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Probe conformational dynamics of proteins in aqueous solutions by terahertz spectroscopy

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ABSTRACT

Proteins solvated in their biologically milieu are expected to exhibit strong absorption in the terahertz frequencies, that contain information on their global and sub-global collective vibrational modes (conformational dynamics) and global dynamic correlations among solvent water and proteins. The dynamics play an important role in enzymatic activities of proteins, but obtaining an accurate and quantitative pictures of these activities, however, is challenging due to the strong absorption of water. In response, we have developed the world's highest precision, highest sensitivity terahertz-frequency domain spectrometer and a standard terahertz-time domain system to probe the collective dynamics of proteins in aqueous solutions. Operating over the frequency range from 5 GHz up to 3 THz, our spectrometers provide an unparalleled ability to probe directly such questions as the hydration level, the dynamics of water and hydrated proteins over the 100 fs to 1 ns timescale. Employing an effective medium approximation to describe the complex dielectric response of the solvated proteins in solution we find that proteins are surrounded by a loosely and tightly held layers of water molecules that behave as if they are an integral part of the protein. The number of water molecules in the protein hydration shells varies with proteins, which can tell us the average surface structure of proteins. These measurements shed light on the macromolecular motions of proteins in their biologically relevant environment.

1. INTRODUCTION

Terahertz vibrational modes typically involve the low frequency, collective atomic motions of macromolecules, which include both inter- and intramolecular interactions. Thus, terahertz spectroscopy of biomolecules in aqueous environments provides an important approach for identifying their global and transient molecular structures as well as directly assessing hydrogen-bonding and other detailed environmental interactions.[1-3] However, a significant challenge in obtaining the terahertz dielectric spectra of aqueous biomolecules and lipid layers is the strong absorption of water in the spectral range of 0.5 – 10 THz. Advances in gigahertz to terahertz technology call for a more thorough study of the dielectric response of such systems. Such a study can act as an important step in understanding the behavior of more complex biomolecular systems in the gigahertz to terahertz frequency range. Terahertz spectroscopy of protein in solution has also been investigated in the past as an alternative approach for probing the collective vibrational motions in solution state. Recent developments in diode based frequency multipliers have improved the accuracy of terahertz measurements by several orders of magnitude, which allows for high-precision measurements of the strong absorption of aqueous solutions.

The dynamics of water in the hydration layer around proteins and other biomolecules play a crucial role in different aspects of biological processes. Some studies have concluded that water molecules in the hydration layer are rigidly attached to surfaces of molecules, resulting in an increase in the effective volume of the molecules.[4] On the other hand, evidence for the dynamic nature of the hydration layer is also abundant,[5] with some suggesting that there are fast and slow dynamic processes within the hydration layer. Some of the dynamical processes in proteins have been suggested as solvent slaved motions.[6] The significance of the hydration layers cannot be overstated in biological process and reactions, as they control the structure and function of biological systems. There are many experimental techniques that allow the investigation of dynamics and structure of hydration water on a biomolecular surface. These include time-resolved fluorescence,[7] dielectric relaxation spectroscopy at gigahertz frequencies,[8]

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nuclear magnetic resonance,[9] X-ray crystallography,[10] neutron scattering,[11] and infrared spectroscopy.[12] Among these techniques, dielectric spectroscopy from gigahertz to terahertz frequencies and computational techniques have advantage to investigate the dynamics of water in confined systems, including interfacial or restricted environments, providing information on the hydrogen bonding, diffusion, and reorientation of water around proteins as well as the dynamics of proteins themselves. In this paper, we investigate interactions of lysozyme protein with water, using the spectrometer in the frequency range from 5 GHz to 1.12 THz.

