Terahertz Dielectric Sensitivity to Biomolecular Structure and Function

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(Invited Paper)

***Abstract*—A review of recent terahertz (THz) dielectric response measurements on large biomolecules is presented. The origin of the dielectric response in this frequency range is discussed. We specifically address the questions of the relative contributions of collective vibrational modes and relaxational contributions. When sample uniformity is controlled and multiple reflection effects included in the analysis, the overall dielectric response of large biomolecules in the THz range is broad and featureless. The response, however, is highly sensitive to hydration, temperature, binding, and conformational change. The origin of these effects is discussed with emphasis on the contribution of surface side chain relaxational loss and how this might be affected by environment, structure, and function.**

***Index Terms*—Biomolecules, biosensing, conformational dynamics, DNA, far infrared, proteins, terahertz (THz), T-ray.**

I. Introduction

With the advent of terahertz (THz) time-domain spectroscopy (TDS) [1], [2], the research community has taken a renewed interest in investigating far-infrared response of biomolecules. The interest primarily stems from the prediction of collective structural vibrational modes at THz frequencies. These modes involving large-scale motion of entire subunits of the macromolecule are dictated by structure and have long been discussed as the dynamics leading to conformational change and biomolecular function. These modes were initially predicted almost 40 years ago based on periodic type modeling for DNA [3]–[9]. In the 1980s, molecular modeling with semiempirical force fields brought the advent of normal mode analysis of large biomolecules such as proteins and short-chain polynucleotides [10], [11]. In some cases, *ab initio* calculations have also been performed on small proteins [12]. Spectroscopic measurement and identification of these collective modes would impact both basic understanding of biomolecular interaction and biosensing applications. Biomolecular function involves structural dynamics to accomplish induced fit binding and to overcome structural barriers to ligand access to the binding site, such as epitomized by molecular oxygen access to the heme group embedded in myoglobin [13]. Recently, it has been shown that major conformational change occurring in enzymes when ligands bind can be well described by a linear combination of only the first few normal vibrational modes of the structure [14]. This suggests that, to understand the function of a particular protein, one would want to characterize these modes and determine how they are manipulated by allosteric interactions and environment. In addition, a protein may have a unique collective vibrational spectrum; thus, analytical identification of biomolecules could be possible.

Various authors contemporary with the early calculations attempted measurements using conventional far-infrared or THz sources ($\lambda = 100\ \mu$m–1 cm, $\nu = 0.03$–3 THz) and spectroscopy systems [15]–[21]. These measurements could be challenging on their own; so, systematic sample variation was rarely attempted, and in some cases, multiple reflection artifacts were misidentified as vibrational resonances. In the early 1990s, THz TDS was introduced [1], [2]. This method of spectroscopy, sometimes referred to as T-ray spectroscopy, allows for direct determination of the complex permittivity over a broad bandwidth (0.1–8.0 THz, [22]) without cryogens and with highly flexible optics and sample control. We note that while systems have demonstrated higher bandwidths, (up to 100 THz [23]), high-precision spectroscopy has been limited to 8 THz and less. Typical systems have a 0.2–3.0 THz bandwidth. This has allowed investigators to examine the complex dielectric response as a function of system size, environment, and function. Fast motions in the picosecond range correspond to collective vibrational modes or very fast small conformational changes. A variety of techniques can be used to study these modes including Raman scattering [24]–[27] and optically heterodyne-detected optical Kerr-effect (OHD-OKE) spectroscopy [28], [29]. The vibrations can be dipole active, and thus, probed using THz dielectric spectroscopy. The dielectric response of the system is related to harmonic and relaxational processes through the dielectric response $\varepsilon(\omega)$:

$$\varepsilon(\omega) = \varepsilon_o + \int \frac{f(\omega')g(\omega')}{(\omega'^2 - \omega^2) + i\gamma(\omega')\omega} d\omega' + \varepsilon_r \int_0^\infty \frac{h(\tau)d\tau}{1 + i\omega\tau} \tag{1}$$

where ε_o is the dc dielectric constant. The second term on the right-hand side refers to a sum of harmonic oscillators with density of states $g(\omega)$, oscillator strength $f(\omega)$, and damping coefficient $\gamma(\omega)$. The third term refers to relaxational processes, assuming a distribution of Debye relaxations $h(\tau)$ and net weighting ε_r. Many investigators have focused mainly on the resonant response, that is, structural vibrational modes with coherent motion of hundreds of atoms. The relaxational response picture is motivated by the observation of a smooth absorption coefficient increasing with frequency reminiscent of the dielectric response

Manuscript received September 15, 2007. This work was supported in part by the ACS Grant PRF 39554-AC6, in part by the NSF CAREER Grant PHY-0349256, in part by the NSF REU Grant DMR-0243833, and in part by the NSF IGERT Grant DGE0114330.

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Digital Object Identifier 10.1109/JSTQE.2007.913424

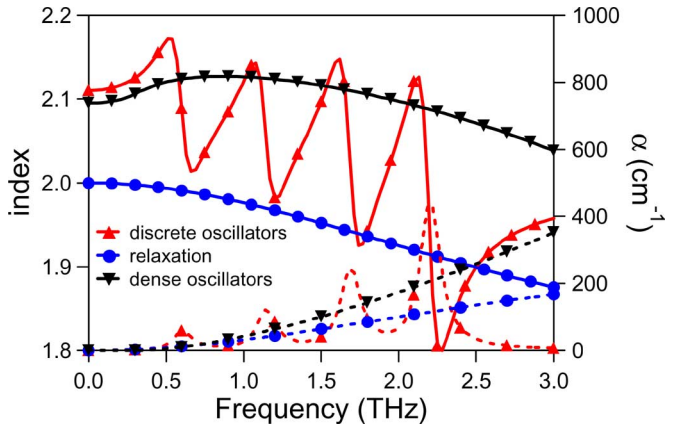

Fig. 1. Comparison of frequency-dependent index (solid lines) and absorption coefficient (dashed lines) for resonant oscillators and a single relaxation response. The resonant response is shown for both widely spaced resonances (▲), a dense oscillator density of states (▼), and a single Debye relaxation (●).

