Terahertz Science & Technology: Sensing Bio-Molecular Nanostructures & Photoinduces Transitions Between Metastable States

**14. ABSTRACT**

Significant progress in experimental and computational sub-THz vibrational spectroscopy has been made in the last 2-3 years to improve the sensitivity of THz spectroscopic characterization of large biological molecules, microorganisms and structural elements for bio-molecular electronics. The correct choice of substrate, concentration of materials in solution or suspension, and material alignment at deposition permitted us to significantly enhance the intensities of modes in solid samples when using a Fourier transform spectrometer with a...
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Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

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(c) Presentations


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- The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields: ...... 0.00
- The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields: ...... 0.00
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**Sub Contractors (DD882)**

**Inventions (DD882)**
Abstract

Significant progress in experimental and computational sub-THz vibrational spectroscopy has been made in the last 2-3 years to improve the sensitivity of THz spectroscopic characterization of large biological molecules, microorganisms and structural elements for bio-molecular electronics. The correct choice of substrate, concentration of materials in solution or suspension, and material alignment at deposition permitted us to significantly enhance the intensities of modes in solid state from 1-2% to 5-10% and the reproducibility of frequencies when using a Fourier transform (FT) Bruker FS66v spectrometer with a moderate spectral resolution of 0.25 cm⁻¹. Sub-terahertz (THz) spectroscopy was 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, and artificial molecules as well. 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. However, it was also demonstrated that the spectral resolution of a Bruker spectrometer (0.25 cm⁻¹) still does not provide a sufficient level of discriminative capability. Implementation of THz vibrational spectroscopy is still impeded because of the absence of spectroscopic systems, which simultaneously satisfy all the requirements of good spectral and spatial resolution, along with high sensitivity. It became clear that further improvement of sensitivity and especially of discriminative capability using sub-THz vibrational spectroscopy as an effective method for characterization of bacterial organisms requires even better spectral resolution. In latest studies, we utilized the CW frequency-domain spectroscopic sensor prototype developed by Vibratess [28], that does not require for cryogenic cooling of the detector. Due to a high sensitivity, spectral resolution, and a spatial resolution below the diffraction limit, this instrument permitted us to observe intense and narrow spectral resonances in transmission/absorption spectra of nano-samples from biological materials with spectral line widths as narrow as ~0.1 cm⁻¹. Demonstrated multiple intense and specific resonance features provide conditions for reliable discriminative capability using sub-THz frequency domain spectroscopy to the level of the strains of the same bacteria that was not possible before.

Scientific Progress

Sub-Terahertz (sub-THz) vibrational spectroscopy for bio-sensing is based on specific resonance features, vibrational modes or group of modes at close frequencies, in absorption (transmission) spectra of large biological molecules and entire bacterial cells/spores. Significant progress in experimental and computational sub-THz vibrational spectroscopy has been made in the last 2-3 years to improve the sensitivity of THz spectroscopic characterization of large biological molecules, microorganisms and structural elements for bio-molecular electronics (bio-molecular nanostructures). Earlier, it was only possible in most cases to obtain intensities of transmission resonance features of only 1-2% when solid samples were characterized. The correct choice of substrate, concentration of materials in solution or suspension, and material alignment at deposition permitted us to significantly enhance the intensities of modes and the reproducibility of frequencies when using a Fourier transform (FT) Bruker FS66v spectrometer with a moderate spectral resolution of 0.25 cm⁻¹. Not only were raw transmission spectra measured, it was also possible to extract absorption coefficient data for quantitative characterization of bio-materials. In this project, sub-terahertz (THz) spectroscopy was 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, DNA from E. coli, and artificial molecules as well. One of the goals of our research was 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 results of this work confirmed 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, which is adequate to the widths of spectral lines, is used.

