# THz Characterization of Lysozyme at Different Conformations

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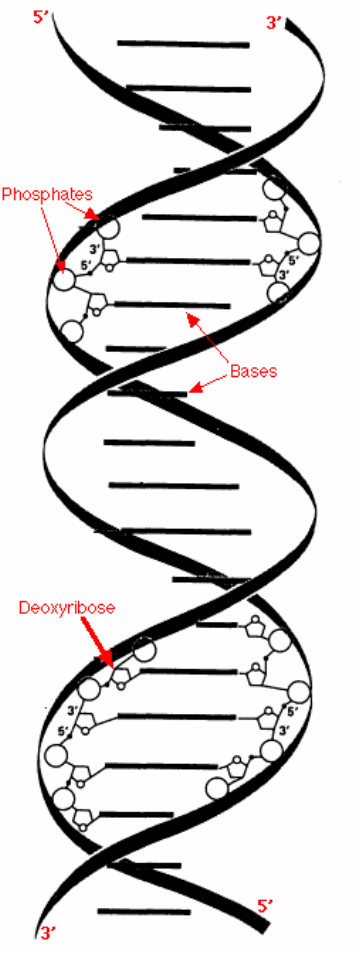
*This work was supported by the U.S. DOD under Contract DAAD19-00-1-0402, and in part by U.S.Army NGIC Contract # DASC01-01-C0009*

# MOTIVATION and GOALS




- There is a general **need for fast and less expensive techniques** that can provide useful **structural information on bio-materials.**
- **The capability of recently emerged THz spectroscopy to detect the hydrogen bond low-frequency vibrations opens potentially many applications for this technique including monitoring of folding-unfolding processes of proteins.**
- Our goal is **to demonstrate** the capability of **THz spectroscopy** to **discriminate between different protein conformations** and to **evaluate it possible application for monitoring folding-unfolding process** of **lysozyme**, a easily available protein and whose **structure** has been **well studied** by other methods.

# LYSOZYME

Lysozime is a globular α+β protein with approximately 45 % of α-helix type secondary structure and ~ 20% of β-sheet structure.

In addition it has ~ 25% various turn conformations that generally exist in globular proteins and ~ 13% of unordered or "random coil" secondary structure [2].

Lysozyme also adopts specific tertiary structure known from X-ray diffraction and nuclear magnetic resonance (NMR).

After many years of study protein folding-unfolding processes still not completely clear.

# Available Techniques for Protein Structure Characterization

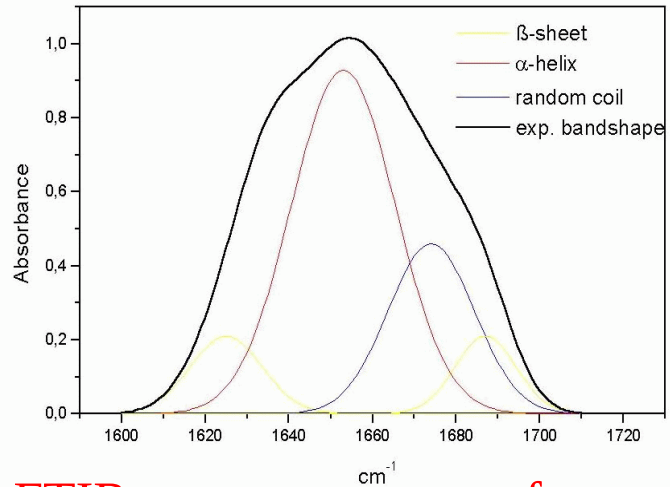
While the three-dimensional structure of proteins is determined by X-ray crystallography, it requires a well ordered crystallyne samples.

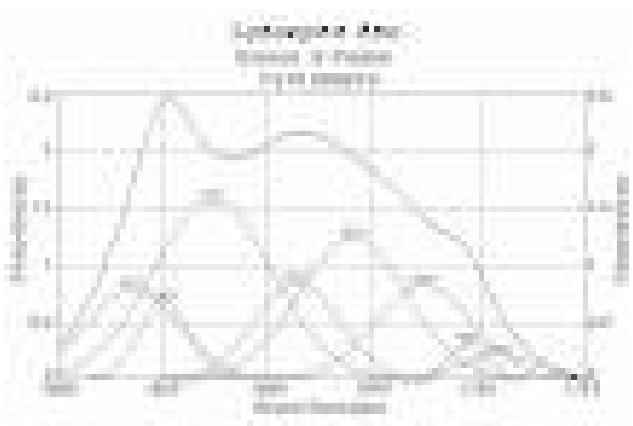
The nuclear magnetic resonance (NMR) spectroscopy can be applied to proteins in solution. However the interpretation is very complex and is limited to small proteins.

Alternative methods, circular dicroism (CD) and vibrational (infrared and Raman) spectroscopy are not able to generate structures at atomic resolution but provide structural information on proteins, especially on secondary structure.

High quality spectra can be obtained with relatively by recently developed Fourier Transform Infrared FTIR spectroscopy

# Fourier Transform Infrared Spectroscopy (FTIR)



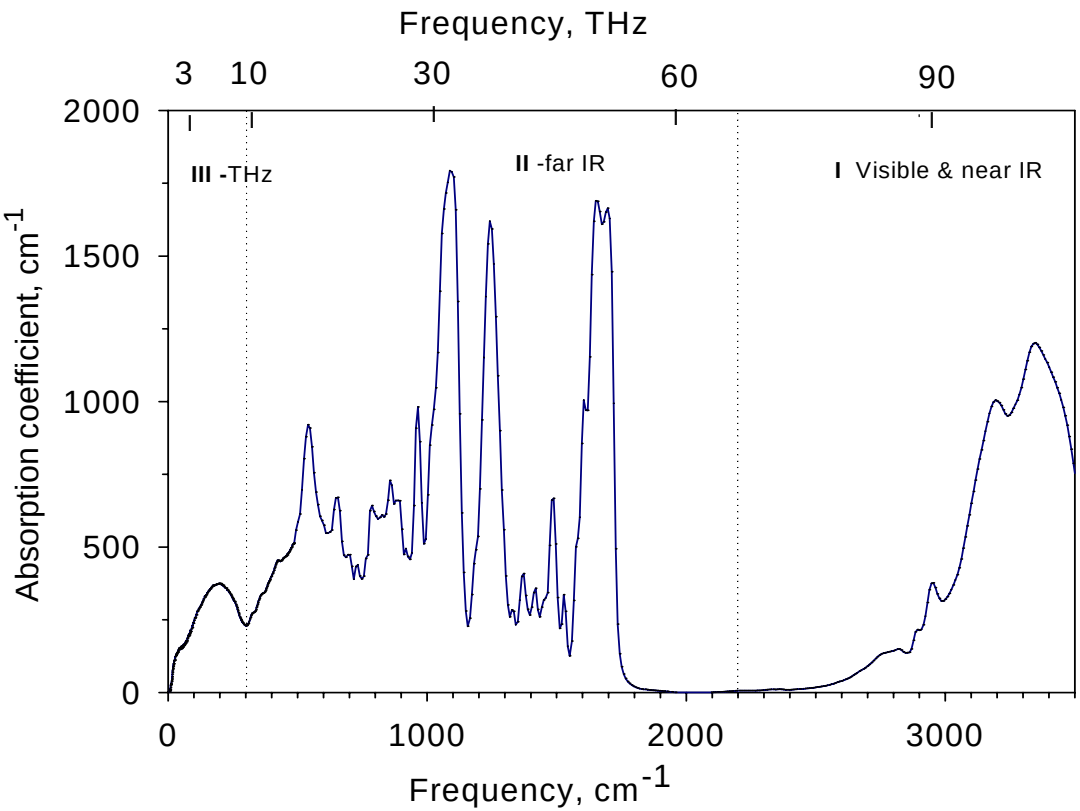
In FTIR spectroscopy, conformation sensitive the amide I band (1600-1700 $cm^{-1}$) is used to determine the type of the secondary structure of proteins.

Each type of secondary structures gives rise, in principle, to a different C-O strech frequency in the amide I region of the spectrum of the peptide linkages in the backbone structure.

Three analytical procedures are used: Fourier self-deconvolution, second derivative, and band curve-fitting. Assignments for deconvoluted Amide I subbands is based on X-ray data to determine the distribution of secondary structures.

The number of sub-bands determined by deconvolution is usually do not exceed 7 with the accuracy of analysis reducing for larger numbers.