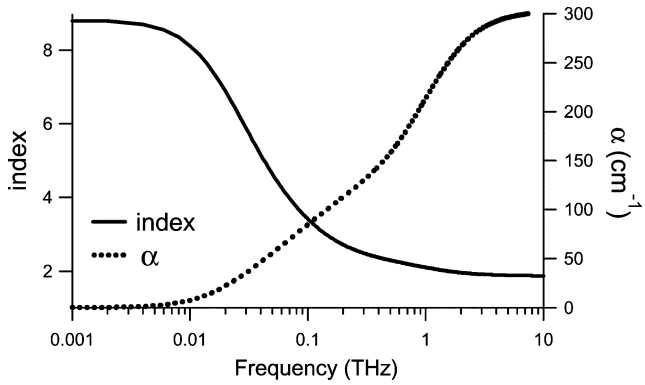

Fig. 2. Bulk water dielectric response using a double Debye model as found in [44].

of glasses [30]. Glasses do not have the phonon spectra seen for crystals as they lack the long range order; however, localized molecular groups with weak hydrogen bonds will respond to an applied field by the alignment of the local dipole [31]. The time for alignment is dependent on the energy necessary to break and reform bonds associated with the rotation. In biological molecules, the amino acid side chains are candidates for this type of dielectric response as the interaction between side chains is through weak hydrogen bonding. The notable exceptions being cysteine disulfide bonds and bonding with embedded chromophores. Karplus and coworkers, using molecular dynamic simulations, examined the residence times for different rotamers within protein fragments, finding residence times as short as $\tau_{\rm res} \sim 5.7$ ps [32]. This residence time can be considered the average time it takes the side chain to jump to a new configuration. This time is a rough approximation to the relaxation time, as discussed by Kauzmann [31]. In Fig. 1, we demonstrate the different frequency dependence of the index n and absorption coefficient α arising from resonant oscillators versus relaxation response. Two resonant oscillator examples are shown: one with only a few widely spaced resonances and a second with a dense distribution of oscillators with increasing density with frequency and a frequency-independent oscillator strength [33] in agreement with recent calculations of the single molecule absorbance of the proteins bacteriorhodopsin (BR) and rhodopsin [34]. For the relaxational response, we use a single Debye relaxation with relaxation time ~2 ps consistent with the Karplus calculations. The figure illustrates that frequency dependence of α is similar for the relaxational and dense resonant oscillator model, and it would be difficult to distinguish the two in the measured α. The frequency-dependent index, on the other hand, is somewhat more distinct. Whereas the index of the dense resonant oscillator model is increasing with frequency at low frequencies, the index is never increasing for the pure relaxational response. This is demonstrated again in Fig. 2 where a sum of two relaxational processes model the bulk water response. As we will discuss, THz TDS determines the complete frequency-dependent complex dielectric response allowing for a direct comparison between the models and the measured result.

In this review, the protocols for performing such measurements and the current understanding based on the measurements undertaken to date are briefly summarized. Efforts were made to include as many contributors as possible, and the author apologizes for any oversight in this regard. An in-depth review of the general spectroscopy of biomolecules, peptides, single nucleotides, short-chain polypeptides, short-chain polynucleotides, chromophores, and pharmaceuticals can be found in an upcoming review by Markelz and Jepsen [35], as well as general aspects of molecular modeling as applied to THz measurements of biomolecules. A review of THz spectroscopy methods can be found in Mittleman's book [36].

II. Materials and Methods

All THz data discussed in this review were confined to that taken in the last 20 years. These data were acquired using either THz TDS or Fourier transform interferometry (FTIR) with broadband sources. FTIR measurements, to some extent, have included the use of bright synchrontron sources [37]. Measurements have also been performed with narrow-band high-power lasers such as free electron lasers [38], [39] and tunable p-Ge lasers. While FTIR can cover very broad frequency ranges, often the optics of commercial systems are not sufficiently robust or flexible to allow for novel measurements of THz response dependence on protein conformation in hydrated environments or to accommodate secondary probes to monitor secondary and tertiary structure during the measurement. Bolometric detection that is standard with FTIR can be cumbersome and requires cryogens, on top of the already formidable task of care for the time and environmentally fragile samples.

THz TDS can, in principle, cover a very broad bandwidth and has very flexible optics allowing for numerous optical pumps and probes of the biomolecular samples for excitation and monitoring during the THz measurement. A significant strength of THz TDS is the acquiring of the magnitude and the phase of the transmitted electric field. This allows for determination of the absorption coefficient and index directly from the magnitude of the transmission and the phase. The complete determination of the complex permittivity allows for modeling the response and places some limits on the extent that collective mode

response must be included or excluded from the analysis [40]. In addition to the complex dielectric response characterization, the pulsed THz spectroscopy allows for time-resolved measurements where, conceivably, the evolution of the collective modes with conformational change can be monitored in real time by synchronizing the THz spectroscopy with the conformational change initiator. For example, visible pump/THz probe measurements could be undertaken for photoactive protein systems. This technique has not been strongly explored to date [41], but with increasing sophistication in sample preparation and experimental design will enable further exploitation of time-resolved THz measurements.