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 a sufficient level of discriminative capability. Improving the sensitivity for spectroscopic characterization of biological materials also remains an important issue in THz resonance spectroscopy. The diffraction limit of spatial resolution is another serious problem in THz spectroscopic characterization (and imaging). Thus, implementation of THz vibrational spectroscopy is still impeded because of the absence of spectroscopic systems, which simultaneously satisfy all these requirements of good spectral and spatial resolution, along with high sensitivity. Experimental characterization still required mg quantities of material and a detector cooled with liquid helium for reliable characterization, and a system under vacuum or purged with dry gas because of very low level of radiation power available from the mercury lamp source. In addition, a simple sample preparation procedure, good reproducibility, and user-friendly operation are also important for wide spread adoption of this technique. It became clear that further improvement of sensitivity and especially of discriminative capability using sub-THz vibrational
spectroscopy as an effective method for characterization of bacterial organisms requires even better spectral resolution. Our experimental results from measurements with high spectral resolution at the beginning of this project had already demonstrated very intense and narrow spectral features from biological molecules and bacteria with the width between 0.05 and 0.2 cm⁻¹. These features were not evident in previous results using a resolution of 0.25 cm⁻¹. The analysis of our results also suggested the coexistence of diverse relaxation dynamics mechanisms relevant to the sub-THz frequency region. Evidences exist for long-lasting relaxation processes for atomic dynamics (displacements), which result in narrow spectral lines and justify the development and application of highly resolved vibrational spectroscopy. For our new studies, we utilize the spectroscopic sensor prototype developed by Vibratess, LLC. This novel CW, frequency-domain spectroscopic instrument with imaging capabilities that operates without the need for cryogenic cooling of the detector is based on a very strong local enhancement of the electro-magnetic field, thus allowing increased coupling of the THz radiation with the sample biomaterials. This enhancement was achieved through the use of an extraordinary transmission of a sub-wavelength-slit conductive structure. Transmission spectra were obtained in the sub-THz region between 315 and 480 GHz for both, macromolecules and biological species. Due to a high sensitivity, good spectral resolution, and a spatial resolution below the diffraction limit, currently restricted by the opening size of a microdetector housing, this spectroscopic instrument permitted us to observe intense and narrow spectral resonances in transmission/absorption spectra of nano-samples from biological materials with spectral line widths as narrow as ~0.1 cm⁻¹. We demonstrated experimental spectra from biological macromolecules and biological cells/spores measured with the new spectrometer and compared the spectra from the protein theoredoxin with MD simulations. Multiple intense and specific resonance features provide conditions for reliable discriminative capability using sub-THz frequency domain spectroscopy to the level of the strains of the same bacteria that was not possible before.

The results of our study have been described in our most recent publications and presentations:

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In this project, sub-terahertz (THz) spectroscopy was applied to characterize lyophilized and \textit{in vitro} cultured bacterial cells of non-pathogenic species of \textit{Escherichia coli} (\textit{E. coli}) and \textit{Bacillus subtilis} (BG), spores of BG, DNA from \textit{E. coli}, and artificial molecules as well. One of the goals of our research was 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 results of this work confirmed 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, which is adequate to the widths of spectral lines, is used.

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It became clear that further improvement of sensitivity and especially of discriminative capability using sub-THz vibrational spectroscopy as an effective method for characterization of bacterial organisms requires even better spectral resolution. Our experimental results from measurements with high spectral resolution at the beginning of this project had already demonstrated very intense and narrow spectral features from biological molecules and bacteria with the width between 0.05 and 0.2 cm\(^{-1}\). These features were not evident in previous results using a resolution of 0.25 cm\(^{-1}\). The analysis of our results also suggested the coexistence of diverse relaxation dynamics mechanisms relevant to the sub-THz frequency region. Evidences exist for long-lasting relaxation processes for atomic dynamics (displacements), which result in narrow spectral lines and justify the development and application of highly resolved vibrational spectroscopy. For our new studies, we utilize the spectroscopic sensor prototype developed by Vibratess, LLC. This novel CW, frequency-domain spectroscopic instrument with imaging capabilities that operates without the need for cryogenic cooling of the detector is based on a very strong local enhancement of the electro-magnetic field, thus allowing increased coupling of the THz radiation with the sample biomaterials. This enhancement was achieved through the use of an extraordinary transmission of a sub-wavelength-slit conductive structure. Transmission spectra were obtained in the sub-THz region between 315 and 480 GHz for both, macromolecules and biological species. Due to a high sensitivity, good spectral resolution, and a spatial resolution below the diffraction limit, currently restricted by the opening size of a microdetector housing, this spectroscopic instrument permitted us to observe intense and narrow spectral resonances in transmission/absorption spectra of nano-samples from biological materials with spectral line widths as narrow as ~0.1 cm\(^{-1}\). We demonstrated experimental spectra from biological macromolecules and biological cells/spores measured with the new spectrometer and compared the spectra from the protein theoredoxin with MD simulations. Multiple intense and specific resonance features provide conditions for reliable discriminative capability using sub-THz frequency domain spectroscopy to the level of the strains of the same bacteria that was not possible before.

