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Protein crowding effects on hydration water dynamics

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ABSTRACT

Water is not only a solvent in which biological molecules are dissolved, but it also plays an essential and active role in the proper development of biological processes within living organism. Water hydration shell surrounds biomolecules and has a plasticizing impact on their backbone, allowing them to execute their physiological activity in biochemical processes and biophysical modulations. Although the essential role of water in biology is evident, the comprehension of hydration water properties remains limited; the mutual influence between the protein surface and the hydration layers, on regulating structure and dynamics, remains a strongly debated question. In particular, the experimental and simulation findings on fast dynamics of hydration water are still controversial. We propose a time-resolved optical Kerr effect study of the structural and vibrational dynamics of the hydration water surrounding lysozyme on a very fast time scale. Measurements as a function of lysozyme concentration makes it possible to distinguish the hydration water contribution from that of both the bulk water and the protein. Our results provide the experimental evidence of the existence of two structural dynamics of hydration water, associated respectively with a hydrogen bond exchange relaxation process and with the reorganization of water molecules induced by protein structural fluctuations. Likewise, we evaluated the vibrational dynamics of the water hydration layer at sub-picosecond time scales. Our study of the dynamics of hydration water reveals a lysozyme clustering phenomenon prompted by the self-crowding environment, which is associated with the liquid-liquid phase separation mechanisms inherent in these protein solutions.

Water molecules that hydrate proteins surface are fundamental for ensuring their structural flexibility, which is among the key requirements to guarantee their ability to perform physiological functions. Protein mobility is modulated by water dynamics, as well as protein motions influence the stability of water hydrogen bonds (HBs). However, the exact role of the hydration layers in regulating the continuous protein motion is still an object of intense debate[1-4]. Even the measurement of the real extent of the hydration shell, (i.e., the number of water molecules whose properties are influenced by the presence of the protein), is the subject of various scientific considerations due to the strong correlation with the spectroscopic observables investigated[4-6].

A widely studied system is that of lysozyme-water solutions. Thanks to the properties of lysozyme, this type of sample provides an excellent testing ground to better understand the physical principles behind several biological phenomena involving proteins. Indeed, this globular protein, which can be easily isolated from the white of a chicken egg, has a remarkably human-like structure and can easily create amyloid aggregates in vitro. This capability is critical for understanding the molecular pathways that lead to the production of amyloid oligomers and mature amyloid fibrils[7-9], which are responsible for many degenerative diseases [10]. Furthermore, lysozyme is one of the few basic proteins with a high isoelectric point, pH 11, which allows for easy charging of the protein surface by varying the pH or using other parameters such as ionic strength and protein concentration and thus shed light on the role of the excluded volume effect and the interaction potential in the formation of transient protein clusters[11-13].

The clustering of proteins in a solution of lysozyme and water is closely intertwined with the phenomenon of metastable liquid-liquid phase separation (LLPS)[14-16]. LLPS leads to the emergence of both a sparse and a densely populated phase, various aggregation structures can quickly emerge with slight alterations in physical parameters like ionic strength, concentration, and temperature. Hydration water plays a crucial role in regulating these clustering phenomena[17,18].

A variety of experimental techniques have been employed to examine the lysozyme-water solution. However, only a limited number have specifically targeted the measurement of hydration water dynamics due to the inherent challenges in distinguishing these dynamics from the contributions of proteins and solvents to the measured signal. Experimental investigations utilizing optical spectroscopic techniques hold promise in accessing ultrafast dynamics and thus in measuring hydration water dynamics. Specifically, optical spectroscopic studies have been conducted on lysozyme-water solutions, exploring both frequency[19,20] and in time[21-24] domain. The findings from these experimental investigations offer interesting insight into the lysozyme interactions and dynamics. Nevertheless, the collected experimental data does not facilitate a detailed examination of hydration water dynamics, so these peculiar dynamics are still not fully disclosed. The earlier time-resolved experimental investigations[21,22] focused on the protein dynamics and do not offer a detailed analysis of water dynamics. The recent studies both in frequency, Perticaroli et al[19,20], and in time domain, Mazur et al[23], do not reveal the articulated nature of hydration water dynamics; the analysed only the structural dynamics reproduced by a simple exponential relaxation associated with the breaking and reformation of hydrogen bonds forming the water network. So, all these studies do not reveal the presence of a second structural dynamic, predicted by simulative work[25,26], and do not characterize the intermolecular vibration of hydration water.

