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ABSTRACT

Lysozyme is a well-known globular protein that can form self-assembled clusters. Here, we report the results of a study on the changes in solvation dynamics upon increasing protein concentration via THz spectroscopy. We find spectral solvation fingerprints in the THz frequency range, which are clearly distinguished from those for liquid–liquid phase separation or liquid–solid phase separation. Furthermore, we carried out supplementary measurements using dynamic light scattering to characterize the size distribution of the protein clusters. This study sheds light on using THz studies of solvation dynamics to characterize and differentiate protein hydration in the case of protein assembly, liquid–liquid phase separation, and liquid–solid phase separation.

INTRODUCTION

The formation of self-assembled clusters and Liquid–Liquid Phase Separation (LLPS) in protein solutions has raised considerable interest due to their implications in the medical field, and these phenomena are, in fact, thought to be possible local hotspots of aggregate formation, which can be traced back to pathologies known as amyloidosis. Lysozyme is a globular protein that can phase separate[1–3] and form equilibrium clusters under specific conditions. The presence of clusters in water–protein solutions has been supported by scattering and NMR measurements[4–8] and supported by theoretical studies that predict the formation of aggregates.[9] The formation of self-assembling clusters may represent an early stage in polymerization or liquid–liquid phase separation. These processes are known to serve as the initial steps in the pathogenesis of diseases such as dementia and Alzheimer's disease. Previous studies have investigated water–protein interactions via Raman spectroscopy,[10] optical-Kerr effect spectroscopy,[11] and dielectric measurements.[12] To understand and characterize the protein aggregation process in more detail, we carried out FTIR-THz measurements accompanied by Dynamic Light Scattering (DLS) experiments.

The THz regime is ideal for studying changes in the water hydration network of proteins without significant interference from protein bands. Previous studies have shown that protein–water interactions contribute substantially to the overall free energy, which drives phase separation and aggregation. We have introduced THz spectroscopy/calorimetry as a new tool to focus on protein–water interactions.[13] In our previous studies on liquid–liquid phase separating proteins, we observed two characteristic spectral features upon phase separation: a decrease in THz absorption around 150 cm^{-1} attributed to the loss of hydration water around hydrophobic groups (denoted as H-cavity wrap water) and an increase in absorption >400 cm^{-1}, as a signature of retained water molecules interacting with hydrophilic groups of the protein (bound water),[14,15] which increases in time due to the increase in probed protein concentration with time. The amplitude of the cavity-wrap band is a measure of both the number of hydration water molecules and the extent of perturbation to the hydration water network. In the case of lysozyme, previous studies have not been able to distinguish between distinct water populations. Here, we focus on changes in the THz spectrum as a function of protein concentration.

METHODS

Sample preparation

Hen egg white lysozyme (HEWL) was purchased from Carl Roth (12 650-88-3). The samples were prepared by dissolving the lyophilized HEWL in Milli-Q water to create a stock solution at the highest concentration. Complete dissolution was obtained using vortex mixing. Samples at lower concentrations were obtained by diluting the stock solution. Three sets of samples were prepared starting from three different stock solutions. The stock solutions were filtered using a 0.22 μm pore size syringe filter to prevent the introduction of dust or undissolved protein. The final concentration of the samples has been tested using a UV–visible spectrometer (V-770 UV–VIS–NIR spectrophotometer), probing the absorption peak at 281 nm.

Experimental setups

For FTIR-THz measurements, we used the attenuated total reflection (ATR) single reflection unit with a temperature-controlled diamond prism of 500 μm diameter (MVP-Pro; Harrick Scientific, Pleasantville, NY) in an FTIR-spectrometer (Vertex 80v; Bruker, Billerica, MA) with a frequency resolution of 2 cm^{-1} in the 50–650 cm^{-1} frequency range. We used a helium-cooled silicon bolometer (Infrared Laboratories, Tucson, AZ) as a detector. We averaged over 64 scans as recorded in 2 min. The details of the ATR setup can be found in Ahlers *et al.*[14] The penetration depth of the evanescent field is frequency dependent [see Eq. (2)].

The THz absorption change $\Delta\alpha$ was deduced from the measured intensity I by Beer's law [Eq. (1)]. We used the following equation for the penetration depth d_p [Eq. (2)] at an angle θ of 45°:[14]

$$\alpha(\nu) = -\frac{1}{d_p}\ln\left(\frac{I(\nu)}{I_{ref}(\nu)}\right), \tag{1}$$

$$d_p = \frac{\lambda}{2\pi\sqrt{n^2_{diamond}\cdot \sin^2\theta - n^2_{sample}}}. \tag{2}$$

The refraction index of the diamond is $n_{diamond} = 2.38$, for $n_{sample} = 1.5$ is assumed.