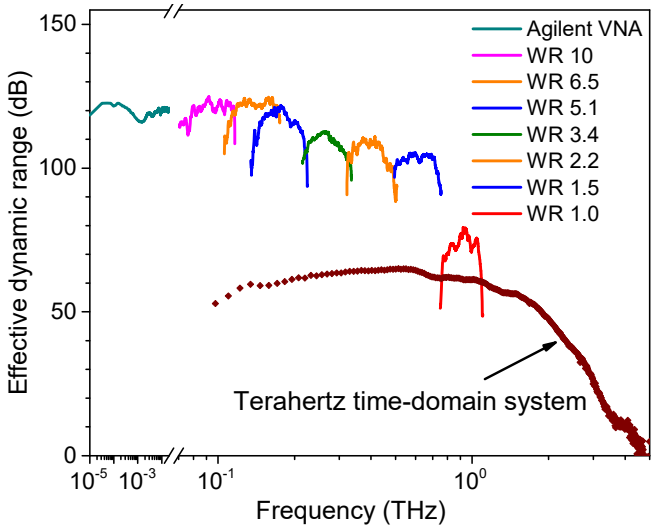

Figure 1: Dynamic range of our gigahertz-to-terahertz frequency-domain spectrometer (Agilent Vector Network Analyzer and frequency extenders from WR10 to WR1.0 systems) is compared with the dynamic range of a typical terahertz time-domain system. For WR10, WR5.1, WR6.5 and WR3.4 bands, we obtain the dynamic range measurements using a DUT with 30 dB loss.

2. EXPERIMENTAL DETAILS

2.1. Experimental setup

In an effort to improve our understanding of the picosecond dynamics of water and solvated molecules, we have built a gigahertz-to-terahertz frequency-domain dielectric spectrometer that supports the simultaneous measurements of absorbance and refractive index of solutions over the spectral range from 5 GHz to 1.12 THz (0.17 to 37.36 cm^{-1} or 0.268 to 60 mm). The signal-to-noise and spectral resolution of this device are significantly improved relative to any previous state-of-the-art instruments. For example, while the dynamic range of a commercial terahertz time-domain spectrometer is just 10^6 and its spectral resolution is several gigahertz, the dynamic range of our instrument reaches an unprecedented value of 10^{15} and the system achieves a spectral resolution of less than 100 Hz (Fig. 1). The system provides a coherent radiation source with a power up to 20 mW in the gigahertz-to-terahertz region. With the high power, we are able to measure thick layers up to 2 mm of liquid water. The temperature of liquid sample can be controlled with high accuracy of ±0.02 °C. Given these attributes, our spectrometer provides unique capabilities for the accurate measurement of even very strongly absorbing materials such as aqueous solutions[1, 13, 14].

Our spectrometer consists of a commercial Vector Network Analyzer (VNA) from Agilent, the N5225A PNA, which covers the frequency range from 10 MHz to 50 GHz, and frequency multipliers and the matched harmonic detectors for terahertz radiation, which are developed by Virginia Diodes, Inc. (Charlottesville, VA). Detailed information of the vector network analyzer frequency extension modules and the mixer process can be obtained elsewhere.[14-16] Instead of using optical sources and mixing down the frequency to access the terahertz range, the terahertz radiation in this case is generated by up-converting frequencies from microwave sources. The frequency multipliers are fabricated using Schottky diode based components.[16] The spectrometer provides a large range of frequencies from gigahertz to terahertz with the output power up to 20 mW. The frequency extenders consist of

commercial frequency extenders and matched harmonic receivers from Virginia Diodes, Inc. including WR10, WR6.5, WR5.1, WR3.4, WR2.2, WR1.5 and WR1.0 to cover the frequency range from 60 GHz to 1.12 THz. The lower frequency bands including WR10, WR5.1, WR6.5 and WR3.4 have high output power up to 20 mW, thus we obtain the dynamic measurements for these bands using a DUT with 30 dB loss. We can easily obtain the dielectric response from 60 GHz to 1.12 THz for liquid samples. For lower frequencies from 5 GHz to 50 GHz, we employ directly the radiation from VNA system into the sample cell designed for WR137 and WR28 waveguide configuration.

We have employed a variable path-length cell setup[2, 17] consisting of two parallel windows inside an aluminum cell, one immobile and the other mounted on an ultra-precise linear translation stage (relative accuracy of 50 nm). Our translation stage from Newport (XMS160 ultra-precision linear Motor Stages) can perform 1 nm minimum incremental motion with a travel range of 160 mm. The linear translation stage has a direct-drive system for ultra-precision and a high accuracy linear glass scale encoder with 80 nm repeatability. The choice of thickness of liquid water sample depends on the dynamics of frequency bands.