# DNA Absorption Spectrum



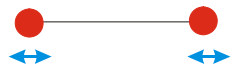
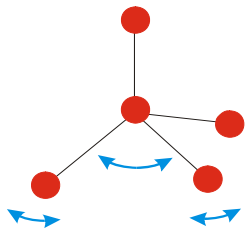
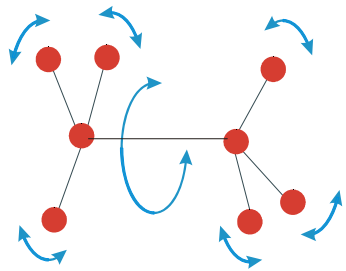

***Absorption spectrum of DNA*** *(our results).*

- **Regions I & II** (Studied well by IR & Raman)- resonances due to short-range, **high energy interactions**

- **in THz Region III – more species specific spectral features of bio molecules are found**

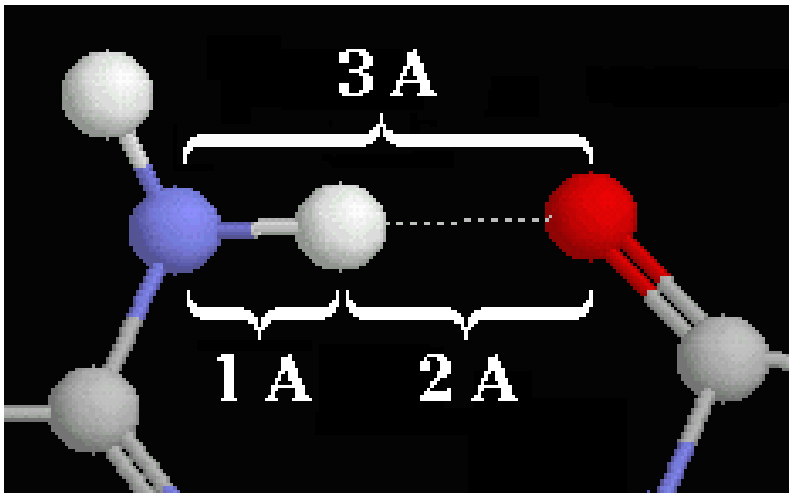
**FTIR** spectroscopy produces high quality spectra **in the region II,** and can **separate overlapping subcomponents** in the spectra

# INTERNAL MOLECULAR VIBRATIONS

| | Vibrations of | | Frequencies |
|---|---|---|---|
| | Bonds |  | >21 THz (700 $cm^{-1}$ ) |
| | Bond angles |  | 6-27 THz (200-900 $cm^{-1}$ ) |
| Region III | Torsion angles |  | < 9 THz **(<300 $cm^{-1}$ )** |

In the THZ region III **(0.1-10 THz or 2 - 300 $cm^{-1}$)**, **absorption spectra** reflect **low-frequency molecular internal motions or vibrations** involving **the weakest hydrogen bonds** and/**or non-bonded interactions** between different functional groups within molecules or even between molecules. The resonant frequencies of such motions – **phonon modes**- are strongly **dependent on molecular structure**

# WEAK HYDROGEN BONDS




Weakest hydrogen bonds, shown by dots:

-C-H ·····O-

-C-H ·····N-

-C ····· H-N-

-C ····· H-O-

-N-H·····O=C-

- **weak** and have only ~ 5% of the strength of covalent bonds

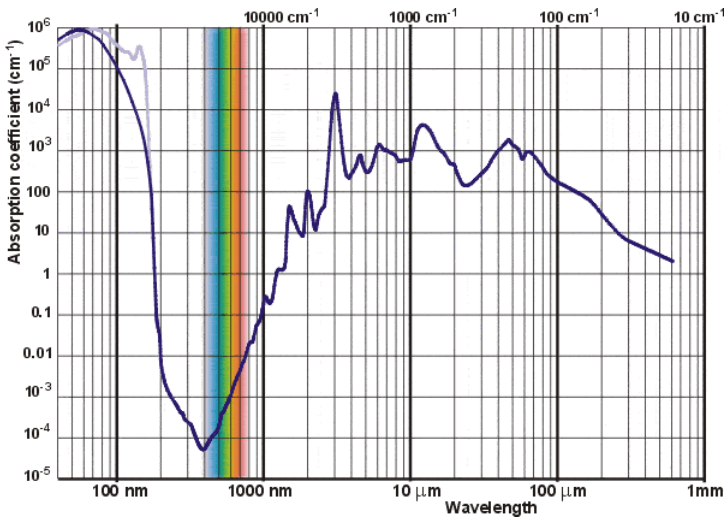
- **multiple** hydrogen bonds **stabilize the structure** of bio-polymers

- **hold** the **two strands** of the DNA double helix **together**, or hold polypeptides together in **different secondary structure conformations**.

# Why THz?

- **THz spectroscopy reveals structural information quite different from all other methods: it is the only technique that can directly detect weakest hydrogen bonds and other non-bonded interactions within biopolymers.**

**Liquid water absorption**




- **Less water absorption (at list 2 orders) compare to IR and far-IR. Less overlap** with water or other analytes **absorption bands. Liquid samples can be characterized.**
- **Absorption bands are more narrow in the THz range than in the IR and overlapping of neighboring bands is less.**

- **The availability of multiple resonances for the sensitive measurement of bio-molecule structure.**
- **Spectra are more species specific.**

# "THE WORLD OF THE DEAD OR OF FUTURE PUNISHMENT"

**M .N. Afsar &K.J.Button, "Infrared and Millimeter Waves," V.12, Acad. Press, 1984**

## "Terahertz gap"

The spectral range between the upper end of the microwave and the lower end of the extreme far IR

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- Low energy of sources.
- Low absorption of biological material requires samples with large area and thickness which is difficult to make because samples are too fragile.
- Poor reproducibility of experimental results due to multiple reflection in measurement systems, responsible for artificial features; difficulties in sample preparation, instability of material.
- The absence of good commercially available laboratory instruments
- Potentially promising laboratory techniques as time resolved spectroscopy and photomixing technology are only recently emerged

# Questions to answer:

- Is there something in the very far IR spectra? (initial prediction of vibrational modes in polymer DNA in the 1-100 cm-1 frequency range [ E.W.Prohofsky, K.C. Lu, L.L.Van Zandt and B. F. Putnam, Phys Lett., 70 a, 492 1979; K.V. Devi Prasad and E.W.Prohofsky, Biopolymers, 23,1795, 1984].

- What are the reasons why researchers for 20 years failed to achieve reproducible results? Experimental results are not reproducible and are contradictive. It was not clear what to expect. Can we improve the results?

- Can we use the observed features for DNA characterization, identification and discrimination between species?

- The key to answer all these questions: we need to know of what we are looking for.

# THEORETICAL PREDICTION OF THz ABSORPTION SPECTRA

Maria Bykhovskaia, B. Gelmont

IR active modes are calculated directly from the base pair sequence and topology of a molecule.

**Initial** approximation was generated and optimized by the **program packages JUMNA and LIGAND** (group of Prof. Lavery, Inst.Biologie Phys.Chim.Paris**).**

Energy minimum → Normal modes → Oscillator strengths → Spectra

**QUESTIONS:**

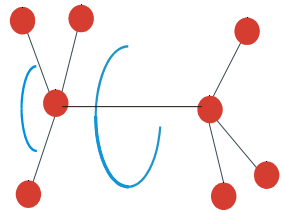
- What do we expect to find in the submillimeter wave range?
- What is the predictive power of the method?
- How sensitive are far IR absorption spectra to DNA structure?

Normal mode analysis is applicable to molecules with less than 30 base-pairs

# ENERGY MINIMIZATION AND NORMAL MODE ANALYSIS
(in internal coordinates of a molecule)

Maria Bykhovskaia, B. Gelmont

Molecular potential energy approximated as a function of dynamic variables(q): torsion and bond angles.