The results of our study have been described in our most recent publications and presentations:

A. Vibrational spectroscopy with a moderate resolution of 0.25 cm\(^{-1}\).

Terahertz (THz) vibrational spectroscopy is an emerging field to examine biomolecular structure and dynamics, and to characterize absorption properties of biological materials 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, and theoretical studies predicted multiple resonances in absorption (or transmission) spectra of biological molecules in the THz frequency (0.1–10 THz ) or millimeter-wavelength (3–300 cm\(^{-1}\)) range [2]. Organic solid systems and relatively small biomolecules like protein fragments have been successfully characterized in this range to demonstrate sharp spectral features determined by their individual symmetries and structures [3-6].

In our study, sub-THz spectroscopy was 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, DNA from E.coli cells, and artificial molecules as well. 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 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°C), radiation, many chemicals (i.e. acids, bases, alcohol, chloroform), and desiccation. One of the goals of our research was to demonstrate that Fourier transform (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.

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.

The significance of this study is justified by necessity for a fast and effective, label free and reagent free optical, reliable, non-invasive method 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 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.

The important motivation of our research was 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 [12, 13]. In parallel with the experimental spectroscopy, the computational modeling technique was developed to simulate vibrational spectra of bacterial cell components using the energy minimization, normal mode analysis and MD approaches. The molecular dynamics simulations were performed using the Amber package as described in [13] with the procedure that was modified to improve the convergence of modeling results for proteins.

In our 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 our 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 [14], but from artificial molecules as well [15-17], thus resulting in more reliable signatures.

The experimental procedure for spectroscopic characterization of large biological molecules and species using a Bruker IFS66v with a moderate spectral resolution of 0.25 cm\(^{-1}\) was described in [12, 14]. Figures 1 and 2 show the current level of the resolution, sensitivity and reliability of this commercially available spectrometer in a sub-THz vibrational region. For reproducibility measurements, different amounts of *E. coli* and *Bacillus subtilis* (BG) cells were dried on substrates and characterized. The signatures in Figure 2 are reproduced down to the smallest discernable details.

**Fig. 1** *Bacillus subtilis* (BG) cells (Bruker FT66v). The spectral resolution is 0.25 cm\(^{-1}\) and the standard deviation for a set of similar samples is better than 0.5%.
Transmission/absorption spectra measured with a FT spectrometer equipped with a detector operating at 1.7°K are rich in well resolved features having spectral widths of ~0.5–1 cm⁻¹. Analysis of the sub-THz absorption spectra revealed some differences between species of *B. subtilis* and *E. coli*. High temperature treatment (~100°C) 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.

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. Some of cellular components of *E. coli*, DNA [18], transfer RNA [19], and protein thioredoxin [20, 13] were also characterized. Absorption spectra of relatively small macromolecules were simulated using MD. The work revealed several important results. Vibrational frequencies from simulated spectra for components correlate rather well with the observed features. These results confirm that observed spectroscopic features are caused by fundamental physical mechanism of interaction between THz radiation and biological macro-mole [12, 14]. At last, the decisive confirmation is obtained in [12] 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-THz 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 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 [12]. Thus, multiple resonances due to low frequency vibrational modes within biological macromolecules, components of bacterial organisms, are unambiguously demonstrated experimentally in the sub-THz frequency range in agreement with the theoretical prediction. 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.