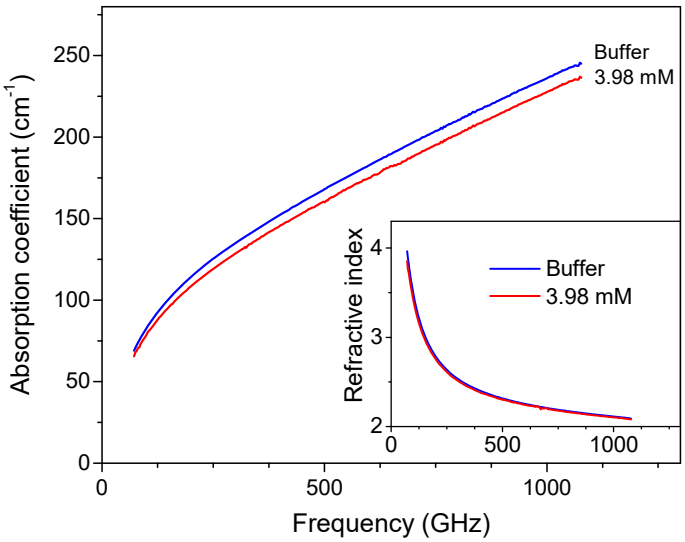

Figure 2: The interaction of lysozyme protein in solution with the gigahertz to terahertz radiation provides insights into the liquid's dynamics over the picosecond to nanosecond timescales. The absorption spectra of both lysozyme solutions and buffer increase monotonically with increasing frequency. The refractive indexes (**inset**) of lysozyme solution and water, in contrast, decrease with increasing frequency. Data were collected at 25°C.

The temperature of the sample cell can be controlled precisely from 0 °C to 90 °C. Peltier cooler plates from Custom Thermoelectric and high power resistors are mounted on the body of the sample cell, allowing precise control of the temperature of the sample. The absorbance and refractive index of water are extremely sensitive to temperature, and thus all experiments are carried out with a measured accuracy of ± 0.02 °C. To mitigate problems associated with multiple reflections of the incident light (standing waves, etalon effect), the thickness of our shortest path-length was selected to be long enough to ensure strong attenuation of the incident radiation (transmission $<10^{-2}$).

2.2. Data Evaluation

Absorption and refractive index measurements.

Using the above-described spectrometer and sample cell, we have measured the change of intensity and phase in aqueous samples as functions of path-length. The absorption process of the terahertz radiation passing through a sample is described by Beer's law:

$$I(l, \nu) = I_0(\nu) \cdot e^{-\alpha(\nu) \cdot l} \quad (1)$$

where ν, I_0, I, $\alpha(\nu)$ and l are the frequency, the incident intensity, the intensity at the detection of the radiation, the absorption coefficient as a function of radiation frequency, and the thickness of the sample, respectively. When the

radiation passes through a material, it will always be attenuated. This can be conveniently taken into account by defining a complex refractive index:

$$n^*(\nu) = n(\nu) - i\kappa(\nu) \quad (2)$$

with the real part, $n(\nu)$, is the refractive index and indicates the phase velocity, while the imaginary part, $\kappa(\nu)$, is called the extinction coefficient and indicates the amount of attenuation when the radiation propagates through the material. The extinction coefficient is related to the absorption coefficient, $\alpha(\nu)$, by

$$\alpha(\nu) = \frac{4\pi \cdot \nu \kappa(\nu)}{c} \quad (3)$$

with c is the speed of light. We have measured the intensity and phase shift of water and aqueous solutions over the three order of magnitude range 5.0 GHz – 1.12 THz as functions of path-length, l.[1, 13, 14] The absorption coefficient is determined by the slope of a linear fit of $\ln I(l, \nu)$ to the path-length, l, without the need for precise knowledge of the sample's absolute absorbance or absolute path-length:

$$\ln I(l, \nu) = \ln I_0(\nu) - \alpha(\nu) \cdot l \quad (4)$$

In parallel, we also fit the observed phase shift $\theta(l, \nu)$ as a linear function of path-length to define the refractive index, $n(\nu)$, of the sample:

$$\theta(l, \nu) = \theta_0(\nu) + \frac{2\pi \cdot \nu n(\nu)}{c} \cdot l \quad (5)$$

where the $\theta_0(\nu)$ is the phase of the reference signal.