We carried out DLS experiments using the DynaPro NanoStar instrument to determine the size distribution of the hydrodynamic radius of aggregates as a function of protein concentration. The detector operates at 90° scattering angles. The data shown are the result of analysis using the cumulants method[16] of the temporal autocorrelation signals measured by the instrument. For each measurement, the analysis is conducted on the average of ten acquisitions.

RESULTS AND DISCUSSION

Due to the intrinsic limitations of the ATR configuration, which prevent an accurate determination of the probed volume and thus of the number of solute moles, together with the possible presence of concentration gradients connected with the heterogeneous nature of the clusters, a quantitative implementation of THz calorimetry[13] is not feasible in the present work. Therefore, our discussion is limited to a qualitative interpretation of the spectroscopic signatures in the intermolecular stretching and librational regions. We record THz (50–650 cm^{-1}) spectra in ATR geometry using a Fourier transform infrared-THz (FTIR-THz) setup to monitor changes in the hydration of lysozyme with increasing protein concentration.

Figure 1 shows the data obtained from the present FTIR-THz investigation. The THz spectra of aqueous lysozyme solutions at different concentrations are reported after subtraction of the bulk water spectrum. With increasing protein concentration, two main changes are observed: a decrease in absorption in the low-frequency range (~90–250 cm^{-1}) and an increase in the high-frequency region (~350–600 cm^{-1}), as indicated by the two arrows. We also want to note that all solutions did not have a cloudy appearance or no indication of the existence of two phases, as in the case of LLPS, but rather were a clear single-phase solution.

In previous studies,[14,15,17] the THz absorption spectra recorded during the evolution of the LLPS process show a strong similarity to the present data. In these studies, the spectra were referenced to the initial measurement; that is, each spectrum was obtained by subtracting the spectrum of the first measurement in the time series. Two main characteristic spectral features in the spectra were observed: a decrease in absorption at 150 and 300 cm^{-1} followed by an increase at 400 cm^{-1}. The former is assigned to the loss of cavity wrap water upon desolvation of protein hydrophobic groups, and the latter was attributed to an increase in protein–water H-bonds due to an increase in protein concentration induced by condensation phenomena.

In our data, the negative absorption difference, $\Delta\alpha$, is redshifted with respect to that observed for proteins undergoing LLPS, being centered at 125 cm^{-1} rather than 150 cm^{-1}. Moreover, it exhibits

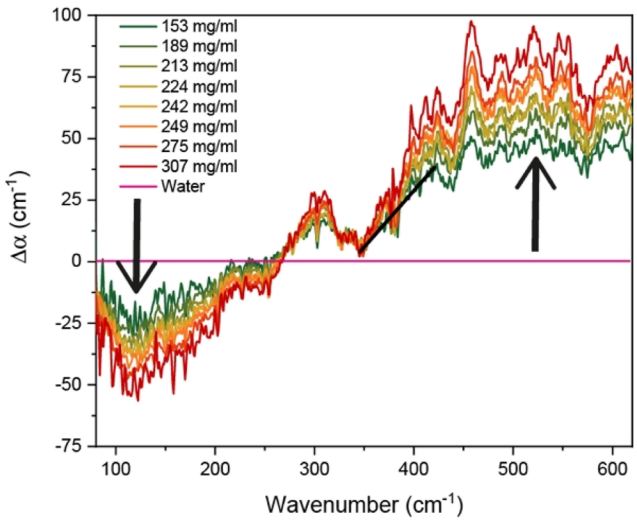

FIG. 1. Difference in THz absorption of lysozyme aqueous solutions at different concentrations compared to the THz absorption of bulk water. We observe the following trends in the frequency range around 125 and >350 cm^{-1} indicated by the two black arrows upon increasing concentration: while the amplitude at ~125 cm^{-1} is decreasing, we observe an increase in THz absorption between 350 and 600 cm^{-1}.

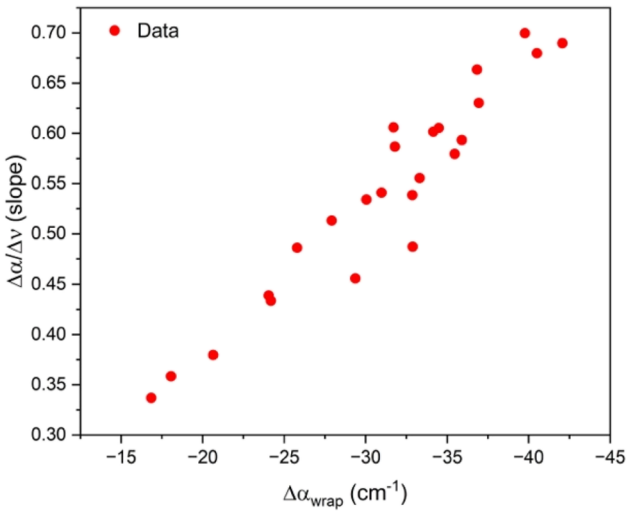

FIG. 2. Plot of hydration water population for lysozyme as a function of protein concentration. Plotted is $\Delta\alpha/\Delta\nu$ in the range between 300 and 400 cm^{-1} against the change in amplitude at 125 cm^{-1}.