Several challenges, however, arise in analyses and understanding of vibrational spectra from biological macromolecules and entire microorganisms. 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

**Fig. 2.** Reproducibility of spectral features in absorption spectra for samples with different amounts of material.
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 causes 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. The width of individual spectral lines and the intensity of resonance features observed in sub-THz spectroscopy are sensitive to the relaxation processes of atomic dynamics (displacements) within a macromolecule. It is clear that the decay (relaxation) time, $\tau$, 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. The suggested range of molecular dynamics relaxation times for processes without bio-molecular conformational change varies from approximately 1.5 ps to 650 ps in different studies [see, for example 21, 22]. The corresponding values for the dissipation factor, $\gamma$, and the width of spectral lines, which are reciprocal to $\tau$, are between 0.05 and 20 cm$^{-1}$. Values of $\gamma$ above 1 cm$^{-1}$ would result in structure-less sub-THz spectra, since vibrational resonances could not be resolved in this case because of the large density of low intensity vibrational modes. The existence of long-lasting dynamic processes responsible for narrow spectral lines have been confirmed by relaxation dynamics of side chains in macromolecules observed by time-resolved fluorescence experiments [23].

The ability to discriminate between the different bacterial species quickly and reliably using sub-THz spectroscopy would provide significant benefits. For example, in the medical field it would enable a faster and more tailored treatment once a bacterial organism is identified as the cause of an infection. At the same time, 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 a sufficient level of discriminative capability. As shown in Fig. 3, some specificity is observed in spectra of different bacteria, including frequency shifts for absorption bands [12]. However, the shape of the curve and the absorption peak intensities are rather close. These results confirm that better spectral resolution is required for more certain discrimination, and new spectroscopic systems (sensors) have to be developed with improved spectral resolution.

![Fig. 3. Sub-THz absorption spectra of E. coli and B. subtilis living cells (PTFE substrate, dry).](image-url)

Our experimental results from measurements with high spectral resolution have already demonstrated very intense and narrow spectral features from biological molecules and bacteria with the width between 0.05 and 0.2 cm$^{-1}$ [24-27]. These features were not evident in previous results using a resolution of 0.25 cm$^{-1}$. The analysis of our results also suggested the coexistence of diverse relaxation dynamics mechanisms relevant to the sub-THz frequency region. It is surprisingly narrow width of spectral lines (or dissipation factor) in transmission (absorption) spectra “that makes them detectable, in spite of their
relatively low absorption cross section compared to polar molecules in the THz region” [28]. For the purpose of detection and discrimination, the spectral resolution has to be adequate to the widths of spectral lines. The diffraction limit of spatial resolution is another serious problem in THz spectroscopic characterization (and imaging) due to the long wave-length of THz radiation, compared to the visible and even IR regions. At last, improving the sensitivity for spectroscopic characterization of biological materials also remains an important issue in THz resonance spectroscopy. In addition, experimental characterization still required mg quantities of material and a liquid helium cooled detector for reliable characterization with the system under vacuum or purged with dry gas because of very low level of radiation power available from the mercury lamp source. Thus, implementation of THz vibrational spectroscopy is still impeded because of the absence of spectroscopic systems, which simultaneously satisfy all these requirements of good spectral and spatial resolution, along with high sensitivity. In addition, characteristics such as small samples requirements, a simple sample preparation procedure, room temperature operation, good reproducibility, and user-friendly operation are also important for wide spread adoption of this technique.

To increase the sensitivity, reliability, spectral and spatial resolution of sub-THz vibrational spectroscopy techniques, Vibratess , LLC, has developed a spectroscopic sensor prototype with imaging capability operating at room temperature, without the need for cryogenic cooling of the detector [29]. This novel CW, frequency-domain instrument is based on a very strong local enhancement of the electromagnetic field, thus allowing increased coupling of the THz radiation with the sample biomaterials [24-26]. This enhancement was achieved through the use of the discontinuity edge effect and the extraordinary transmission of a sub-wavelength-slit conductive structure [30-33]. This instrument was utilized in our latest research on highly resolved vibrational spectroscopy of biological materials.