**Conformational energy**

including long distance interactions :

$$E_{total} = E_{Van\ der\ Waals} + E_{Electrostatic} + E_{HBonds} + E_{Torsion} + E_{Bond\ angles}$$

Van der Waals and electrostatic interactions; the energy of hydrogen bonds deformations; torsion rotation potentials; stretching deformations of bond angles and of bond length

Two **B-helical conformation DNA** fragments **(TA)12** with different base pair sequences:

AAAAAAAAAAAA
TTTTTTTTTTTT

ATATATATATAT
TATATATATATA

and the **A-helix of double stranded RNA Poly[C]·Poly[G]**

# LOW FREQUENCY NORMAL MODES

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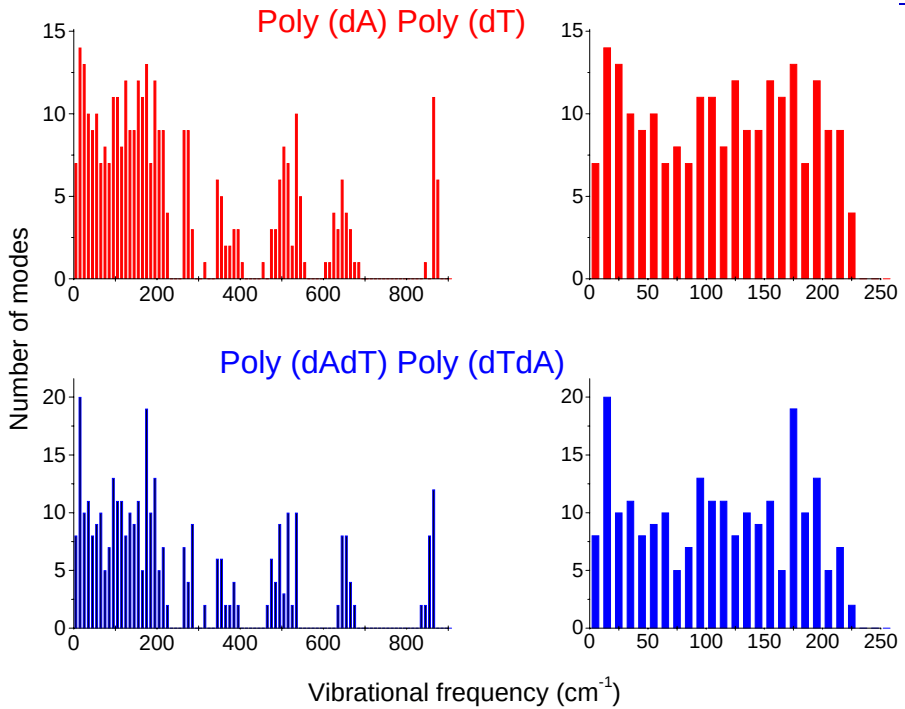
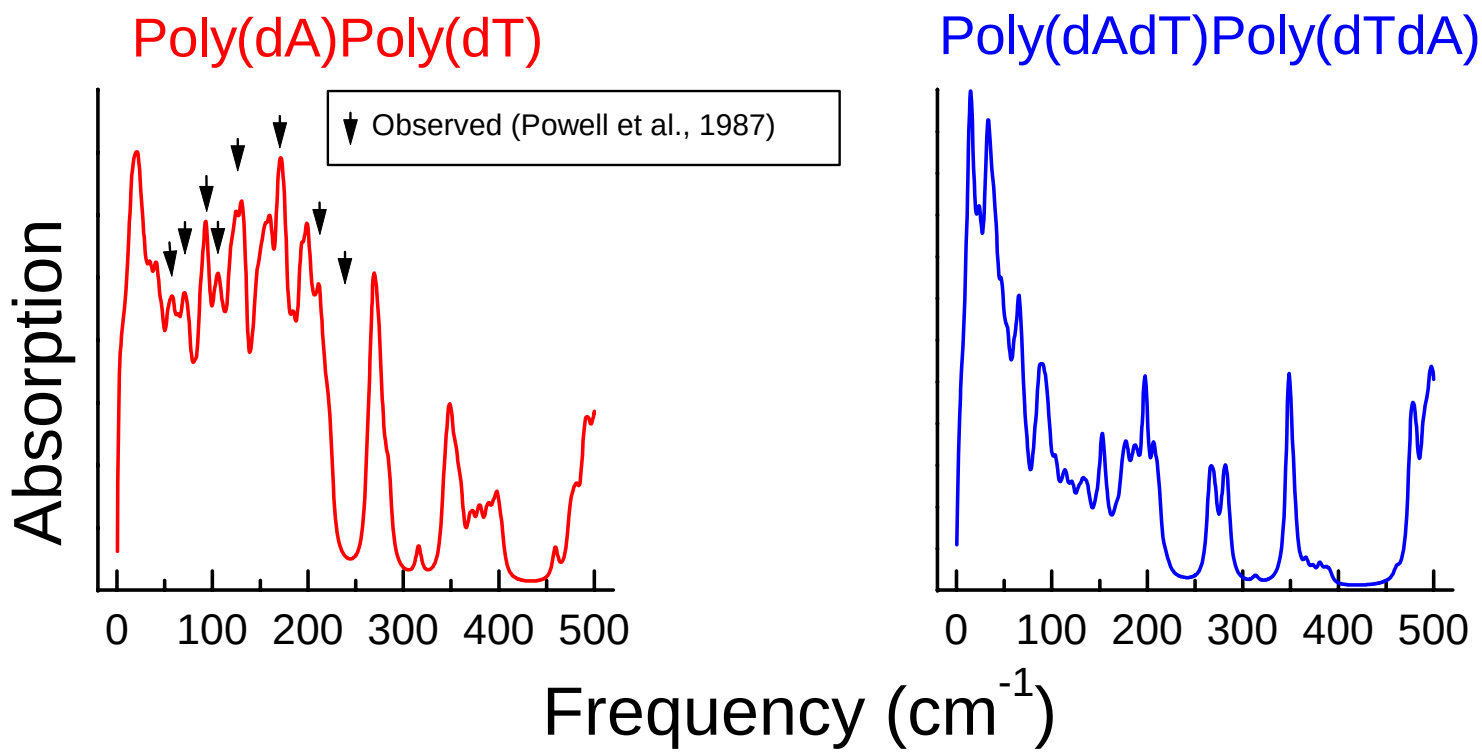



360 normal modes were found for each sequence with the density higher than 1 mode per $cm^{-1}$. There is almost no overlap of weak bond modes with vibrations of covalent bonds which have frequencies above 750 $cm^{-1}$.

# Absorption Spectra vs. Base Pair Sequence

$\alpha(\omega) \sim \gamma\omega^2 \sum (p^k)^2 / ( (\omega_k^2 - \omega^2)^2 + \gamma^2 \omega^2 )$, the oscillator decay $\gamma_k = 2\ cm^{-1}$ ,

the dipole moment $\mathbf{p} = \sum_i e_i \mathbf{a}_i / \sqrt{m_i}$ ,




The **spectrum of optical activities** is very **sensitive to the DNA base pair sequence**



Maria Bykhovskaia, B. Gelmont

# Experiment and Modeling

Many of the initial successful measurements of the THz absorption properties of biological materials have been performed at the University of Virginia.

**Evidence** of multiple **resonances** in THz transmission **spectra** with a high degree **confidence in recognition of bio- molecules** has been demonstrated.

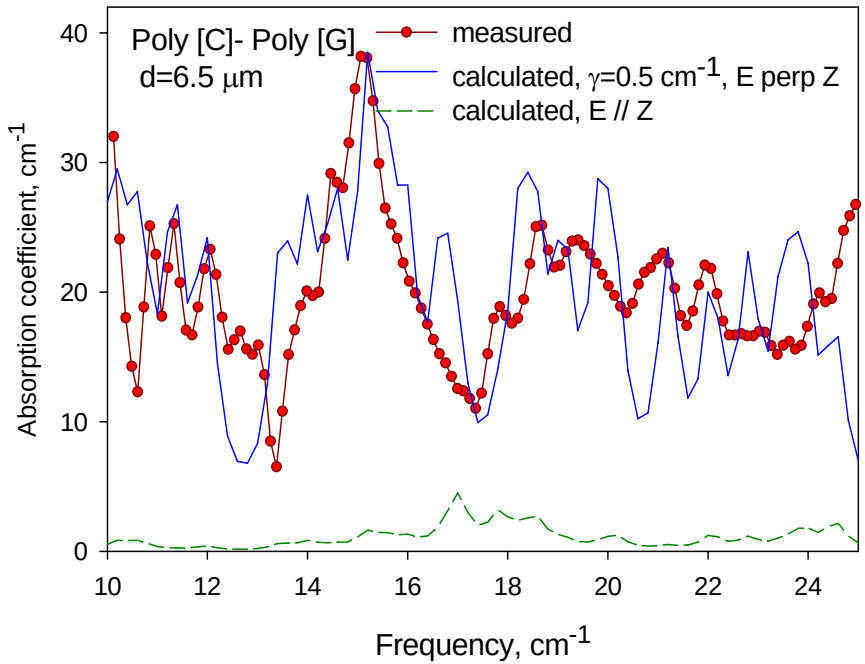
**Direct comparison of experimental spectrum** (red) **with theoretical prediction** (blue) **for a short chain DNA fragment with known structure.**

Reasonably **Good correlation validates both, experimental and theoretical results**.