a weaker dependence on concentration compared to condensation phenomena.[14] These differences can be attributed to the distinct nature of the proteins and processes investigated. Lysozyme is a globular protein which hydrophobic patches are much less exposed to water than that exposed in intrinsically disordered proteins. Thus, we expect a smaller loss of cavity-wrap water from hydrophobic patches upon desolvation. Accordingly, the loss of cavity-wrap water due to lysozyme clustering is expected to be much lower than that induced by protein condensates of α-elastin and FUS,[14,17] in the same probed sample volume. This can be attributed to both the lower surface exposed area and the lower protein concentration in the single-phase solution.

The spectra reported in Fig. 1 show high-frequency slopes apparently similar to the feature observed upon LLPS. Nevertheless, they occur over different frequency ranges: between 350 and 600 cm^{-1} and between 400 and 600 cm^{-1}. We want to point out that the rise in absorption starts already at 280 cm^{-1} instead of at 450 cm^{-1}. In fact, we observe a positive band in $\Delta\alpha$ around 300 cm^{-1}, which we attribute to an intramolecular protein–water absorption band[17] based on the linewidth and the linear increase with protein concentration. The overlap of a positive intramolecular band with the other negative $\Delta\alpha$ feature might cause this earlier overall increase. The increase around 500 cm^{-1} is the signature of an increase in hydrogen-bonded hydration water population.[18] This increase in absorption with respect to bulk water with increasing protein concentration is expected, as the number of hydration water molecules on the interface of the clusters increases with more proteins being probed. Both the decrease in $\Delta\alpha$ between 90 and 250 cm^{-1} and the increase between 350 and 600 cm^{-1} grow linearly with increasing protein concentrations (see Fig. 2).

We attribute the decrease in cavity wrap water to the formation of shared hydration shells with increasing protein molecules at higher concentrations. Because with increasing concentration, the protein molecules move closer to each other, and during this, we expect the loss of wrap water molecules, which is an entropically favorable process. Hence, along with the loss of wrap water, we observe that an increase in the number of bound water molecules interacting with hydrophilic groups on the surface of the protein increases linearly with concentration as there is an overlap of hydration shells. A graphic illustration of this is shown in Fig. 3.

As a further test of protein aggregation upon increasing the protein concentration, we carried out DLS measurements. The data were analyzed using the cumulants method.[16] According to this approach, the signal decay can be attributed to the presence of monodisperse protein together with two main populations of lysozyme clusters in aqueous solution, characterized by different sizes. The corresponding hydrodynamic radii are as follows: $R_1 \approx 1.5$ nm, associated with lysozyme monomers; $R_2 \approx 25$ nm, corresponding to small clusters; and R_3, related to the distribution of larger clusters. The R_3 values are shown in Fig. 4.

We find that for higher concentrations, the cluster population becomes highly dispersive, indicating the formation of a broad distribution of clusters with radii spanning from tens to hundreds of nanometers. Our results are in agreement with previous studies reporting evidence of equilibrium protein clusters at high protein concentrations.[4,9] This behavior has been attributed to a balance between electrostatic repulsion and attraction forces.

We can add the present data in our phase diagram [Fig. 5] (for details, see Refs. 15 and 19); we can clearly distinguish the effect of increasing the protein concentration for globular proteins, leading to cluster formation, from the formation of liquid droplets upon LLPS or solid protein aggregates in intrinsically disordered

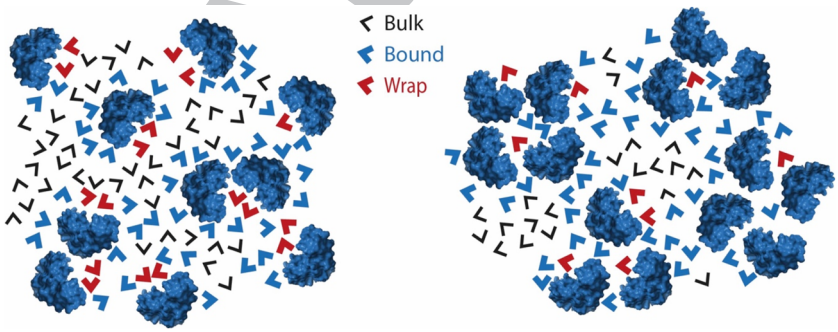

FIG. 3. Graphical representation of the water–lysozyme system at low (left side) and high (right side) concentrations. Bound and wrap hydration water molecules are represented in blue and red, respectively. The bulk water molecules are shown in black.