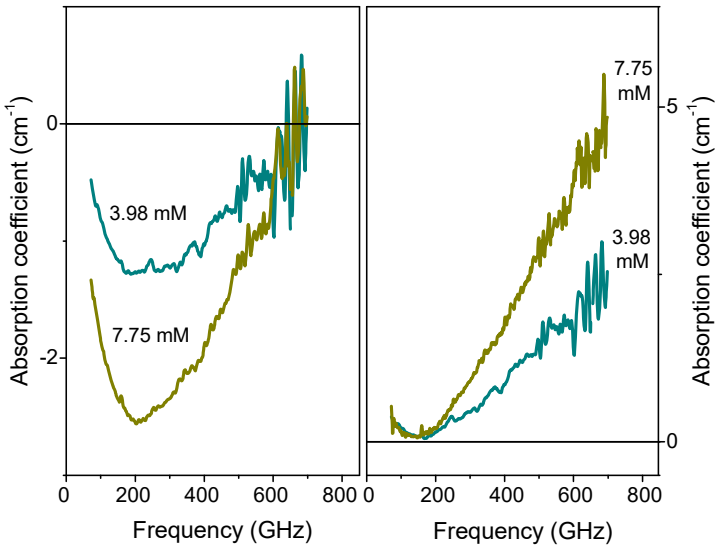

Figure 3: The absorption of solvated lysozyme provides a measure of the protein's low-frequency vibrational dynamics. (**left**) Shown is the absorbance of lysozyme (minus the absorbance of the relevant buffer blank) without any correction for the protein's hydration shell. This leads to negative absorption, which is unphysical. (**right**) Scaling of these spectra such that their absorbance minima reach zero suggests that 153 ± 15 water molecules in a hydration shell around each lysozyme no longer behave as if they were bulk solvent in terms of their gigahertz absorption.

We have collected complex dielectric response spectra from lysozyme solutions in a large range of frequencies, from 60 GHz to 1.12 THz using the gigahertz-to-terahertz spectrometer. The very high dynamic range of the frequency extenders together with the precise sample thickness controller allows us to obtain the most highly precise and accurate gigahertz-to-terahertz dielectric response spectra reported so far for this frequency range (Fig. 2).

Complex dielectric response of solutions.

With simultaneous measurements of the absorption and refractive index, the complex refractive index of a material can be expressed as

$$n^*(\nu) = n(\nu) + i\kappa(\nu) \tag{6}$$

where ν is frequency, $n(\nu)$ is the refractive index of the solution, and $\kappa(\nu)$ the extinction coefficient of the solution. $\kappa(\nu)$ is related to the absorption coefficient, $\alpha(\nu)$, by $\kappa(\nu) = c\alpha(\nu)/(4\pi\nu)$ with c being the speed of light. Similarly, the complex dielectric constant of a material can be expressed as

$$\varepsilon^*(\nu) = \varepsilon'(\nu) + i\varepsilon''(\nu) \tag{7}$$

where $\varepsilon'(\nu)$ and $\varepsilon''(\nu)$ are the dielectric dispersion and dielectric loss components. Since our experiment can simultaneously measure both the absorption and refractive index of a material, $\varepsilon^*_{sol}(\nu)$, the complex dielectric response can be calculated from the following relations:[18]

$$\begin{aligned} \varepsilon'_{sol}(\nu) &= n^2(\nu) - \kappa^2(\nu) = n^2(\nu) - (c\alpha(\nu)/4\pi\nu)^2 \\ \varepsilon''_{sol}(\nu) &= 2n(\nu)\cdot\kappa(\nu) = 2n(\nu)c\alpha(\nu)/4\pi\nu \end{aligned} \tag{8}$$

From absorption and refractive index measurements, we have determined the dielectric spectra of lysozyme solutions.