In this context, we performed an extensive experimental spectroscopic analysis employing time resolved Optical Kerr Effect techniques on lysozyme-water solutions. Our aim was to investigate the rapid dynamics of hydration water and detect any crowding effects caused by different protein concentrations. Our work succeeds to give detailed information on the structural and vibrational dynamics of hydration water investigating a very wide range of concentrations. Furthermore, we sought the establish correlations between these observations and the reported occurrence of LLPS in existing literature.

Our findings experimentally confirm the presence of two relaxation times associated with the hydrogen bond exchange relaxation process and with the reorganization of water molecules induced by protein structural fluctuations Furthermore, we sought the establish correlations between these observations and the reported occurrence of LLPS in existing literature.

The Heterodyne Detected Optical Kerr Effect (HD-OKE) technique is a non-linear time-domain spectroscopy[27-31]. In brief, a first non-resonant laser pulse with linear polarization (pump pulse) generates a transient anisotropic modification of the refractive index in an optically transparent and isotropic sample. The response of the material to the perturbation produced by the pump pulse is investigated with a second, circularly polarised pulse (probe pulse). The experimental control of the time delay between these two pulses enables the direct measurement of sample dynamics in the time-domain, i.e., the relaxation of the non-equilibrium coherent state, induced by the pump pulse in the molecular ensemble, towards the equilibrium state. Thanks to the femtosecond laser pulses employed, the accessible time window of this technique covers an extended time range, spanning from a few femtoseconds to tens of picoseconds.

The signal measured in the HD-OKE experiment can be expressed as[31]:

$$S(t) \propto \int_{-\infty}^{+\infty} [\gamma\delta(t-t') + R(t-t')]G(t')dt' \qquad (1)$$

$$R(t) \propto -\frac{\partial}{\partial t}\langle\chi(t)\chi(0)\rangle \qquad (2)$$

where $\gamma\delta(t)$ is the instantaneous electronic response and $R(t)$ is the third order nuclear response of the sample and $G(t)$ is the instrumental function. The latter represents the temporal cross-correlation of the laser pulse intensity. An accurate evaluation of the instrumental function it's a critical element of an HD-OKE measurement for a correct extraction of the sample response function[31,32]. The response function $R(t)$ is directly related to the time derivative of the correlation function of first order equilibrium susceptibility $\chi(t)$, see eq.(2). The $\chi(t)$ is defined by the dynamics of the entire molecular system investigated and includes many intra- and inter-molecular motions; so, its expression can be very complex and hardly can be described by a rigorous molecular model. Nevertheless, in the case of protein-water solutions, a general separation of the dynamic time scales is present[33]; in the fast sub-picosecond time scale the dynamics are mainly due to the vibrational dynamics of intra-molecular modes of protein and inter-molecular H-bond modes of water, while beyond the picosecond time scale, the dynamics are prevalently associated with the overall water structural relaxations. The dynamics of the protein structural transitions and rotational diffusion are very slow and not accessible in the time window probed in the present experiment.

The Fourier transform of the HD-OKE response function, $R(\omega)$, is directly connected with the spectrum measured in a Depolarized Light-Scattering (DLS) experiment. The imaginary part of $R(\omega)$ $Im[R(\omega)]$ is obtained by Fourier transform the measured HD-OKE signals after the deconvolution processes from the instrumental function: $Im[R(\omega)] = Im[S(\omega)/G(\omega)]$.