With regards to sample preparation, biology occurs in water. While the nature of water in cells is certainly not yet clear, many studies suggest that it is at least in part bulk like. This poses a challenge for THz measurements of biological systems as molecular rotation time in bulk water is $\sim$1 ps, and water has an 80 Debye dipole moment [42]. These lead to substantial dielectric relaxational loss in the THz range [43]. Typically, authors describe the dielectric response of bulk water for biological systems with a single Debye response, with $\varepsilon(\omega) = \varepsilon_\infty + (\varepsilon_{\rm dc} - \varepsilon_\infty)/(1 + i\omega\tau)$. However, recent measurements have suggested that two relaxation times are required to accurately reproduce the results [44]. This response is shown in Fig. 2.

As seen in the figure, the imaginary part of the permittivity is substantial at THz frequencies; thus, most measurements of protein solutions have a higher transmission than neat buffers simply because the solvent is displaced from the interaction path by the protein [38], [39]. Recently, careful measurements of this effect have suggested that, in fact, one can use THz measurements to determine the effective extent of the so-called biological water from the surface of the biomolecule [45]. However, if one is interested in measuring the THz response of the biomolecule itself and not primarily the buffer, then, in general, the total amount of water in the sample is limited. There are several methods that various authors have used to achieve this. The most popular is using lyophilized, or freeze-dried powders [30]. It has been demonstrated that, in some cases, lyophilization changes structure and removes function [46]. In some cases, lyophilized powders contain crystallites of the protein that can be sufficiently large to warrant grinding of the sample to increase uniformity [47]. The optical densities of many proteins can be sufficiently high in the 1–3 THz range that preparing pellets directly from the biomolecules make pellets so thin that they easily deform and cause spurious refraction effects effecting the frequency-dependent transmission. To counteract this, many investigators mix the powders with a polyethylene filler to make the pellet sufficiently thick to both provide a uniform, robust sample with etalon fringe spacing below the resolution limit of the measurement. This method has been used to investigate denaturing effects [48] and hydration effects [30], [49]. In the case of hydration, measurements made on formed pellets exposed to various hydration conditions showed no significant hydration effects [30], a result in disagreement with later measurements on hydrated films [33], [40]. The early pellet hydration studies demonstrated that the hydration of pressed pellets is not easily manipulated or controlled. On the other hand, measurements performed on pellets formed from hydrated powders showed the characteristic hydration transition [49] in agreement with hydrated film measurements.

The use of pressed pellets is not conducive to measurements relevant to most biochemists and biologists. Biology occurs in hydrated systems, with enzymatic response ceasing below critical hydration levels in some cases [50], and practical biosensing instruments should avoid the requirement of pressed pellets of lyophilized samples. For THz measurements of the dielectric response evolution with biological function, samples must be sufficiently hydrated and secondary probes must be available to monitor the protein state such as mid-IR, UV/Vis, fluorescence, or circular dichroism spectroscopy. Such optical monitors can only characterize the surface of pellets at best, and thus, cannot be used to characterize the bulk of the sample used in THz measurements.

Hydrated films provide a method of control hydration to functional levels and allow for other spectroscopic probes of the entire sample. A number of THz measurements have used hydrated films of DNA [51], RNA [52], and proteins [33], [40], [53]–[57]. For the DNA and RNA samples, free standing films were formed by depositing solutions on substrates, and the dried films lifted from the substrates after drying. Protein film measurements used substrate-supported films made by pipetting starting buffered solutions on water-free quartz substrates and drying in a controlled fashion to ensure uniformity. We will discuss the issue of film uniformity at more length in Section VII-A. These transparent films have thicknesses in the range of 60–200 μm. While the proteins directly adjacent to the substrate will very likely be denatured, those several molecular layers away are sufficiently isolated from the surface to have native state structure and function. UV/Vis transmission and fluorescence measurements have demonstrated native state conformation, and in the case of photoactive proteins, exhibit native state photocycling when sufficiently hydrated. Hydration control of the films is realized by mounting the sample in a closed cell with circulating air from a dew point generator. In the case of oxidation studies of cytochrome c, pure nitrogen is used with the dew point generator for preventing the oxidation of reduced samples.

Another method used to reduce the bulk water background absorption is to measure below the water liquid–solid phase transition to significantly slow down the water rotational motions giving rise to the loss. This was done in the case of lysozyme binding measurements [56] and measurements of the dynamical transition at 200 K [55]. This technique of simply measuring the protein solutions below 273 K guarantees a hydration environment similar to *in vivo*, and eliminates sample uniformity issues that occur for the films. It is known that protein function continues below 273 K [58]–[60]; thus, native state response is assured.

A more thorough discussion of sample preparation and signal analysis is included in an upcoming review [35]; however, one issue should be discussed before summarizing data to date. One of the chief sources of artifacts can be multiple reflection effects in either the sample or elsewhere in the system giving rise to interference fringes in the data, referred to as Fabry–Pérot fringes,

or etalon. For the sample preparations used for the DNA, RNA, and protein measurements, the majority of investigators use a few standard techniques to alleviate or eliminate multiple reflection effects. In the case of pellets, investigators either use very thin pellets, where the fringe spacing is larger than the entire bandwidth of the measurement, or very thick pellets so that the fringe spacing is below the resolution of the measurement. In this case, using THz TDS makes this particularly easy in that the scan length is limited to times before the arrival of the first reflected pulse. For films, again the thickness can be sufficiently small, so the interference fringes are beyond the bandwidth. For the protein films on substrates, slight imperfections and nonuniformities appear to be sufficient to eliminate interference effects, but it will be shown that one can achieve uniformity in these films where very clear Fabry–Pérot fringes corresponding to the film index and thickness are observed. In the case of solution cells, it is found that the protein solutions have near index matching with the water-free quartz cell windows; however, the etalon for the empty portion of the cell used as a reference is found to be an ideal method to allow the precise determination of the cell spacing between windows, and therefore, sample thickness, and thus, the index and absorption coefficients. This precise determination is essential in the case of comparison of absolute absorption change with binding. Using the expression for the field transmission including multiple reflection effects is