**From the width of the vibrational modes:**

**oscillator decay** $\gamma = 0.5\ cm^{-1}$

**relaxation time** $\tau = 7\ 10^{-11}$ **s**



# Experimental set up

- Bruker IFS-66 spectrometer (Hg- lamp source, He cooled Si-bolometer @ 1.7 º K). Vacuum systems are not shown.

- Attachment for reflection measurements.
- Resolution 0.2 $cm^{-1}$.
- Range of interest throughout 10 $cm^{-1}$ – 25 $cm^{-1}$.

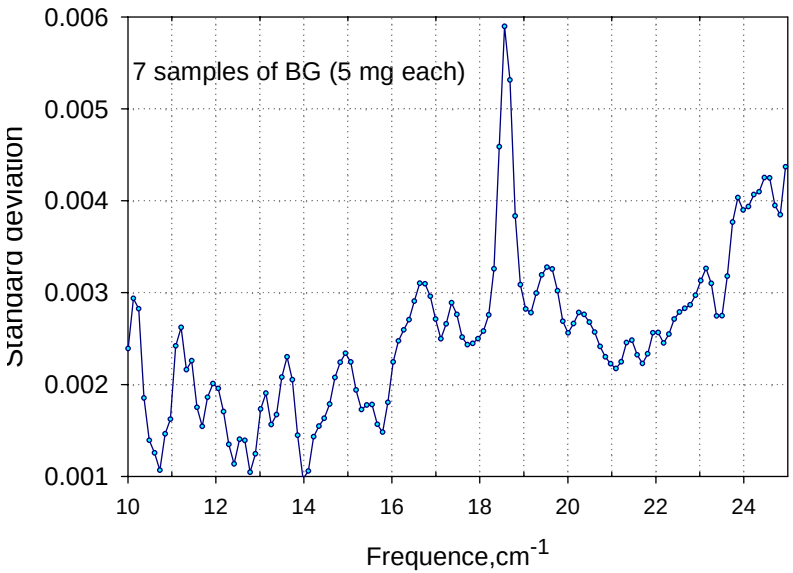
# THz Fourier-Transform (FT) spectroscopy

## What is important?

Good Instrument performance:

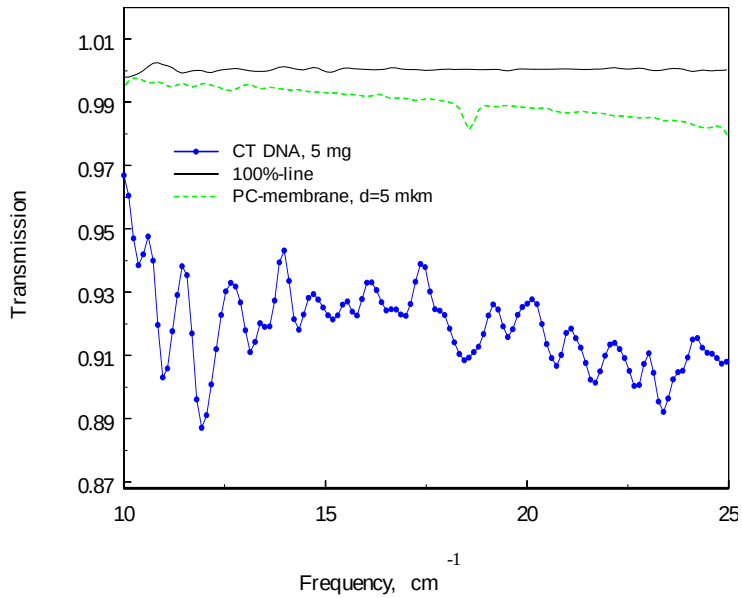
- **Spectral resolution at least 0.25 $cm^{-1}$ to measure features with 0.5 $cm^{-1}$ band width**
- **High sensitivity (signal to noise) and reproducibility to provide standard deviation better than 0.3% to measure small signals**

**Sensitivity Resolution**




7 different samples

- **High transparency of substrates**

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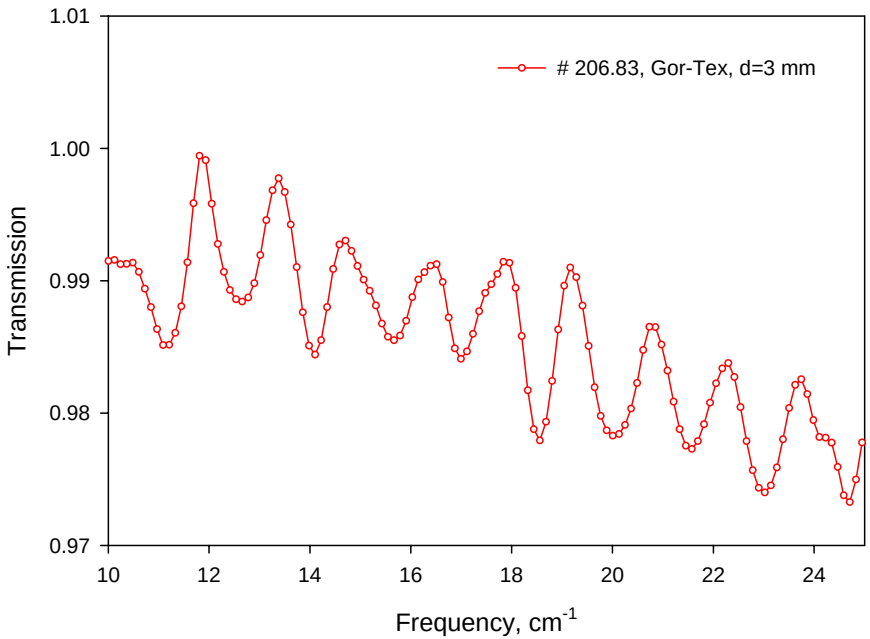
# Challenging Problem

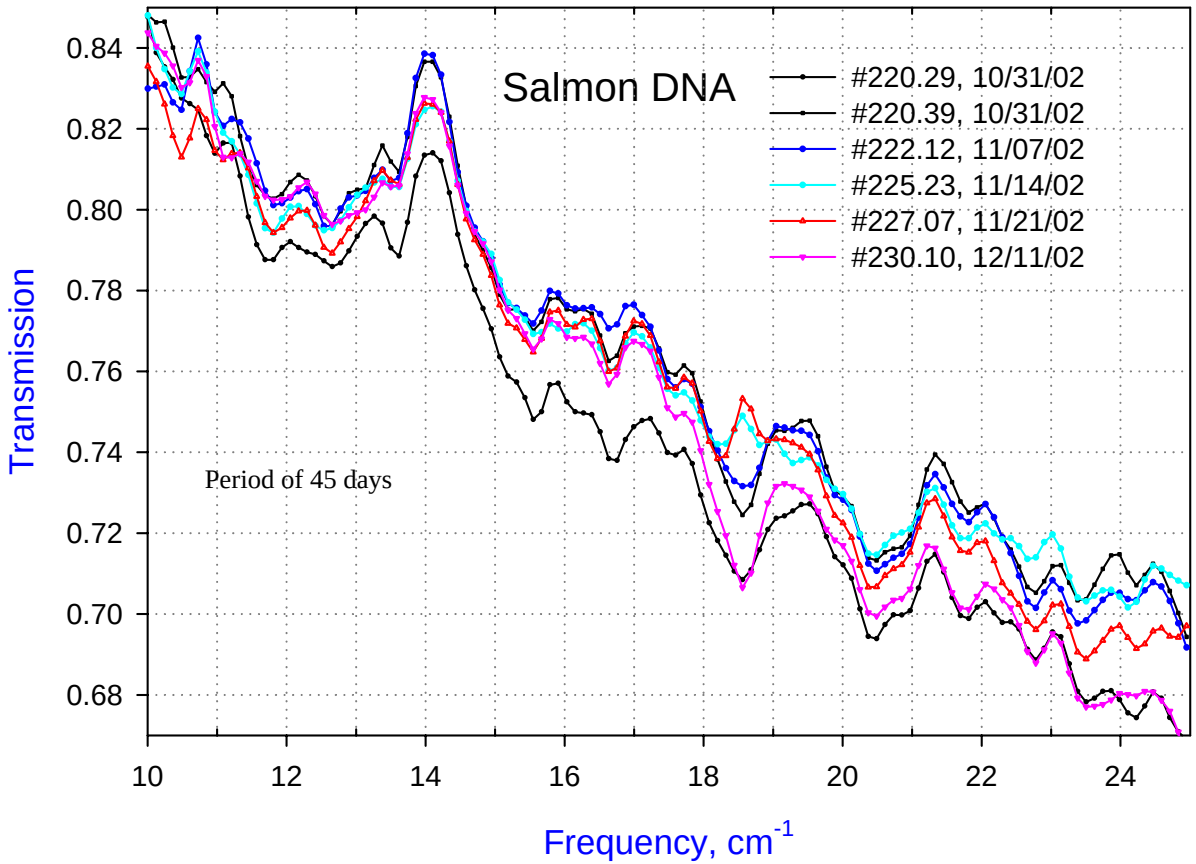
Serious problem at THz **- all kinds of multiple reflections** or **standing waves** in most of measurement systems because of large wavelengths of radiation.
These effects cause **artificial false resonances**.