3. RESULTS AND DISCUSSION

Using our high resolution and dynamic terahertz frequency-domain spectroscopy and a variable-thickness cell, we have determined the absorption coefficient and refractive index of lysozyme solutions along with that of pure water (Fig. 2). A quick glance at the absorption as well as the refractive index data indicates these are strong functions of frequency, increasing and decreasing with increasing frequency, respectively. It is evident that the most prominent effect of addition of solvent is a monotonic decrease in absorption with increasing solvent concentration. This is primarily due to the fact that the higher absorbing solvent is replaced by the solute having a much lower absorption.

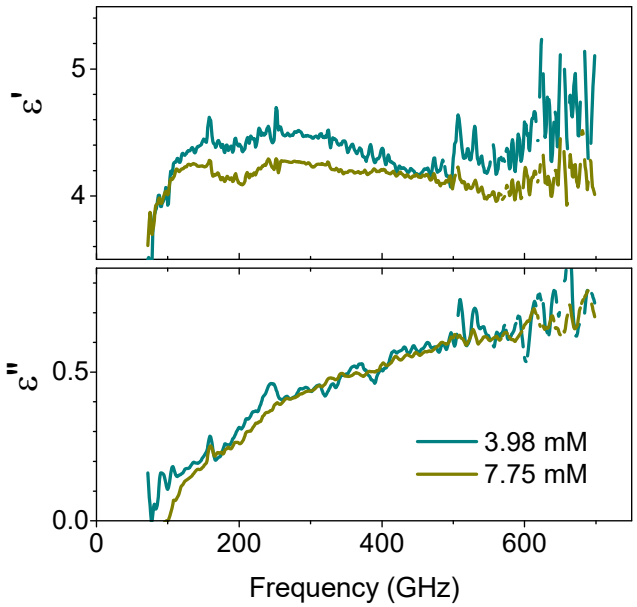

Figure 4: Dielectric loss, $\epsilon''(\nu)$, and dispersion, $\epsilon''(\nu)$ spectra of hydrated lysozyme in solutions at 25°C in the THz frequency range provides insights into the collective motions of lysozyme using the Bruggemann effective-medium approximation. From the effective-medium approximation, we have found out that 165 ± 15 water molecules in the hydration shell around DPC micelles that no longer behave as bulk water.

In order to study the interaction of the surface of lysozyme protein with water molecules, we first look at the change in absorption with the addition of solute after taking into account the displacement of water molecules. As a first approximation, we can consider the lysozyme protein solution as a two-component system with proteins and surrounding bulk water. If we consider the total absorption of the solution as a weighted average of its two constituents, we can express the absorption as

$$\alpha_{\text{sol}} = \sigma_{\text{wat}} M_{\text{wat}} + \sigma_{\text{lyz}} M_{\text{lyz}} \quad (9)$$

where α_{sol} is the total absorption of the solution, σ_{wat} and σ_{lyz} are the apparent molar absorptions, and M_{wat} and M_{lyz} are the molarities of water and lysozyme, respectively. Following this approach, the absorption of water is subtracted from each of solution absorption. The displacement of water from the solution is taken care of by using the appropriate molarities of bulk water calculated for each solute concentration. This is done by calculating the volume of lysozyme in each of the samples according to volume calculations outlined in the sample preparation. The absorption for lysozyme turns out to be negative.

This seemingly unphysical result can be explained by the fact that the hydrophilic surface of lysozyme interacts with water and bind some of the waters adjacent to it. Therefore, this water layer immediately surrounding lysozyme proteins cannot take part in dipole relaxation, or in other words, they are removed from the frequency window. It should be noted that this does not necessarily mean that these water molecules are immobile. Assuming the minimum of the curves in Fig. 3 left is zero, we can estimate how many water molecules are removed in this way. This is done by calculating the fractional decrease in molarity of water that has to be applied in the calculation of absorption in Fig. 3 right until the minimum value of absorption is zero. An average of 153 ± 15 water molecules is found to be bound to lysozyme protein using this method. It is worth noting that this value is less than a single layer of water molecules around the clusters.