The Vibratess’s Spectroscopic Sensor Prototype shown in Figures 4 and 5 operates in the frequency range between 315 and 480 GHz with an average output power of ~0.2 mW. It includes a sub-THz electronically tunable source based on Schottky frequency multipliers (Virginia Diode, Inc) and a sub-micron precision motorized stage for three dimensional scanning capability of a detector subsystem relative to a multi-channel chip sample holder. The detector system entailed a micro-detector (Schottky diode, VDI) with a replaceable beam lead microprobe (Lichtenberger Consulting) and a planar microdetector circuit [29] mounted in a custom micro-waveguide housing. The microfluidic chip placed in the sample holder, contained an array structure of micro-channels to generate the edge effects necessary for enhancement of the EM field of the THz radiation and to provide regions for holding biomaterial.

A high magnification optical imaging system with a long-working-distance objective allowed accurate positioning of components. Data collection options included signal vs. frequency, time, and the three position coordinates. The waveguide and microdetector circuit were designed using HFSS.
simulation to separate THz radiation from a dc Schottky diode signal [29]. Once the instrument was built, testing demonstrated better than 200 µm spatial resolution, currently restricted by the opening size of the microdetector waveguide (150x200 µm²). Spectral resolution better than 1 GHz was achieved, along with high sensitivity, a signal/noise ratio of ~10³ (depending on frequency) and at room temperature operation without the requirement for air evacuation, or purging with nitrogen.

B. Highly resolved vibrational spectroscopy (0.03 cm⁻¹)

In our latest work, transmission spectra were obtained in the sub-THz region between 315 and 480 GHz for both, macromolecules and biological species. Due to a high sensitivity, good spectral resolution, and a spatial resolution below the diffraction limit, this spectroscopic instrument permits us to observe intense and narrow spectral resonances in transmission/absorption spectra of nano-samples from biological materials with spectral line widths as narrow as ~0.1 cm⁻¹. We demonstrate experimental spectra from nanogram quantities of biological macromolecules and biological cells/spores measured with the new spectrometer and compare the spectra from the protein thioredoxin with MD simulations. Demonstrated multiple intense and specific resonance features provide conditions for reliable discriminative capability using sub-THz frequency domain spectroscopy to the level of the strains of the same bacteria that was not possible before.

For sample preparation, a 0.1-0.3 µl drop of solutions/suspension of biomaterial was micropipette in one spot of the array of microchannels in the sample holder. Measurements were taken at 10 min following droplet placement, after the sample was dried. The probe for the detection system was positioned several microns above the array. Only ~20 ng of biomaterial is required as the sample in our system as compared to the mg sample size required in the previous work done on the Bruker spectrometer. With the complete development of a sealed micro/nanofluidic chip sample holder, liquid samples will be utilized, and the amount of biomaterial required for characterization will be further reduced ~10 to 100 times, thus opening the way for single bio-molecule characterization.

To demonstrate the capabilities of the spectrometer, transmission spectra from bacterial cells and some of their molecular components (DNA, thioredoxin) were measured and recalculated for absorption coefficient per 1 mg of material, as described in [12]. Reliability of the transmission results was verified by measurements of samples with different amounts of material and at different coordinates in the microchannel array. Figure 6 demonstrates transmission changes for two samples of E.coli cells with different masses. The frequencies of spectral features are reproduced with scaling in the samples with different amounts of materials, indicating that the absorption is from the sample. Transmission and absorption spectra of E. coli DNA are shown in Figures 7 and 8. Very narrow spectral lines are detected, with a width of ~0.1-0.2 cm⁻¹. In many cases, the intensities of normalized absorption peaks calculated per 1 ng