$$t = e^{ik_o d[(n_s-1)+i\kappa_s]} \frac{t_{ws}t_{sw}}{t_{wa}t_{aw}} \frac{1 - r_{aw}^2 e^{i2k_o d}}{1 - r_{sw}^2 e^{i2k_o d(n_s+i\kappa_s)}} \qquad (2)$$

where $k_o = 2\pi/\lambda$ is the THz radiation wave vector and t_{ij} (r_{ij}) are the Fresnel coefficients. The subscript s (a, w) stands for the sample (air, window), e.g., t_{ws} (r_{ws}) is the Fresnel transmission (reflection) coefficient for light incident from the window side of the interface. The variables n and κ are related to the relative permittivity ε and complex index N through $N_i = \sqrt{\varepsilon_i} = n_i + i\kappa_i$. The exponential term on the right-hand side of (2) is the usual propagation term through a sample with thickness d. The second term in the product is the Fresnel transmission term. The third term arises from multiple reflections within the cell, the Fabry–Pérot term. The n and κ are solved for at each frequency using (2) and the measured magnitude and phase of field transmission. The fitting of the Fabry–Pérot oscillations for both $|\tau|$ and ϕ allows for precise determination of the spacer thickness d. The agreement with the experimental results with fringes is sensitive to small changes in the thickness, allowing for a precise determination of the cell thickness, with uncertainties typically of 2%.

We will now turn to a general review of the data presented to date. Again, this discussion is limited to polypeptides and polynucleotides. A thorough discussion on amino acids, nucleosides, nucleotides, drugs, and chromophores will be included in the review by Markelz and Jepsen.

III. Spectroscopic Signatures

A wide variety of measurements on proteins and long-chain DNA suggests that the overall response in the 0–6 THz range is broad and featureless. Several reports have suggested narrow-band features [37], [61]–[63], but unlike in the case of THz measurements of explosives [64]–[68], the narrow linewidth results do not appear to be reproducible group to group. In the reports on biomolecular fingerprinting absorbance features, the absorbances often have periodic structure and Beer's law is not demonstrated for these features. The broad response measured by a majority of investigators is not surprising given the high density of states calculated by normal modes analysis [54], [69], [70]. The calculated intensities for these collective modes tend not to have strong features [34]. Further, the features that do exist for these intensity calculations very likely will be washed out by the heterogeneous broadening of the macroscopic samples measured. In addition to the collective mode distribution calculated by normal mode analysis, one expects a significant contribution from overdamped rotational motions of the side chains. This relaxational loss may, in fact, dominate the overall THz dielectric response for the large biomolecules. In spite of this general trend of a lack of narrow-band signatures of specific collective modes, the frequency-dependent response is not independent of protein or even for a single protein in a different functional state. Most measurements of lyophilized powders pressed into pellets tend to have linear frequency dependence [71], [72]; however, in the case of collagen for example, we found that the frequency dependence was nearly quadratic [30]. Others have shown a similar difference in the frequency dependence power law for single amino acids versus homogeneous polypeptide chains [71]. In our measurements on hydrated films, we found that the absorption coefficient of lysozyme films was nearly linear [40], whereas for cytochrome c films, it was nearly quadratic [33], and this frequency dependence was also dependent on whether the sample was oxy-cytochrome c or reduced-cytochrome c. Each protein likely does have its unique THz response, but this uniqueness is somewhat subtle and does not lend itself to biomolecular identification. On the other hand, the broadband THz response does not interfere with THz sensitivity to biomolecular binding and the universal THz binding sensitivity has application potential for biosensing, as will be discussed.

IV. Terahertz Sensitivity to Environment

A. Hydration

There have been several demonstrations for the hydration dependence of biomolecular THz dielectric response [30], [49], [73]–[75]. Measurements on powders showing a strong hydration effect were performed by first hydrating loose powder, and then, pressing the pellet from this [49], [74]. Earlier measurements attempted to change the hydration continuously of pressed pellets by exposure to different relative humidities (RHs) [30]; however, the THz measurements on these pellets did not show a substantial change, indicating that the compacted powder does not permit ready diffusion of the water into the interior of the pellet. The environmental humidity is controlled either by saturated salt solutions or dew point generators. The actual water content for a given sample is determined by either a full determination of the isotherm relating RH to absorbed water, or single-point mass measurements for a given RH [76]–[78]. It is

worthwhile to determine the entire isotherm as the general form of the water content as a function of RH is somewhat similar for various proteins and a significant deviation from the curves in the literature can indicate a referencing problem for the water content. Often, the referencing difficulty arises from inaccuracies in either the zero hydration weight determination and/or lack of control of the RH for the actual mass determination. That is, for loose powders and films, the hydration equilibration times are very fast, and even a brief exposure to a different RH for the purposes of weighing will cause variance in the results. For hen egg white lysozyme (HEWL) film, samples we found a strong increase in both the absorption coefficient and index at 23% g water/g protein [40]. This hydration level is near the filling of the first solvation shell and suggests significant increases in biomolecular flexibility at this hydration level. The rapid increase in the THz response is somewhat similar to that seen for hydrated samples when the temperature is increased through the so-called dynamical transition temperature ~200 K. The film samples and optical setup did not accommodate hydrations higher than 0.4 g water/g protein. Up to this level, the change with hydration had not yet saturated indicating that HEWL had not yet reached its solution phase dynamics and additional water was still profounding influencing the picosecond motions.