**Check for false resonances using thick plates from material with low absorption is important. Ideally spectrum is close to cos form.**

# Long term reproducibility

- Improved technique for solid film sample preparation:
  - good alignment
  - reduced amount of material from 15-20 mg to 1- 3 mg for one sample
  - reproducibility better than 0.5%

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# Material for study

**Hen egg white lysozyme (HEWL)** has been purchased from Sigma (97% pure, balance primarily sodium acetate; catalog no. L6876; lot 65H7025).

## ❑ Salt precipitation

➢ **Native** lysozyme, HEWL was resuspended in 0.1M phosphate buffer to a protein concentration of 50-150 mg/mL.

➢**Unfolded** lysozyme have been prepared by **salt (KSCN) precipitation**. HEWL was resuspended in 0.1M phosphate buffer to a protein concentration of 50 mg/mL and then KSCN was added (0.2M in solution, pH adjusted to 2.14 using 1M HCl) **to induce unfolding and precipitation** to form slurry.

➢**Refolded** lysozyme by **removing of KSCN** from slurry of unfolded lysozyme by centrifuging, **dissolving in 0.1M sodium acetate** buffer solution (pH 3.8 with 1M NaOH), second centrifuging to remove precipitate, ultrafiltration and desalting using size exclusion chromatography.

# Lysozyme Denaturation. Sample Prep.

**Two standard procedures** were used to prepare samples in unfolded conformation:

- **The thermal denaturation** at $95^0$C for at least two hours.

In water the secondary structure of lysozyme undergoes a cooperative thermal unfolding transition .

- **Denaturation by dissolving in 6M guanidine cloride (GuHCl),**

pH 4.6, **results in substantially unfolding state** in which **little** persists **secondary or tertiary structure** and eliminates refolding process in unfolded lysozyme.

Simple techniques has been developed to fabricate large area, thin, stable samples

Thin, **air dried** protein **films** were prepared **on polycarbonate** (PC) filter membrane. **Heat denaturated** lysozyme was **drying at 95°C**. Material in the **form of gel** have been prepared by placing 100-200 μl of **solution between two polyethylene films** (PE )and stored in the freezer at -20 °C

# Material texture

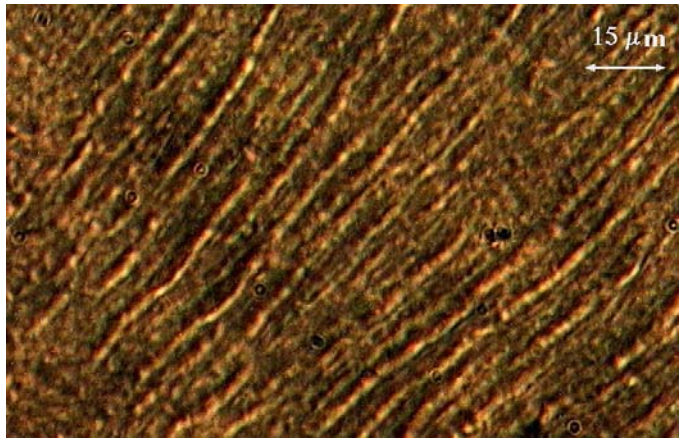



Image of the Salmon DNA sample in polarizing microscope (free standing).

Film **thickness** about **10 μm**.

Gel concentration 1:10.

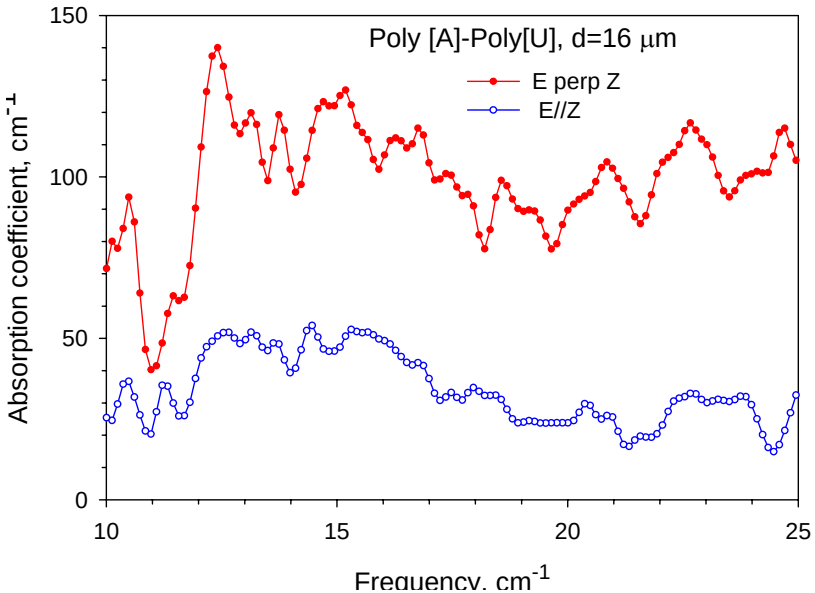
DNA, as a **rod-like polymer**, **spontaneously** forms **ordered liquid crystalline phases** in aqueous solution with the **long molecular axis preferentially aligned in one direction.**

In drying process, DNA solution undergoes a series of transitions and **film samples are characterized by their microscopic textures** with periodic variations in refractive index and **fringe patterns observed in polarizing microscope.**

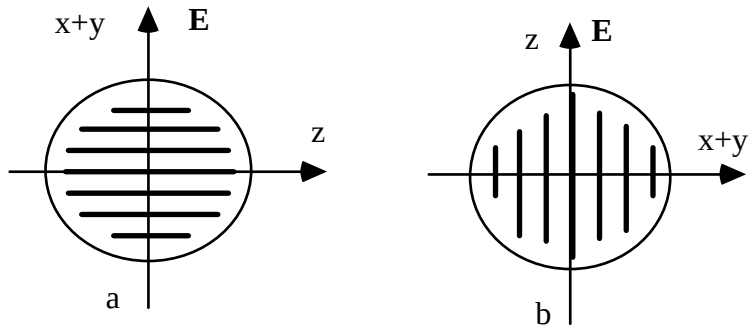
The film **texture** depends on the **concentration of molecules in solution and on drying conditions.**

# Change with sample rotation.

- Documented **strong anisotropy of optical characteristics** of biological molecules at THz.



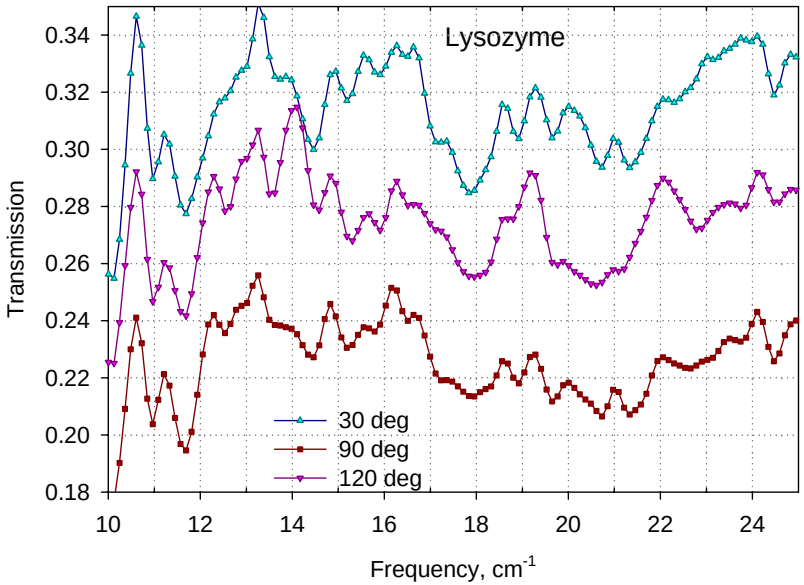

**Absorption coefficient at two orientations**




Sample position with electric field (a) perpendicular and (b) parallel to the long-axis of the molecule z.

