performed to examine how and to what extent the DNA might contribute to the transmission spectra of an entire cell. Spectra with approximately equal mass of the biomaterial were analyzed for the similarities and differences. Since the amount of the DNA inside the cells is less than the amount in the DNA only samples, we expect to see stronger spectroscopic features in the DNA only samples. Although DNA may have strong features, some of them can be masked inside the cell and also altered as the DNA interacts with the cytoplasm and the other molecular structures.

Fig. 17 below shows a spectrum of *E. coli* DNA (2.1 mg) and a spectrum of *E. coli* cells (3.3 mg). Although, the mass of the DNA sample is somewhat smaller, its spectroscopic features are stronger, and both samples have a comparable transmission level. New features can be seen in the DNA spectrum, just as expected. Nevertheless, the similarity of two spectra and so the contribution from the DNA to the spectrum of cells are obvious. This result can be understood since many other components of a cell are much less absorbing at THz than DNA.

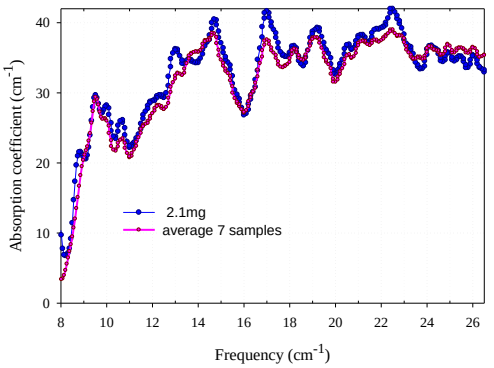

Figure 16. Absorption spectra of *E. coli* DNA

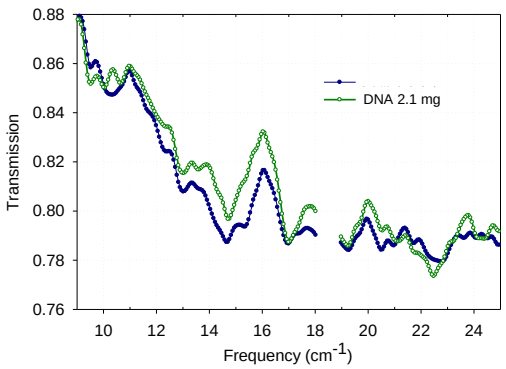

Figure 17. Transmission spectra of *E. coli* cells and DNA on PTFE substrate

4. MD Simulation and Discussion.

We could not compare experimental spectra from the *E. coli* DNA shown in Fig. 16 with modeling since a large size of this macromolecule prevents direct application of MD simulation at the current level of computational capabilities. However, some components from bacterial cells and spores are relatively small macromolecules, and the absorption spectra of these entire molecules can be calculated directly from MD simulation of their vibrational modes. One example is shown in Fig. 18 for the tyrosine transfer RNA ($tRNA_{tyr}$) from *E. coli*, where the simulated spectrum is compared with experimental absorption. THz spectrum of this molecule (74 bases and about 40000 atoms including water) was simulated using MD [20], and a similarity between measured and calculated spectra is obvious. Proteins are more difficult to perform the simulations. Nevertheless, rather good results were received for the protein thioredoxin from *E. coli* (see Fig. 19) after improving the convergence of simulation[22], thus validating both, the experimental and theoretical results. Therefore, multiple resonances due to low frequency vibrational modes within biological macromolecules, components of bacterial organisms, are demonstrated in the sub-THz frequency range experimentally in agreement with the theoretical prediction.

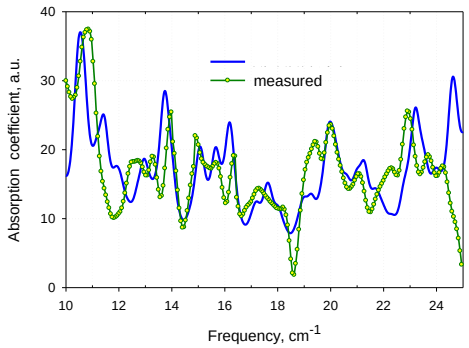

Figure 18. Sub-THz vibrational absorption coefficient spectra of $tRNA_{tyr}$(*E. coli*) in water: spectra simulated and measuredwith spectral resolution of 0.25 cm^{-1}[20]