B. Temperature

As mentioned earlier, one expects linewidth to be thermally broadened due to in part temperature-dependent damping. Thus, it is possible that a broad nearly featureless spectrum may reveal distinct bands when the system is cooled. For example, measurements on retinal had precisely this result, where narrow-band absorbance are nearly indistinguishable from noise at room temperature, but clear resonances in the absorption are accompanied with anomalous dispersion features at 10 K [79]. A similar result was found for measurements of crystalline serine and cysteine [80]. Naturally, there have been attempts to see if some structure could be resolved for large biomolecules in this way, but these did not reveal any distinct modes. Rather, a somewhat interesting result was observed [55]. For cytochrome c solutions, the imaginary part of the THz permittivity was seen to rise rapidly as the temperature was raised, with the rapid increase occurring at 200 K. This rapid change in the permittivity is a manifestation of the so-called dynamical transition for proteins, and the observation of this at THz frequencies is in contradiction to other measures of the dynamics. An example of this result is shown in Fig. 3.

The temperature dependence of protein dynamics has been under study since the 1980s. Using various measures of the average atomic mean square displacement $\langle x^2 \rangle$ such as neutron quasi-elastic scattering [81], X-ray Debye Waller factors [82], and Mössbauer measurements of isotopically substitute Fe^{57} in heme prosthetic groups of myoglobin and cytochrome c [83], investigators have demonstrated that $\langle x^2 \rangle$ increases rapidly in the 180–240 K temperature range. This rapid increase is hydration dependent and the cause of the rapid increase is controversial [83]–[86]. The time and energy scales of the motions

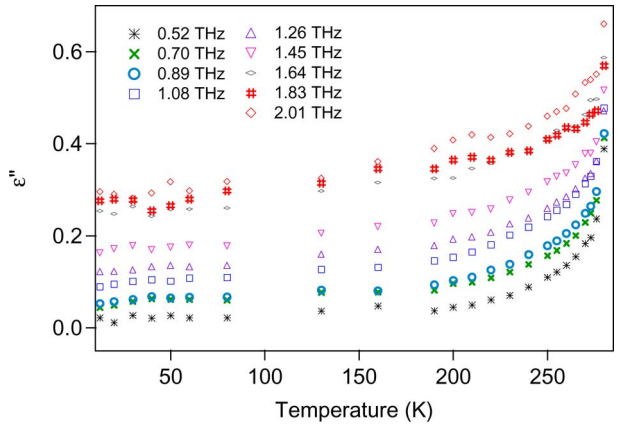

Fig. 3. Temperature dependence of the imaginary component of the permittivity for a 200 mg/ml reduced cytochrome c solution. The rapid increase at 200 K is associated with the protein dynamical transition.

involved have been dictated by the particular measurements, but, in general, have a low time cutoff with integration over all faster motions. Mössbauer measurements, for example, have a low-frequency cutoff of 7 MHz [83], and all faster motions contribute to the measurement. Regardless of the precise explanation of the effect, there is some agreement that the temperature dependence seen by the various techniques indicates that, below the transition temperature, the dynamics are dominated by harmonic motions, and above the transition, anharmonic motions come into play. These anharmonic motions may be the biomolecule accessing different conformations, for example. Recent measurements have, indeed, suggested that the dynamical transition is absent for motions faster than 5 ps [87]. The appearance of the dynamical transition in the THz dielectric response contradicts these earlier results and gives some support to the transition being merely a manifestation of thermally activated surface side chain diffusive motion with activation barriers that are hydration dependent.

V. Terahertz Sensitivity to Binding

As discussed, measurements of proteins and polynucleotides have yet to demonstrate reproducible narrow-band features that may be used for spectroscopic fingerprinting; however, several authors have considered the sensitivity of THz to biomolecular binding as a method of biosensing. Nearly all biosensors to date work by chemical specific binding of the target with a probe, and transduction of that binding into a measurable signal either by attachment of a tag to the probe, such as a fluorophore or a measurement of an intrinsic physical change with binding. If biosensing can take place without the need of tagging would greatly simplify biosensing. For example, tags need to be appropriately tailored to ensure sensitivity to the binding. Intrinsic changes are, therefore, preferable, and allow for universal systems that can be applied to any target, as long as a binder can be found. Among the most popular are surface plasmon resonance [88] and microcantilever methods [89] that detect index change and mass change with binding, respectively. However, both cases require that the probe be attached to a surface, again

requiring specific chemistry to be developed for detecting the specific biomolecule of interest. This surface attachment requirement is lifted in the case of binding detection through collective mode spectroscopy. The structural vibrational modes of the biomolecule will be strongly influenced by changes in structure commensurate with binding. Thus, if THz dielectric response is sensitive to the overall density of states, binding should be detectable. Indeed, investigators have shown in a variety of systems that THz can detect biomolecular binding. The only major challenges remaining are increasing sensitivity so that the required sample size is sufficiently small for large-scale application.

A. DNA

Pioneering work on the demonstration of THz sensitivity to biomolecular function was performed in the Kurz group where they demonstrated a significant change in both the absorption and index for single-stranded versus hybridized DNA [90]. Even more significant was the demonstration of the sensitivity to single-point mutations [91]. Again, there was an overall change in the dielectric response, without strong frequency dependence. Measurements were performed on thin films deposited on transmission lines.

B. Proteins

There have been several demonstrations of the sensitivity to binding in proteins. Specifically, Menikh and coworkers showed a clear contrast with a high-sensitivity sampling technique for films of biotin bound to avidin [92], [93]. We found that, for solution phase samples, we could detect a decrease in the dielectric response when tri-acetylglucosamine was bound to HEWL both at room temperature and below the freezing point of water [56]. These results were somewhat surprising since a contrast above the freezing point was expected to be masked by the bulk solvent.

VI. Terahertz Sensitivity to Conformation

If the response that is measured for the polynucleotides and polypeptides does primarily come from the collective vibrational modes associated with the 3-D structure, then altering the structure, either by denaturing or during function, should induce a change in the collective vibrational mode density of states. Here, we review several attempts to measure such a change.