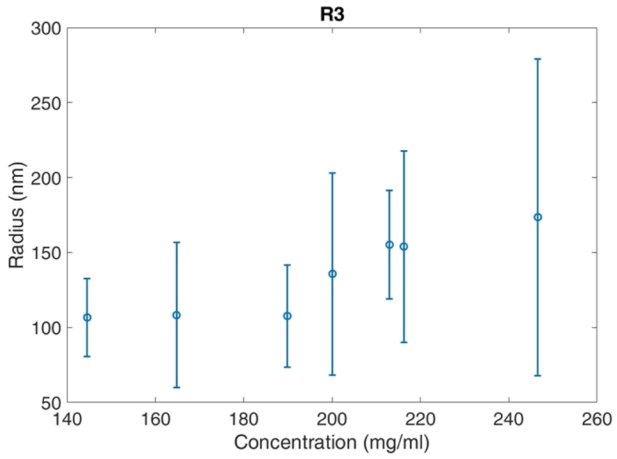

FIG. 4. Hydrodynamic radius of larger clusters (R_3) in water–lysozyme samples obtained from DLS measurements. The bars indicate the polydispersity detected by the instrument. As the concentration increases, the average radius increases and, in particular, the polydispersity increases dramatically.

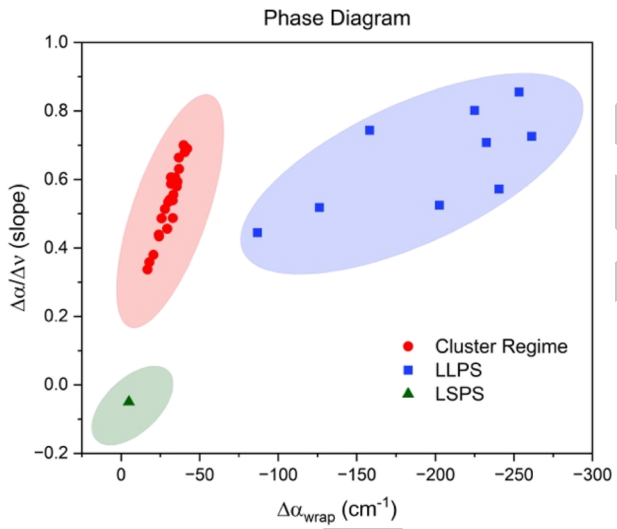

FIG. 5. THz phase diagram for protein solutions. The data presented in this work relating to a lysozyme clustering regime are shown in red, the data extracted from Pezzotti *et al.*[15] relating to the LLPS transition are shown in blue, and the data extracted from Ramos *et al.*[19] relating to an LSPS transition are shown in green. The three phenomena occupy three distinct regions of the phase diagram, demonstrating the efficiency of THz calorimetry in studying the effects of protein reorganization on water.

protein solutions. A comparison of the results obtained with those observed in the case of LLPS reveals that, during the formation of high-concentration droplets, there is a proportionally greater release of wrap water molecules than is observed during cluster formation. This is evident in the phase diagram.

CONCLUSION

We present THz and DLS studies of an aqueous solution of hen egg white lysozyme at high concentrations. The DLS studies point to the onset of protein clusters and provides an estimation of the distribution of cluster hydrodynamic radii at increasing protein concentration. The THz measurements provide insights into the nature of the hydration processes. As the protein concentration increases, the population of bound water (i.e., water molecules interacting with the hydrophilic patches of lysozyme) increases, whereas the number of water molecules solvating hydrophobic patches or less attractive regions of the protein surface decreases.

This behavior is consistent with the hydration phenomena observed during LLPS processes. However, the THz phase diagram, Fig. 5, shows that the changes in solvation phenomena of protein clusters can be clearly distinguished from those proteins that undergo LLPS or liquid–solid phase separation (LSPS).

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Author Contributions

L.C. and S.S.N. contributed equally to this work.

Luigi Caminiti: Data curation (equal); Formal analysis (equal); Investigation (equal); Writing – original draft (equal). **Sanjana S. Nalige**: Data curation (equal); Formal analysis (equal); Writing – original draft (equal). **Renato Torre**: Conceptualization (equal); Funding acquisition (equal); Project administration (equal); Supervision (equal); Writing – review & editing (equal). **Martina Havenith**: Conceptualization (equal); Funding acquisition (equal); Supervision (equal); Writing – review & editing (equal).

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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