![Fig. 6. Transmission of E.coli cells: two samples with different amounts of material.](image)
are reducing for larger sample amount (Fig. 8). As it is demonstrated in this work and earlier [12], spectral lines are sharper in thin samples and significant broadening and damping of spectral features is observed in bulk samples, probably due to less oriented material. Very thick samples usually do not demonstrate well resolved spectral features in the sub-THz region. This effect can explain the dependence of calculated absorption coefficient on the thickness of sample material in the channel, as demonstrated in Figure 8, as well as the sensitivity of sub-THz spectroscopic characterization to specificities of sample preparation techniques. Further development of the microfluidic sample holder will provide increased reproducibility and efficiency, as the sample presentation in the spectrometer detection region will be more accurate and controlled.

Additional testing and evaluating sensitivity and discriminative capability of a new frequency domain spectrometer developed by Vibratess was conducted jointly with Dr. Raphael Moon (ECBC) and Dr. Ashish Tripathi (SAIC) during their visit to Vibratess on April 11 & 12. Several sample materials, including B. atropheus spores were prepared in advance at ECBC.

B. atrophaeus is a close relative to B. subtilis. The strain was var. niger. All the bacterial samples were triple washed with DIW (with subsequent centrifuging for 5 mins at 5000 RPM to recover the bacterial sediment). Samples were prepared just before measurements as a water suspension and a small drop with diameter of 1 to 2 mm was put on a substrate of a sample holder. Background was recorded before deposition of material. Measurements started after material dried (~10 min). Only a small portion of material inside narrow channels in the substrate contribute to transmission measurements.

Phenotypically, Bacillus atrophaeus is indistinguishable from the type strain of Bacillus subtilis except by virtue of pigment production on certain media [35]. “Bacillus atrophaeus is a gram-positive, aerobic, endospore-forming, rod-shaped bacterium whose description is virtually identical to that of Bacillus subtilis except for the production of a pigment on media containing an organic nitrogen source. Many of the isolates belonging to this species were previously classified as Bacillus subtilis var. niger, Bacillus niger, or even earlier as Bacillus globigii. Several of these strains are used in industry as sterilization control organisms or sources of restriction endonucleases, but specifically within the biodefense research and testing community, some of these strains are used extensively as nonpathogenic surrogates for Bacillus anthracis. Isolates used in this latter capacity are still commonly referred to by their historical designation of B. globigii or simply BG” [35].

The goal of this our work was to demonstrate detective sensitivity and discriminative capability of our recently introduced experimental technique utilizing a new frequency domain spectrometer developed by Vibratess. Highly resolved spectra in the sub-range between 11 to 16.8 cm\(^{-1}\), rich in resonance features were obtained from measurements of 10-40 ng of sample materials in a virtually dry
condition (Figures 9,10). Scaling in transmission spectra for 0.1 and 0.2 µl of suspension in water is demonstrated (Fig. 9). Spectral features that are frequencies of the transmission minima (or absorption maxima), correlate very well. The level of transmission reduced for larger amount of material. This room temperature instrument enhances the signature significantly (at least 20 times) compared to commercial Bruker FTIR spectrometer operating with liquid He bolometer. Discriminative capability of characterization technique using Vibratess Spectrometer is clearly demonstrated: the frequencies of resonances (transmission minima) in Figure 10 are different for most spectral features of two species.

![Transmission spectra from B. Atropheus measured on Vibratess spectrometer.](image1)

**Fig. 9.** Transmission spectra from B. Atropheus measured on Vibratess spectrometer.

![Comparison absorbance spectra from B. Atropheus and BG spores](image2)

**Fig. 10.** Comparison absorbance spectra from B. Atropheus and BG spores.

**Fig. 11** compares the averaged transmission spectra of *E.coli* cells, as measured on the Bruker FTIR spectrometer, with the results from the Vibratess spectrometer. Note the different transmission scales for the two instruments and significantly reduced amount of sample used in the new instrument. Most spectral features, like transmission minima or shoulders in the spectrum of *E.coli* cells measured by the FTIR instrument, are resolved by the Vibratess spectrometer as one or several more narrow and intense spectral lines. There is a shift in the major transmission minimum position from 14.6 cm\(^{-1}\) to 14.9 cm\(^{-1}\). This 0.3 cm\(^{-1}\) shift to a higher frequency can be explained by a different orientation of the cells in the two set-ups, since spectra are sensitive to the orientation of biomaterial with respect to the electric field vector in THz radiation [12].