A. Denaturing

If the 3-D tertiary is entirely removed such as by denaturing, one expects a dramatic change in the collective vibrational mode density of states. Several attempts have been made to look for this effect. Early measurements using bovine serum albumin in pressed pellets used irreversible thermal denaturing to look for any effect [30]. The net change was somewhat subtle with the chief surprise that the THz response was not eliminated. This result demonstrated that the THz response for the biomolecules could not be arising entirely from the 3-D structural vibrational modes alone. As shown in (1), an additional term in the response is the relaxational response, coming from, for example, the rotational motions of the side chains. For the native state, the interactions of the internal side chains serve to form and stabilize the tertiary structure. Presumably, this internal bonding limits their rotational motion and the primary contribution to the THz response is due to the surface side chains that do not have strong bonding except with the solvent, if present. It is possible that the removal of internal contacts with denaturing allows more side chains to have rotational motion contributing to relaxational loss, compensating for the loss of THz collective vibrational mode density of states. This may be the reason for the surprising result seen by investigators of cytochrome c denatured by pH [72]. This result, suggesting little sensitivity to conformation, is in contrast to the somewhat significant change seen for cytochrome c films as the central iron of the attached heme group changes its charge state [33]. The measurement of chlorophyll proteins, in fact, showed an *increase* in the THz absorbance with denaturing [48] and a similar result was found for HEWL films [94]. These results both indicate that the response for the denatured samples must arise from the relaxational contribution in (1). Investigators using a microwave technique have demonstrated that denaturing can also be detected through the change in the nature and extend of the bound water [95].

B. DNA

The conformation of DNA found most commonly *in vivo* is B type (3.4 Å helical rise per base pair) that can be converted to A type (2.3 Å helical rise per base pair) by lowering the RH of a sample below 92% RH [96]. While a significant number of measurements have been performed on nucleosides and some nucleotides and disordered samples without precise control of the sample or environment, a recent measurement has demonstrated both a slight polarization dependence and a significant hydration dependence for oriented DNA films [51]. Systematic measurements of these samples showing an abrupt change in THz response with critical hydration for B form would help to determine if these early results are due to conformational change, or are more akin to the hydration transition observed for the proteins [40], [49].

VII. Terahertz Sensitivity to Function

A. Bacteriorhodopsin

BR is a photoactive membrane protein from the archaebacteria *Halobacterium salinarum*. This archaebacteria can exist in extremely high salt concentrations and is found, for example, in salt marshes. BR is a seven α helix transmembrane protein with the chromophore retinal attached to the interior by a Schiff base linkage to the lysine-216 residue. The main photocycle serves to pump protons from the intracellular to extracellular side of the membrane as a method of energy storage for anaerobic metabolism. The photocycle consists of the resting state bR, and then, a series of photointermediate states K, L, M, N, O, and returning to the resting state bR. These intermediate states have unique UV/Vis spectra by which one can determine the population distribution of the sample. The M state, in particular, is

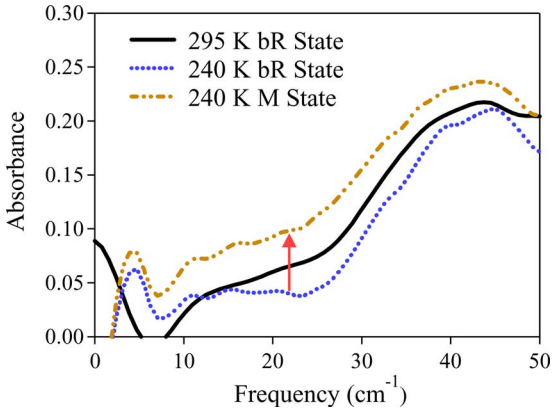

Fig. 4. THz absorbance sensitivity to conformational state of BR. The M intermediate state is thermally captured by reducing the temperature to 240 K and illuminating with $\lambda \sim 568$ nm light.

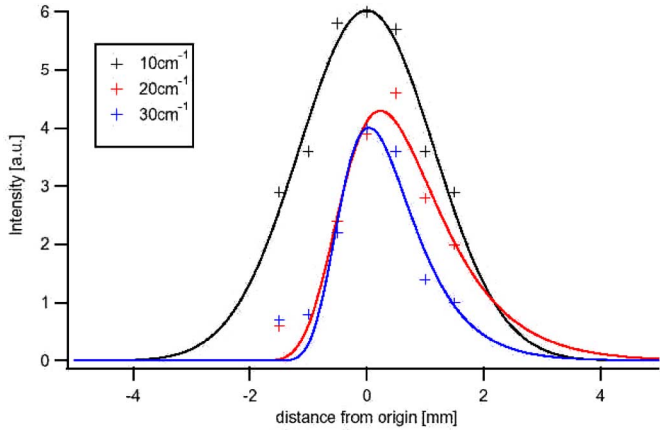

Fig. 5. Power profile across focus in a typical THz TDS system for several frequencies. The majority of the power is at lower frequencies, but the high-frequency content is spatially shifted relative to the main power peak.

associated with a substantial conformational change and can be thermally captured, that is, the lifetime significantly increases (more than days) by cooling the sample to approximately $-40\,^{\circ}$C. This system provides an excellent opportunity to examine how protein conformation effects THz dielectric response. The ability to perform the entire experiment on a single sample greatly reduces possible artifacts due to variance in sample preparation, and *in situ* UV/Vis monitoring determines whether the sample is still functional and characterizes the sample state. The results reported previously will not be discussed extensively here [54], rather this particular measurement brings out several overall points: 1) THz response sensitivity to protein conformation; 2) effects due to sample nonuniformity; and 3) etalon effects within a film sample. In Fig. 4, we show data for one of the wild-type (WT) BR films.