# THz spectra of Biopolymers in water (gel)

Importance: **all living matter is in a liquid form**

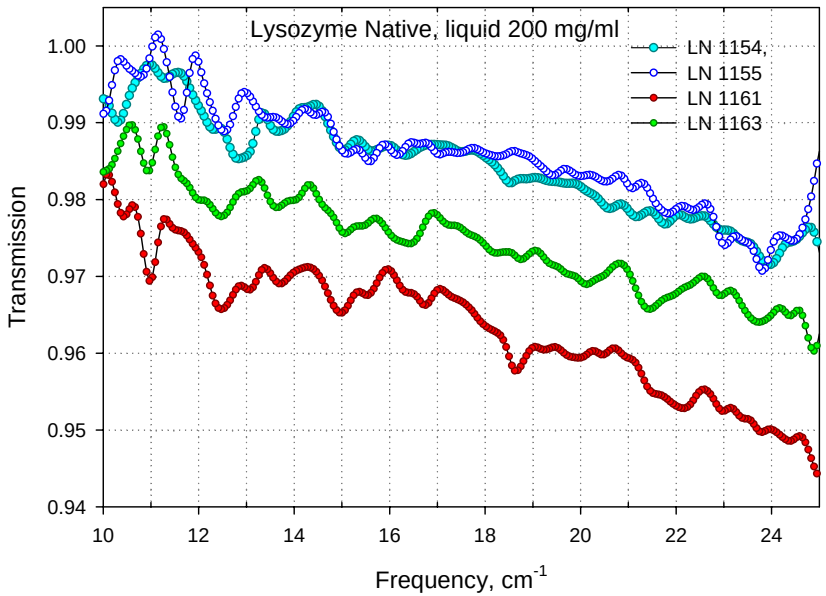



Disturbance at 18.6 $cm^{-1}$ is due to absorption of water vapor in air.

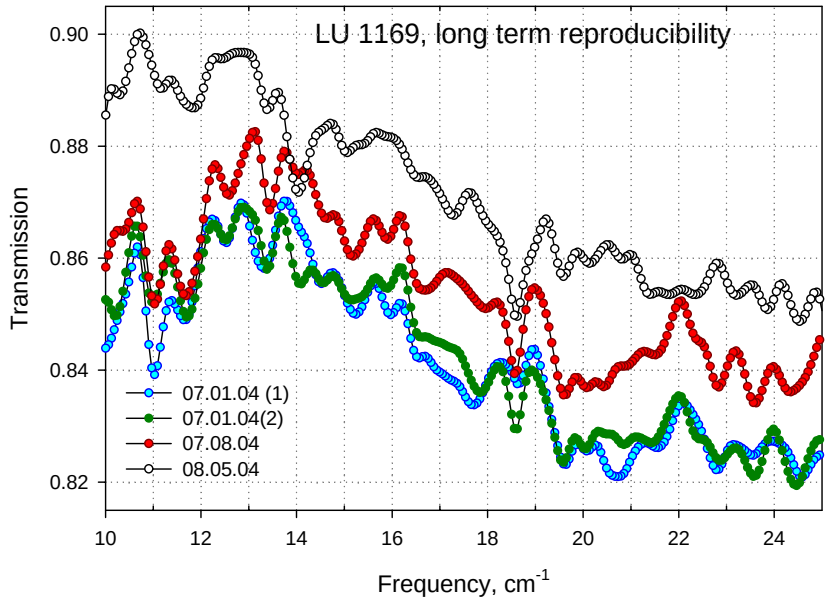
- Biological materials in **an aqueous form (or gel)** can be characterized as well as in solids.
- **Signature is strong.** Relative **change in the peak** up to 10-30%.
- **Spectra are not disturbed** by water absorption **at THz (except at 18.6 $cm^{-1}$)**.
- **High sensitivity of spectra to orientation**.

- Possible applications: **structural characterization of proteins and DNA at THz; monitoring biological processes.**

# LYSOZYME Native and Thermally Unfolded




*4 samples of LN by diluted HCl*




*Spectra of thermally denaturated (LU) lysozyme*

A big **difference** observed between transmission **spectra of native lysozyme** obtained by diluted HCl and **thermally denaturated** lysozyme with the same amount of dry material

- Resonance **structure** in four **folded samples (LN)** in **diluted HCl** is very **weak** with the intensity of peaks ~0.5% - 1%, and the probability of errors is high.
- **Unfolded lysozyme has higher absorption** (less transmission) with **higher intensity of resonances.** Spectra of unfolded samples demonstrate a good long term reproducibility at the same orientation. However, after storing for a month, significant changes in the measured spectrum observed.