RESULTS

All the experimental measurements were acquired and analysed in the time domain. Figure 1A reports, in a semi-log scale, the OKE signal of some investigated lysozyme concentrations, while Figure 1B shows the same data in the Fourier transformed domain. To perform a compared data analysis of the vibrational and structural contributions of all the samples, a specific normalization procedure is needed. The normalization accounts for the signal intensity change due to the different concentrations of lysozyme in solution. This procedure is deeply described in the Supporting Information. Figure 1B shows the normalized response function spectra of some of the recorded data. The lysozyme concentration increase causes an increment of the signal intensity and a change of the spectral shape due to the structural and vibrational contributions.

In Figure 1C, we report an HD-OKE signal kinetics and its fitting function for the measurement at a 250 mg/ml protein concentration. The experimental signal of this sample (red points) shows a long structural exponential decay of tens picoseconds, while the inset in the short time range (< 2 ps) shows the oscillations due to the intermolecular vibrational dynamics in solution of both protein and water.

In order to fit the measured signal by a relatively simple function we utilized a phenomenological model based on the multi-Damped Harmonic Oscillators (m-DHO) approach[32]. It should be noted that unlike the majority of OKE measurements in literature [22,23], we performed all the data fitting in the time domain. The fitting process enables to disentangling the different contributions coming from bulk water, hydration water, and protein. The HD-OKE response function is composed by the bulk water response, $R_{bulk}(t)$, and by the hydration water and lysozyme the response function, $R_{h-ly}(t)$; so that:

$$R(t) = F_{bulk}R_{bulk}(t) + R_{h-ly}(t)$$

The bulk water response $R_{bulk}(t)$ it is simulated by an analytical function that is fixed and defined by the independent data analysis of the pure water OKE signal[31]; this response is weighted by the bulk water volume fraction, F_{bulk}, present in each lysozyme sample considered. This fraction parameter was calculated following the estimate made by Camisasca et al.[26], where the hydration water corresponds to the first hydration layer around each lysozyme molecule (see Supporting Information). $R_{h-Ly}(t)$ is the response function related to the protein and hydration water contributions. This includes two components: a structural, $R_{struct}(t)$, and a vibrational response, $R_{osc}(t)$:

$$R_{h-ly}(t) = R_{struct}(t) + R_{osc}(t) \qquad (3)$$

According to our data analysis, the structural response can be reproduced by a bi-exponential decay, characterized by two structural relaxation times: τ_1 and τ_2. The vibrational response can be simulated by a series of Damped Harmonic Oscillators (DHO), and it requires a minimum of four DHO.