We will discuss the origin of the apparent frequency dependence shortly. As seen in the figure, the absorbance slightly decreases when the sample is cooled, and then, increases significantly after the sample is illuminated by a 568 nm light. We note that the change in the THz is persistent after the illumination is turned off as the thermal motions necessary to complete the photocycle are frozen out. The illumination was with a flashlight and a low-pass filter focused through the cooling cell windows and no heating effects were observed. Typically, we achieved 30% conversion to the M state for illuminating in this manner.

We now discuss the frequency dependence seen in Fig. 4. This measurement was performed multiple times on multiple samples. While the trend with illumination was reproducible, a net increase in THz absorbance with M state population, the frequency dependence varied from sample to sample. The cause of the variance was found to originate from the nonuniformity of the films coupled to a spatial dependence in the frequency content at the THz focus. In Fig. 5, we show a mapping of the THz focus for several different frequencies. The main power for this particular system peaks around 0.3 THz, and the profile at this frequency appears Gaussian. The peak power distributions for higher frequencies are displaced from the peak in the low-frequency region, and their distributions are not Gaussian. This type of aberration can easily occur by misalignment of the

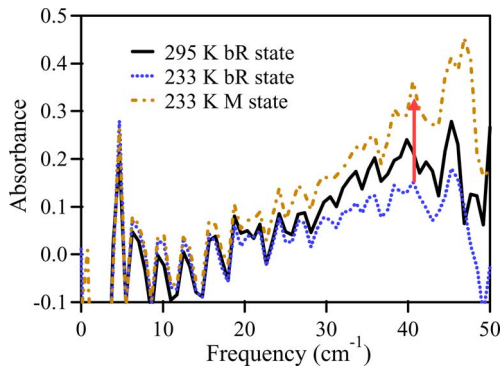

Fig. 6. Same experiment as in Fig. 4, but with a highly uniform BR film. Etalon fringes occur due to multiple reflection effects within the film; however, on average, the overall frequency dependence is nearly linear similar to that seen for many proteins measured in pellets. Arrow indicates the change in the response due to population transfer to M intermediate state for the cooled sample.

standard 90° off-axis parabolic mirrors used with THz systems. A slight displacement of the incident beam from the center of the mirror and a slight angular deviation from the 90° configuration can result in a nonuniform focus. This effect will be compounded with multiple paraboloids. The spatial nonuniformity in the frequency distribution coupled to spatial nonuniformity of a sample can give rise to spurious frequency dependence in the transmission. As an example, suppose a sample has a uniform absorbance over the entire frequency range; however, the sample distribution is such that it is thickest in the region where the high-frequency distribution is nearly absent, the transmission at the higher frequency transmission will be larger than the lower frequencies solely due to the nonuniformities and not the actual sample response. When care is taken to ensure sample uniformity, this frequency dependence for the BR films is no longer present, as seen in Fig. 6. The film in this case is sufficiently uniform that etalon fringes are clearly present due to interference affects within the film. Again, we see for this sample that there is a net increase in THz absorbance in the M state.

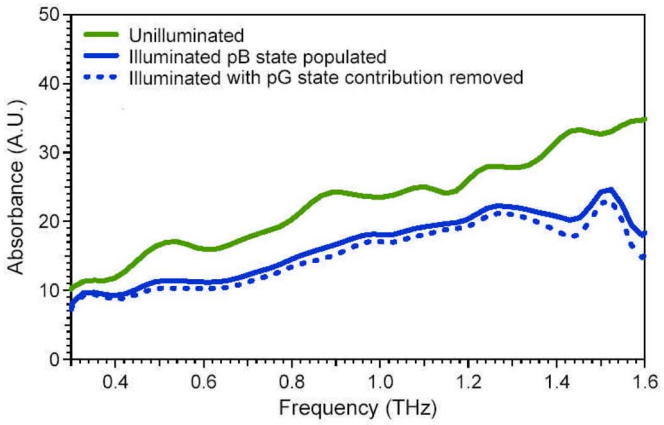

Fig. 7. Conformational dependence for photoactive yellow protein. A net decrease in the THz absorbance is observed when the population is transferred to the photointermediate pB.

B. Photoactive Yellow Protein

As another example of THz sensitivity to conformational state, we show results for another photoactive protein, photoactive yellow protein (PYP). Again, the photocycle consists of a series of intermediates states, the primary of which are the resting state pG, the red shifted intermediate pR, and the long-lived blue-shifted intermediate pB. Again, the relative populations of the intermediate states can be determined by UV/Vis spectroscopy, and for films sufficiently hydrated, the photocycle is the same as that in solution. The details of the measurements can be found elsewhere [57], but follow a similar method as for BR, except PYP photocycling time is so long that nearly 100% conversion is achieved with continuous illumination. In Fig. 7, we show the THz dependence on the pB state population. In this case, the steady-state population of the intermediate state results in a decrease in the THz absorbance. This result was somewhat surprising, as the pB state is associated with a partial melting of the structure. Intuitively, one might expect that the flexibility of the system would increase with partial melting. Nevertheless, the measurement again demonstrates THz sensitivity to protein conformation.

C. Heme Oxidation State

There are a significant number of heme proteins, where the heme prosthetic group with its central iron serves as the mechanism of the protein function. In the case of myogloin, the Fe binds molecular oxygen for transport to muscle tissue. The charge state of the Fe can also be altered by the binding of an internal water going from reduced ferro (Fe^{2+}) to oxidized ferri (Fe^{3+}). Similarly, cytochrome c's heme group participates in an electron transfer step as part of the mitochondrial metabolic pathway. X-ray diffraction studies on myoglobin and cytochrome c show the $\langle x^2 \rangle$ of the protein increases for the ferri form. We have performed measurements on cytochrome c films as a function of heme oxidation and find that the THz dielectric response has an overall increase in the ferri state, consistent with an increase in flexibility that the $\langle x^2 \rangle$ suggests [33]. We have found a similar result for myoglobin, as shown in Fig. 8.