In dealing with the heterogeneous system, the dielectric response of dispersed solvent molecules in a bulk solvent is employed, rather than assuming that overall absorption is the sum of the absorption of its constituents. Lysozyme solutions are a mixture of water and lysozyme protein and their complex dielectric response were determined from the experimental observables (Fig. 2). We assume that (i) lysozyme proteins in solution are spherical with a radius of R_{lyz} and have a spherical hydration shell with a thickness of d; (ii) the water molecules in the hydration shell are a part of lysozyme; (iii) solvent outside of the lysozyme has the same dielectric property as that of pure water. Since the size of lysozyme protein with hydration water is orders of magnitude smaller than the wavelength of the probing electromagnetic radiation, the medium can be considered homogenous with an effective dielectric response. A more elegant method has been employed for the effective-medium approximation, such as the Bruggeman model,[19] which effectively treats both low and high concentration mixtures or Maxwell Garnet,[20] Wagner and Hanai approximations,[21] which are for low concentration limits. Following the Bruggeman approximation, the complex dielectric response of the solution can be determined from:

$$f_{\text{lyz}} \frac{\varepsilon^*_{\text{lyz}} - \varepsilon^*_{\text{sol}}}{\varepsilon^*_{\text{lyz}} + 2\varepsilon^*_{\text{sol}}} + \left(1 - f_{\text{lyz}}\right) \frac{\varepsilon^*_{\text{wat}} - \varepsilon^*_{\text{sol}}}{\varepsilon^*_{\text{wat}} + 2\varepsilon^*_{\text{sol}}} = 0 \quad (10)$$

where $\varepsilon^*_{\text{wat}}(\nu)$ is the complex dielectric response of water; $\varepsilon^*_{\text{lyz}}$ is the complex dielectric response of hydrated lysozyme described as the process of forming an aggregate with hydrophilic regions in contact with surrounding solvent; $f_{\text{lyz}} = (N_{\text{lyz}}/V)(4\pi/3)(R_{\text{lyz}} + d)^3$ is the volume fraction of the lysozyme with hydration water, and N_{lyz}/V is the concentration of the lysozyme in solution.

When we performed the Bruggemann effective-medium analysis, we found that each lysozyme entraps a hydration shell composed of 165 ± 15 water molecules. Unlike the absorbance-based method used above which estimate the size of the hydration shell by assuming that the macromolecule's absorption falls to zero at its minimum, this method of estimating the size of the tightly-bound hydration shell requires only the well-founded assumption that the protein's absorption falls to zero at zero frequency.[1] The value of 165 water molecules for the THz-defined hydration shell corresponds to a monolayer on the surface of the lysozyme protein. Specifically, if we approximate lysozyme as a ~2 nm diameter sphere, a solvent layer with one molecule deep will contain 350 water molecules. Using the number of water molecules in the hydration shell related to the scaled filling factor and the measured $\varepsilon^*_{\text{wat}}(\nu)$ and $\varepsilon^*_{\text{sol}}(\nu)$, we employ eq. 10 to obtain the dielectric spectra for several hydrated lysozyme concentrations (Fig. 4). These water molecules become an integral part of lysozyme and cannot move easily; instead, they are held in the tightly-bound hydration layer.

Understanding how lysozyme dynamics and structure are connected to the chemical composition and geometry of the surfactants offers a considerable challenge. Here, we directly probe the collective dynamics of hydrated lysozyme protein with terahertz radiation using the Bruggemann approximation to exclude contribution from bulk water for several lysozyme solutions (Fig. 4). Upon doing so, we found that these spectra are characterized by a rising

dielectric loss and a broad maximum of the dielectric dispersion component. For lysozyme concentration below 12 mM, the measured dielectric response extracted from the effective-medium approximation is independence from concentration, suggesting that the size of the tightly-bound hydration shell is likewise independent of lysozyme concentration.

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