![Transmission spectra of *E.coli* cells measured with two different instruments: Bruker FTIR (FS66v) and Vibratess’ frequency domain spectroscopic sensor.](image3)

**Fig. 11.** Transmission spectra of *E.coli* cells measured with two different instruments: Bruker FTIR (FS66v) and Vibratess’ frequency domain spectroscopic sensor.
Comparison of parameters and conditions in the new spectroscopic sensor with the Bruker spectrometer FS66v in the sub-THz operation range (11-17 cm\(^{-1}\)) already demonstrated an order of magnitude better spectral resolution and 10-15 times higher peak intensities. This high sensitivity allows two orders of magnitude less sample to be deposited in a spot of 1-2 mm\(^2\), with a sample area of only 0.12 mm x 0.15 mm under the detector waveguide opening actually interrogated in each analysis. With the development of a microfluidic device with channels sealed for liquid material flow, the amount of material for sampling will be additionally reduced by 1-2 orders.

Since overlapping of individual resonances is still possible, the estimates for the dissipation factor from the width of spectral lines in Figures 6-10 provides us the upper limit for \(\gamma\) between 0.1 and 0.2 cm\(^{-1}\). These are significantly less than the 0.5-1 cm\(^{-1}\) values measured on the Bruker spectrometer, which has a spectral resolution of 0.25 cm\(^{-1}\). The lowest limit for corresponding relaxation time scale, \(\tau\), of molecular dynamics processes is between ~160 and 330 ps based on these better resolved spectral features. These values are close to results from time-resolved fluorescence experiments [23].

To further confirm the reality of the observed narrow and intense resonance features in the sub-THz transmission/absorption spectra of biological materials as measured with the new spectroscopic sensor, we compared in Fig. 12 the spectrum from the *E.coli* protein theoredoxin with computational modeling results using molecular dynamics (MD) simulations with a damping factor of \(\gamma = 0.12\) cm\(^{-1}\) (relaxation time 275 ps). Again, due to possible contributions from several different modes occurring at close frequencies, the width of spectral lines gives us an upper limit of \(\gamma\). As seen in Figure 12, not all peaks are reproduced in the measured and simulated spectra, since simulation parameters have not yet been optimized. Besides, the same value of \(\gamma\) was used to calculate absorption for all vibrational modes, and that is not necessary the case. However, the overall correlation between the theory and experimental data confirms again the existence of intense and narrow absorption lines, which can be used for discrimination between different bacteria and strains. The existence of such long-lived vibrations was confirmed in recent experimental results on relaxation time scales in thioredoxin from femtosecond-resolved fluorescence spectroscopy [23]. In addition to the dynamical processes on the time scale of 95-114 ps, which are very close to the relaxation times in our experiments with the Bruker spectrometer, the authors observed 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 widths of 0.12-0.054 cm\(^{-1}\).

One of the goals of this project was to apply improved technique for sensing and characterization of artificially designed structural elements and bio-molecular architectures developed in research groups of collaborators. Our latest research was focused on application of highly resolved spectroscopy described above to study transmission/ absorption spectra of artificial DNA crystal grown by Rahman Masudur from the group of Professor Michael Norton (the Department of Chemistry at Marshal University, West Virginia).

![Fig. 12. Absorption spectrum of protein thioredoxin from *E. coli*: MD simulation and experimental results as measured using Vibratess spectroscopic sensor.](image-url)
Virginia). The DNA was purchased from IDT HPLC and purified Tm 43.1C, MW 4088.7. The DNA sequence is

5’ GGA CAG ATG GGA G 3’

A picture of a crystal is shown in Fig. 13.