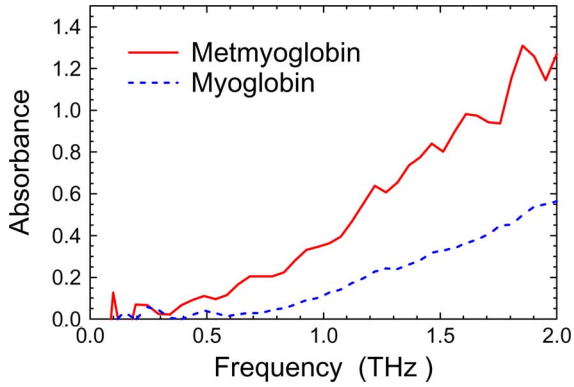

Fig. 8. THz absorbance for myoglobin films as a function of iron oxidation state. The Met form of myoglobin film was verified with UV/Vis transmission measurements. Films at $<5\%$ RH.

VIII. Discussion

A. Biomedical Applications

Not long after THz imaging systems were developed, they were applied to tissue samples to determine if there was any sensitivity to pathologies. If such sensitivity could be established, then THz could be used as an alternative to more invasive histology or repeated X-ray exposure. These measurements were performed in reflection. There was some success in finding tooth decay [97] and basal cell carcinoma [98], [99]. The skin cancer detection is particularly intriguing since the protein measurements suggested that the heterogeneity of the protein content of cells along with the broadband response would completely wash out any specificity for cancer cells. The contrast mechanism is still not resolved, although there is some discussion that the contrast arises from a difference in the nature and extent of the water in cancerous cells. The hydration-dependent measurements on proteins along with the dynamical transition measurements suggesting that the relaxational times are strongly influenced by the solvent in part support this contrast mechanism.

B. Biosensing Applications

As discussed, no robust fingerprinting of proteins or DNA has been demonstrated using THz spectroscopy. Reports of narrowband structure are not reproducible, do not follow Beer's law, and have not demonstrated narrowing with reduced temperature. But this does not imply that THz biosensing is not possible. On the contrary, a number of researchers are addressing the need for tagless and universal binding indicators that do not require processing of probe immobilization. Biosensing through chemical specific binding is well established and accepted in the community, but for each sensing target, the appropriate chemistry must be established for probe immobilization.

THz sensitivity to biomolecular binding has been demonstrated [90], [92] *and it has been demonstrated in solution* [56]. There is no question if it works, and there is no question that it does not require probe immobilization. But can THz biomolecular binding detection have sufficient sensitivity and be sufficiently convenient to motivate a wide-scale technology shift by the pharmaceutical and bioresearch community?

More systematic measurements need to be carried out in order to gain interest and acceptance in the biochemical community that is currently far more willing to accept the processing requirements of SPR and fluorescent labeling before moving to a radically different technology. Various groups are pursuing engineering of sampling structures to minimize sample volume needs [91], [100]–[105]. If these groups are successful, then sufficiently simplified THz spectroscopic systems coupled to these sampling structures will have widespread applications.

C. Origins of the Biofunctional Sensitivity

The demonstration of sensitivity to binding both in solution phase and in dry films is intriguing. Why is THz dielectric response so sensitive to binding? Naturally, we would expect the original motivation for THz studies to be the answer, that is, the change in structural vibrational modes with binding. This may indeed be the entire answer. To date, the modeling of the change of the density of states with biomolecular binding has not been reported for any system. Before assuming that this is the case, let us consider other possible contributions that may be affected by the binding. For example, how is the relaxational contribution affected by binding? The interactions between the surface side chains that may inhibit rotation are strongly effected by the solvent. In addition, it is not entirely clear what the THz response is of the water immediately adjacent to the biomolecule, that is, the so-called biological water. Havenith's groups suggests that, in fact, the dielectric loss from this solvation shell is larger than bulk [45]. If this is correct, then changes in the solvent shell structure due to binding may also result in the apparent THz sensitivity to binding. Thus, the relaxational loss can be effected by binding through the change in the solvent exposure of the side chains and the change of the solvent shell structure is due to a change in side chain exposure. One may argue that the binding sensitivity that colleagues have measured in dry films points to the fact that solvent interactions cannot play a strong role, and in these cases certainly, the binding contrast must be originating from a change in the vibrational density of states. However, if we consider our measurements on solutions of 3NAG binding to HEWL, these noncollective mode contributions may be the dominant ones [56].

Similarly, for the functional response measurements made for BR and PYP, as discussed in Section VII, the films are sufficiently hydrated that the diffusive motions on the surface may make a substantial contribution, and this contribution will be strongly effected by side chain exposure and burial with the conformational change of the intermediate state. The modeling of these changes is currently underway. Understanding of the contrast mechanism is necessary for further optimization of the sensitivity.

IX. Conclusion

To conclude, THz dielectric spectroscopy has been established as a technique to probe biomolecular hydration, binding, conformational change, and oxidation state change. The contrast mechanisms are not yet established, but the application of the technique to specific questions of picosecond protein dynamics and to the development of tagless biosensors is underway. Biomedical applications are being developed. There is much work to be done and some emphasis should be made on applying THz to address questions of importance to the biomedical community.

Acknowledgment

The author acknowledges the support from the University at Buffalo MRIF clean room facilities. She is also deeply indebted to her collaborators; in particular, recent Ph.D. graduates J. R. Knab and J. Y. Chen. She would like to also acknowledge all students and postdoctorates who have worked in her laboratory at Buffalo: S. Whitmire, S. Ye, S. Feigh, S. Moses, S. Sachs, D. Wolpert, Y. He, F. Lipps, W. Cox, and B. Moeller. She is also indebted to her collaborators R. Birge, S. Gregurick, and W. Hoff.

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