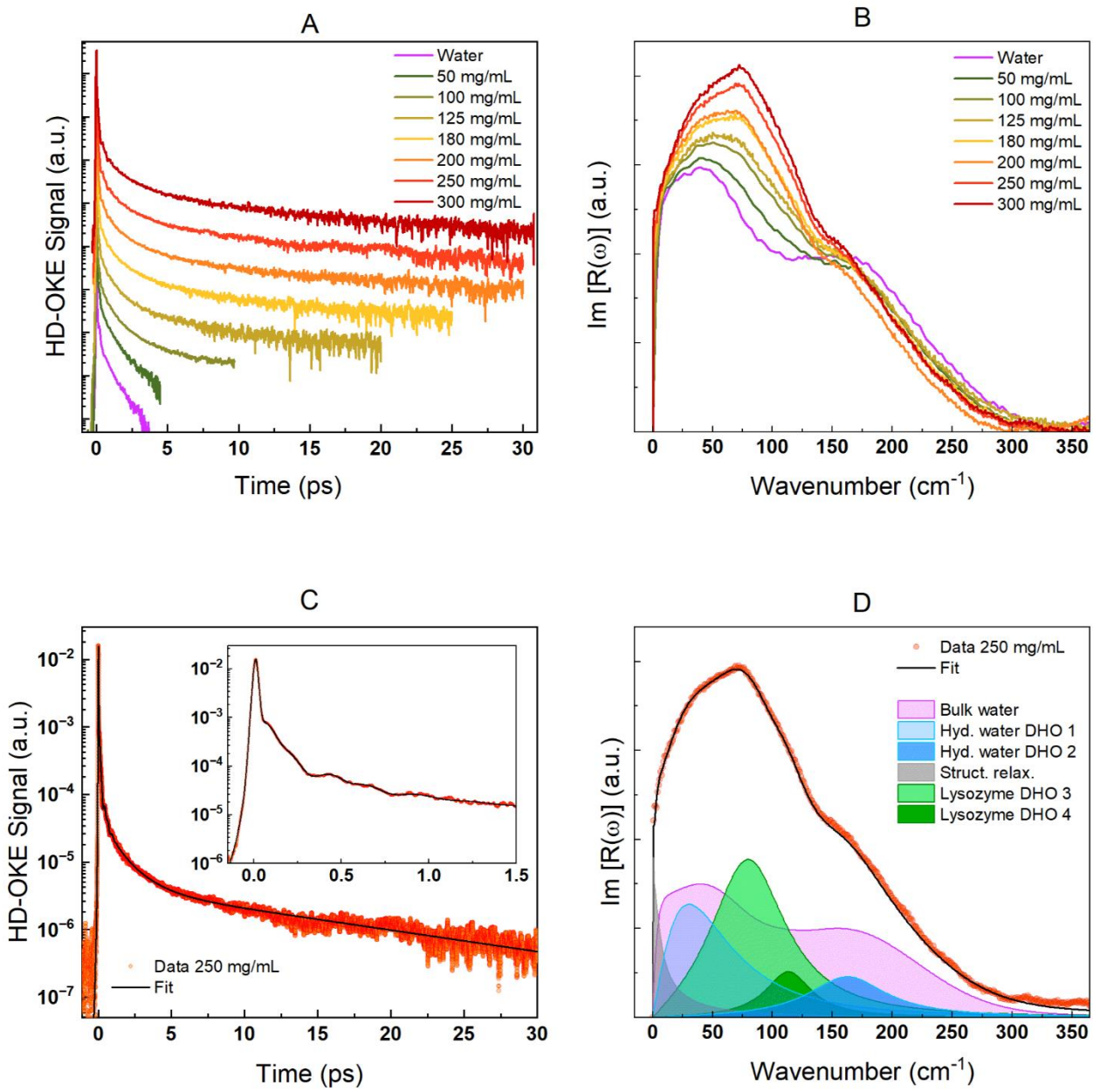

Figure 1. (A) HD-OKE experimental data of 50, 100, 125, 180, 200, 250, and 300 mg/mL (from green to red) lysozyme solutions, compared with that of pure water (purple trace). Each curve is presented multiplied by an appropriate factor to avoid overlapping. (B) Response function spectra of some of the kinetics of panel A after the deconvolution from the instrumental response and the normalization procedure described in the Supporting Information. (C) Semi-log experimental signal of the 250 mg/mL (red points) lysozyme solution and its time fitting function (black line). The inset shows signal oscillations due to vibrational dynamics in the short time range (< 2 ps). (D) Spectrum of the response function of 250 mg/mL lysozyme solution. The experimental data (red points) and the relative fitting curve (black line) are compared with the bulk water contribution (purple shaded curve) imposed to the fitting function ($F_{bulk}R_{bulk}$) and with the other spectral contributions. In detail: a biexponential relaxation (grey shaded curve) and two damped Lorentzian oscillators associated with the hydration water response (shaded blue curves) and other two damped Lorentzian oscillators associated with the protein response (shaded green curves), all extrapolated from the fitting function (R_{h-ly}).