Because the crystals have a lot of water inside channels in the unit cell structure, the crystals were shipped in a special buffer solution, containing salts (MgCl2 and NaCl), some organic species, and some excess DNA strands. In order to keep the crystals wet (not in contact with a gas bubble, which could allow dehydration), the crystals have been sent in a fully filled, small capillary tube, capped with a wax top to be sealed until measurements. A drop of solution with crystals was extracted from the 2 mm diameter capillary tubes using sharpened wooden stick and placed on a sample holder with array of channels. The big crystals (shown in Fig. 13) are too big for our micro-channels, and only small crystals fit the inside channels with the 10 mkm width. Measurements were conducted at once when crystals are still wet, and continued after crystals drying. Fig. 14 shows transmission spectrum of a big DNA crystal changing with time, Fig. 15 demonstrates transmission spectra of two different small crystals, and Fig 16 compares absorbance of DNA crystal with that of E.coli DNA (5mg/ml in water). Transmission is

![Image](image_url)
growing when the sample is drying (Fig. 14). Reproducibility of signatures in transmission spectra from two different crystals is rather good (Fig. 15). Transmission minimum or absorption peak maximum near the frequency of 14 cm$^{-1}$ was very often observed in other DNA materials. As in other cases, the intensities of spectral lines is sensitive to the thickness of sample material, and it is usually higher in thinner samples.

Although absorbance spectra of two DNA sample materials in Fig. 16 are different, the intensities and the widths of spectral lines are approximately the same.

![Absorbance spectra from DNA crystal and from E. coli DNA](image)

Fig. 16. Absorbance spectra from DNA crystal and from E. coli DNA (0.1 µl with 5 mg/ml in water)

**Conclusions**

Significant progress in experimental vibrational THz spectroscopy was demonstrated in the last 2-3 years. Reliable information was received for transmission/absorption spectra from biological materials measured with a moderate spectral resolution of 0.25 cm$^{-1}$ using Bruker spectrometer FT 66v with liquid helium cooled detector. Sub-terahertz (THz) spectroscopy was 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*, and artificial molecules as well. We demonstrated that this technique 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 results of this work confirmed 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. However, it became clear, that further improvement of sensitivity and especially of discriminative capability using sub-THz vibrational spectroscopy as an effective method for characterization of bacterial organisms requires even better spectral resolution.

We tested and evaluated an automated transmission frequency-domain THz spectroscopic system prototype developed by Vibratess that is operating at room temperature. The developed prototype provides spectral resolution better than 0.035 cm$^{-1}$, and improved detection sensitivity and reliability in the sub-THz operation range over a commercially available spectrometer with liquid helium cooled detector by more than an order of magnitude. Spatial resolution of the instrument is currently restricted by the opening size of the microdetector waveguide. Highly resolved transmission (absorption) spectra from only 10-20 ng of biological macromolecules and bacterial cells/spores were demonstrated. Thus, a new
sub-THz vibrational spectroscopy technology with high spectral and spatial resolution was experimentally demonstrated.

The experimental results measured with high spectral resolution demonstrate very intense and narrow spectral features from biological molecules and bacteria with spectral widths between 0.1-0.2 cm\(^{-1}\). This corresponds to much longer scattering time values of ~330-150 ps as compared to those previously detected using a spectrometer having a resolution of 0.25 cm\(^{-1}\). The results provide completely new information about the interaction between THz radiation and biological materials, confirming diverse relaxation dynamics mechanisms relevant to sub-THz spectroscopy. This demonstration of multiple intense and specific resonance features provides conditions for reliable discriminative capability of frequency domain spectroscopy to the level of bacterial strains in extremely small sample volumes. Additionally it creates the basis for the development of new types of advanced biological sensors. Integrating the developed spectroscopic instrument with a microfluidic platform will open the way for reliable and accurate detection of nanograms of biological materials using optical, highly sensitive biosensors. Finally, this new instrument can be used for monitoring interactions between biomaterials and reagents, and for studying conformational change and biomedical processes in a near real-time.

REFERENCES