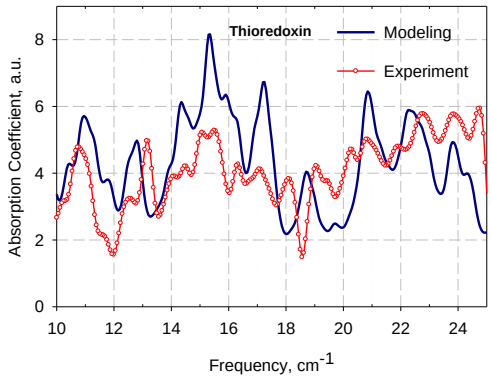

Figure 19. Sub-THz vibrational absorption coefficient spectra of protein thioredoxin from *E.coli* simulated and measured with spectral resolution of 0.25 cm^{-1}[22]

Thus as observed in Figures 16, 18 and 19 and in our other experiments on Bruker spectrometer FTS-66v taken with a relatively good resolution of ~0.25 cm^{-1} in the sub-THz range, absorption spectra of bio-molecules reveal multiple resonances - vibrational modes or groups of modes with close frequencies - that reflect their low frequency internal molecular motions.

The width of individual spectral lines and the intensity of resonance features are sensitive to relaxation processes of atomic dynamics (displacements) within a macromolecule, and in particular to the relaxation time. The experimental spectral line width of resolved features is approximately estimated as ~0.5 cm^{-1}. Due to possible contributions from different modes occurring at close frequencies, this estimate gives us the upper limit of damping factor γ, and the lowest limit for the time scale of low energy vibrational motions in the sub-THz as ~ 70 ps. Our estimate for a damping coefficient is of the same order as $\gamma = 1 cm^{-1}$ found in[3-6] at higher frequencies (~1 THz) in experiments based on photomixing technology. In our MD simulations, the damping factor was taken as a parameter, and comparison between experimental and calculated spectra was used to adjust its value. The experimental value of γ=0.5 cm^{-1} gave us the best correlation between the spectral line widths in simulated and experimental absorption spectra.

The similarity between sub-THz spectroscopic signatures of *E. coli* DNA and of *E. coli* cells as demonstrated in Fig. 17 permits us to make a conclusion that multiple resonances due to low frequency vibrational modes within biological macromolecules, components of bacterial organisms, do contribute to the spectroscopic features of biological cells.

Long relaxation times in our experiments are also confirmed by results from recent work using femtosecond-resolved fluorescence spectroscopy and the other techniques to break down the complex protein dynamics into four elementary processes and determine their relevant time scales[32, 42]. Among these processes the authors determined “a robust dynamical process in 95-114 ps” that is very close to the relaxation time in our experiments with Bruker spectrometer, and also much longer quenching dynamic processes with time scales of 275-615 ps at a hydrogen bond distance, which can give local fluctuations with vibrations spectral line width of 0.12-0.054 cm^{-1}. The results suggest the existence of diverse relaxation dynamics mechanisms in complex biological molecules.

The gamma factor can depend on frequency, and we expect the lower value in our sub-THz range compare to higher frequency THz region. However in our MD calculations we used the gamma factor frequently independent as a first try since our frequency range is rather narrow, from 10 to 25 cm^{-1}. More accurate calculations of absorption coefficient spectra from MD simulations results require the better knowledge of damping factor and its possible difference for relaxation processes of individual bonds.

5. Conclusions

Significant progress in experimental THz spectroscopy has been made in the last 2-3 years to improve the sensitivity of THz spectroscopic characterization of bacterial cells and spores to secure reproducible results. The correct choice of substrate, material concentration in solution or suspension and material alignment at deposition permitted us to significantly enlarge the intensities of modes and the reproducibility of frequencies when using Bruker IFS66v with the spectral resolution of 0.25 cm^{-1}. Our results demonstrate the contribution from the cellular molecular component to the vibrational spectrum of the entire cell and confirm that observed spectroscopic features are caused by fundamental physical mechanism of interaction between THz radiation and biological macromolecules.

It became clear, however, that further improvement of sensitivity and especially of discriminative capability using sub-THz vibrational spectroscopy, as en effective method for characterization of bacterial organisms requires even better spectral resolution. There are evidences that the processes with different relaxation time, including long lasting processes, do exist, thus justifying the application of highly resolved spectroscopy. This promises characterization with better spectral resolution to become possible, and we work in this direction in our next study.

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