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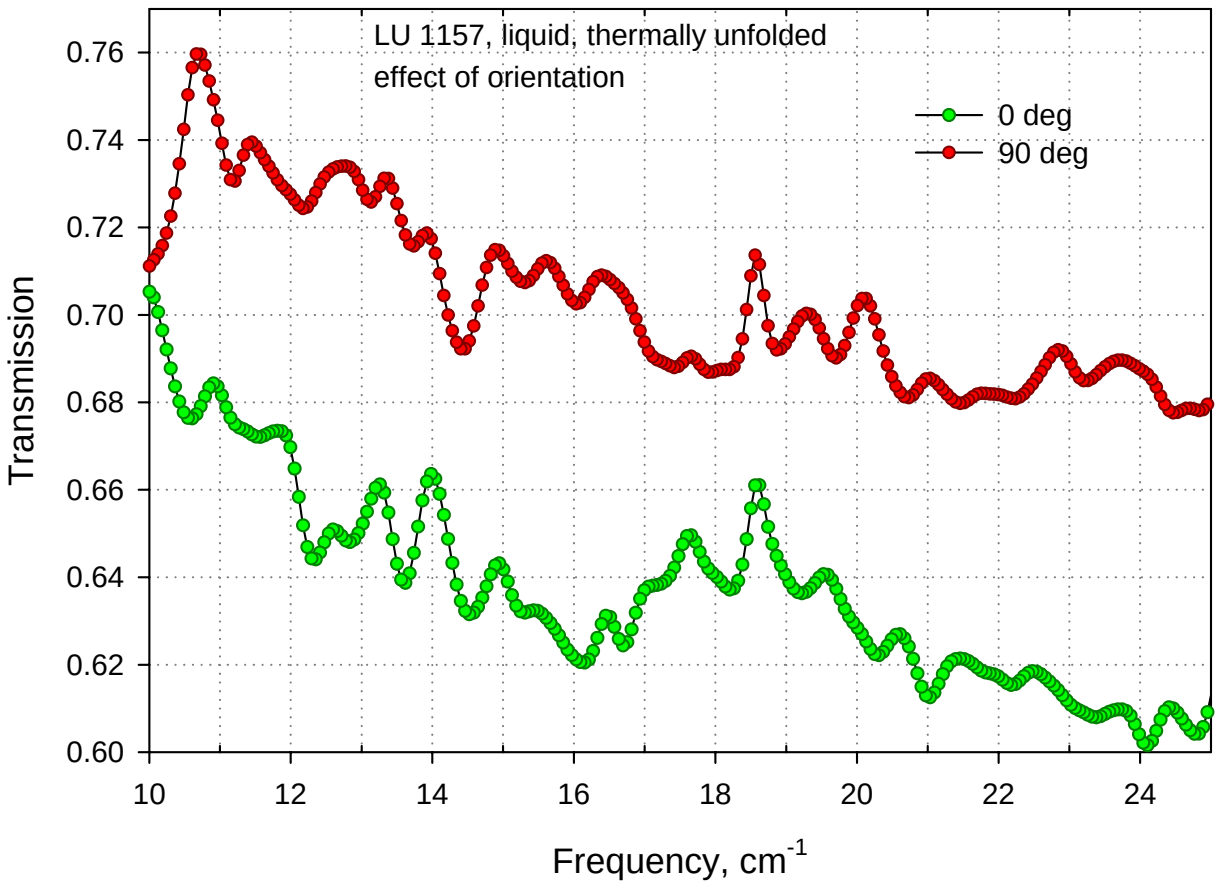
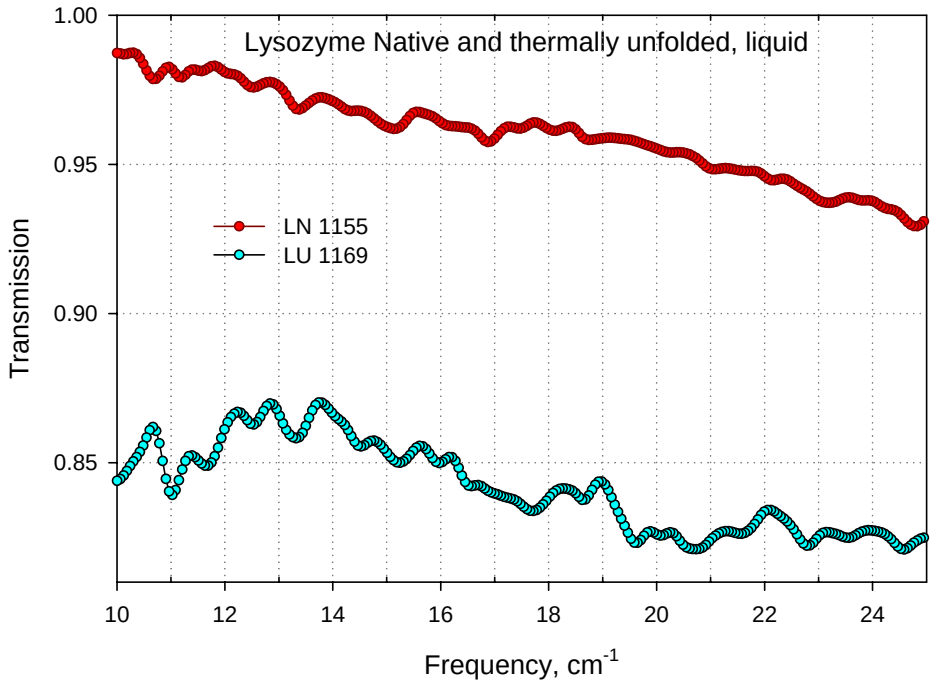
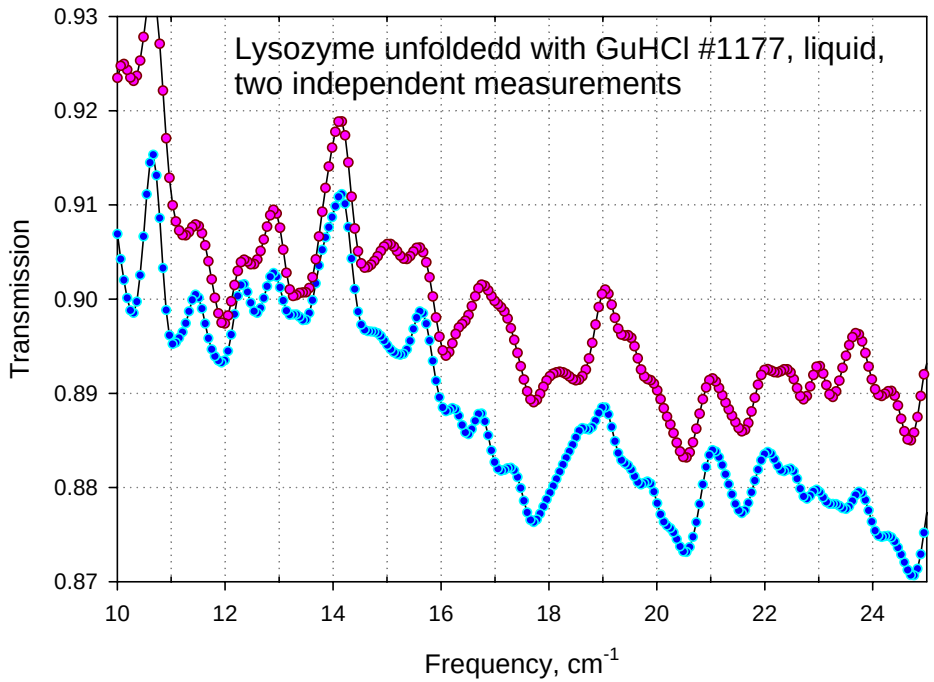
# LYSOZYME Thermally Denaturated (Unfolded)




***Spectra of unfolded lysozyme are sensitive to orientation.***

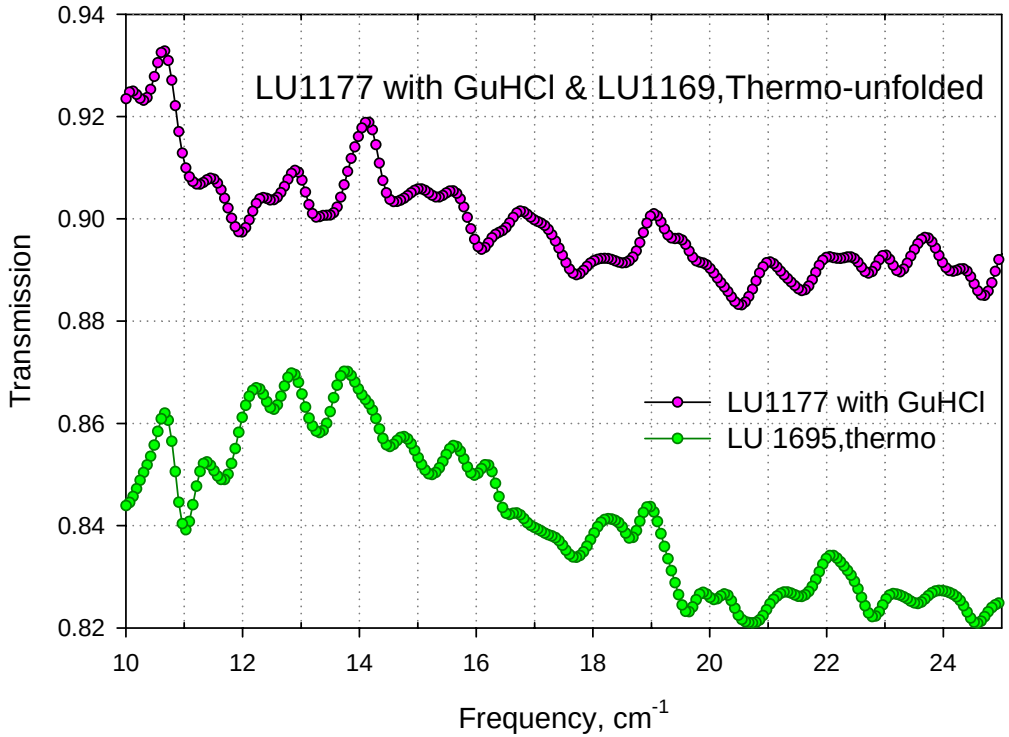
# LYSOZYME Native and Denaturated with *GuHCl*




Spectra of a lysozyme sample **unfolded with GuHCl** differ from **thermally unfolded.**

# LYSOZYME Unfolded with *GuHCl* and thermo-Unfolded




*Spectra of thermally unfolded and GuHCl unfolded lysozymes.*

2 spectra show very close correlation with almost all resonant frequencies present in both cases, however there are some differences: clearly additional absorption peak at 19.6 $cm^{-1}$ in thermo-denaturated sample, and difference between two at frequencies 23-24 $cm^{-1}$.

# Possible applications of proteins THz spectroscopy

Wide-range of biomedical applications based on close relationship between structure and spectra, including **monitoring changes in molecular conformation in real time**

- real-time analysis of protein binding for transport
- protein binding with antibodies,
- specificity interactions between proteins and nucleic acids
- binding stability studies of drug-protein and vitamin-protein systems
- disease diagnostic

# Conclusions

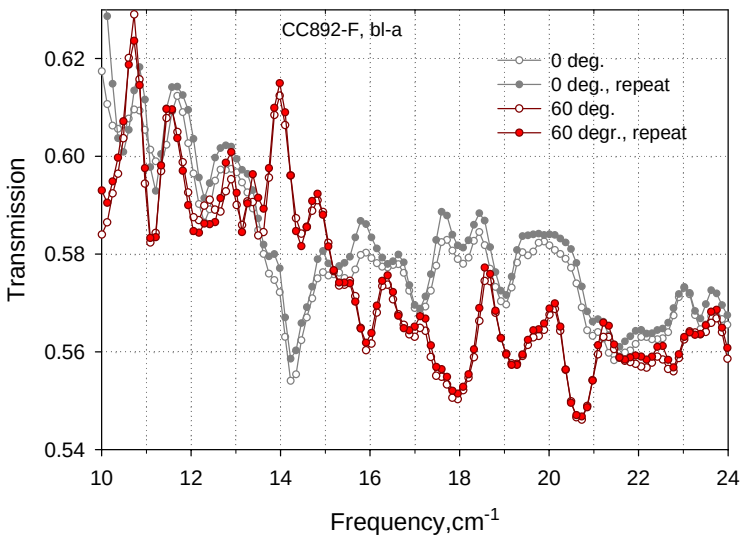
- THz spectra are sensitive to conformation change in proteins.

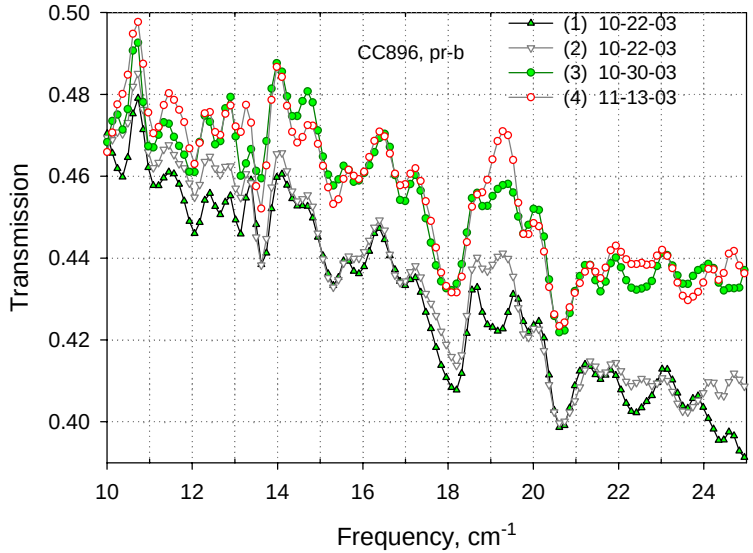
- Absorption of unfolded lysozyme is higher in comparizon with that of a native lysozyme.
- Spectra of unfolded lysozyme are much more sensitive to orientation.
- Resonance features in THz spectra of unfolded lysozyme are stronger and differ in frequencies from those of native lysozyme.

- The sensitivity of THz spectroscopy is high enough to discriminate between unfolded samples prepared by different techniques reflecting different degree of unfolding process.

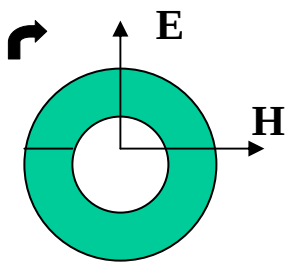
- THz spectroscopy apparently can be used for monitoring folding-unfolding process of proteins.

# Experimental Results on Cancer Cells




Typical transmission spectra of cancer cells. **Resonance structure** is **sensitive** to **orientation** of a sample **relative to the electric field** of radiation.

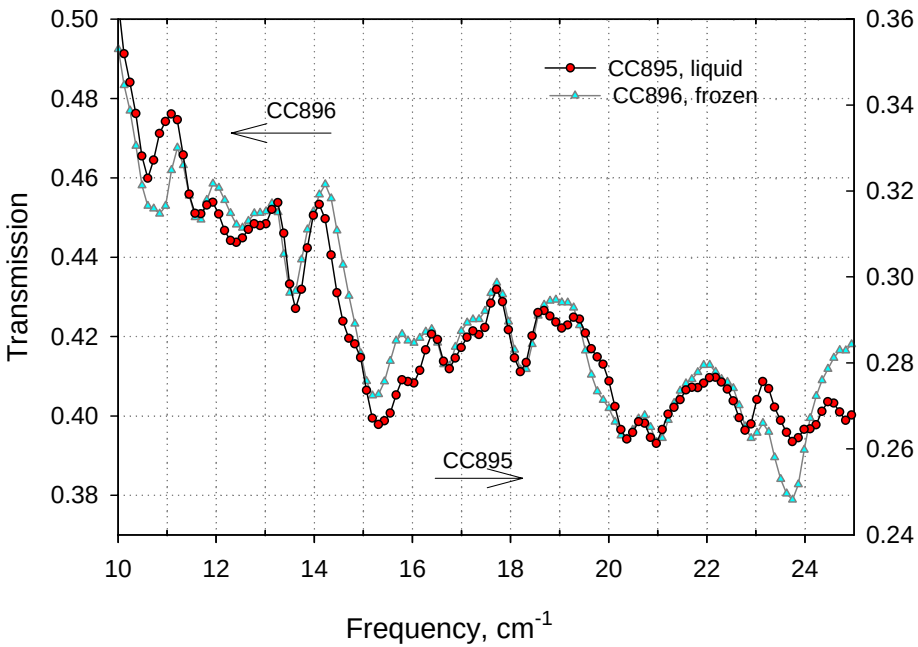



**Long term reproducibility** of transmission spectra of prostate cancer cells measured **at the same orientation** after storage of the sample in frozen condition **for several weeks**.

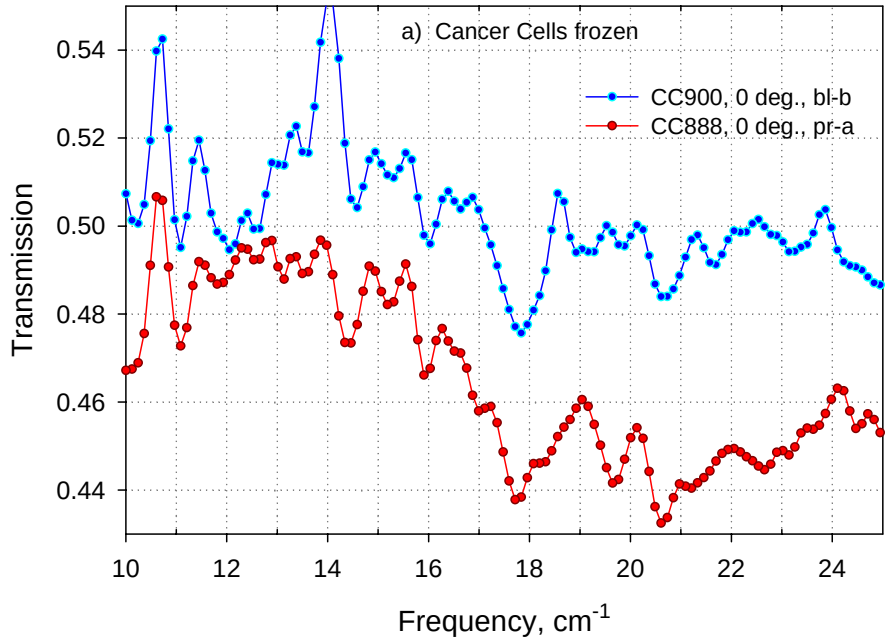
Radiation is 80% polarized

# Prostate and Bladder Cancer Cells

Structure around 14 cm-1 have been chosen to control material orientation, and spectra from different samples **having the same orientation** have been **compared using the structure at 14 cm$^{-1}$ as a key**.




**Liquid and frozen samples** measured at close orientation have **a similar pattern.**




Spectra of prostate and bladder cancer cells

There are **noticeable difference** in spectra of prostate and bladder cancer cells:

# CONCLUSIONS

- **Cancer cells revealed vibrational modes** in THz transmission spectra with **the resonance structure that is sensitive to the orientation** of cells relative to the electromagnetic field of radiation.

- **Spectral features,** like position of transmission minima which correspond to the maxima of absorption, demonstrated **good long-term reproducibility** and had almost the same pattern after storage of samples for 2 weeks at 0° C or below **when** sample **orientation** was maintained **a constant**.

- Comparison of transmission spectra for a **prostate** and a **bladder** cancer **cell lines** measured **at the "same orientation"** revealed **spectral features** which can be used **to discriminate between** them.

- **THz spectroscopy** appears to be **a promising approach** toward the **characterization of human tumor histology**, and with further refinement, may be **capable of discriminating between different tumor phenotypes** and biologic potential.

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