Through our rigorous data analysis, we have concluded that these are the minimum number of functions that align with the physical model and are necessary to accurately reproduce the HD-OKE data.

The errors associated with the fitting parameters are primarily influenced by the exact reproducibility of the experimental conditions and measurements. As a result, these errors are estimated by fitting a series of measurements and it is determined by comparing the dispersion of values from numerous measurements of the same sample.

Figure 1D shows the data of the 250 mg/mL lysozyme solution in the frequency domain (red points) and the Fourier Transform of the fit (black line), which was performed in the time domain. In addition, figure 1D shows all the components contributing to the fit. These are the $R_{bulk}(t)$ function, weighted by the F_{bulk} factor, and the $R_{h-ly}(t)$ function, composed of the bi-exponential contribution (grey shaded curve) and the four damped harmonic oscillators (blue and green shaded curves). In the following section, we provide a deeper description of these dynamic contributions present in the HD-OKE signal.

STRUCTURAL RELAXATION

Here we analyse the relaxation dynamics contained in the $R_{struct}(t)$ fitting function, i.e. the bi-exponential decay, characterized by two structural relaxation times, τ_1 and τ_2.

These relaxation dynamics are limited to tens of picosecond time range and can be addressed only to the hydration water dynamics since the protein relaxations, both structural and rotational, are characterized by much slower time scales[33].

Our HD-OKE data reveals that hydration water dynamics is characterized by two separate relaxation processes, each operating on distinct time scales: one occurring within a few picoseconds, and the other spanning tens of picoseconds. This observation aligns with the predictions of computer simulation studies of the lysozyme-water system[25,26] and findings from time-resolved fluorescence spectroscopic studies of protein solvation in other protein-water solutions[34,35].

According to these prior investigations, these two relaxation processes are related to two distinct dynamical phenomena:

- the rapid relaxation, observed in our data to be approximately 1.6 picoseconds, is driven by the breaking and reforming of hydrogen bond networks associated with OH large-amplitude jumps[4,25]. This phenomenon mirrors the α-relaxation process in the bulk water[25,26] but is slowed down by a factor of about 3 due to the binding of water molecules to hydrophilic protein residues[19,26];
- the slower relaxation, measured in a range from about 13 picoseconds to 22 picoseconds, is linked to a structural dynamical phenomenon influenced by rearrangement dynamics driven by the structural fluctuations of the protein. This dynamic is absent in bulk water.

The amplitudes, $\mathrm{A_{exp1}}$and $\mathrm{A_{exp2}}$, of both these relaxation times are reported in the supplementary information. Figure 2 reports the values of the relaxation times obtained by the biexponential fit, $\boldsymbol{\tau_1}$ and $\boldsymbol{\tau}_2$, of the experimental data at variable concentrations. The first relaxation shows a nearly constant time of $\boldsymbol{\tau_1}$ ~ 1.6 picoseconds at variable concentrations (i.e., this dynamic appears to be very weakly affected by the increasing of the lysozyme concentration in solution).

Differently, the slower relaxation process reveals a slight acceleration with increasing protein concentration. Additionally, an anomalous dynamic crossover is observed at a concentration of about 225 mg/mL, which separates two dynamic regions related to increasing protein concentration. As we introduced previously, the lysozyme-water solutions are affected by the liquid-liquid phase separation (LLPS) that defines in the phase diagram a binodal line with a critical point taking place at a concentration of about 200 mg/mL, typically at temperature lower than ambient temperature and depending on the chemical physics parameters of the solution[14-16].

Moreover, this concentration range was even connected with the establishment of weak protein-protein interactions, whereby the delicate balance between short-range attraction potential and long-ranged electrostatic repulsion results in the formation of transient protein clusters in dynamic equilibrium with each other and with protein monomers[11,13,36].

VIBRATIONAL DYNAMICS

The analysis of the vibrational dynamics region (from 0.01 to 0.1 ps) turns out to be quite complex and it generates a debate on the assignment of the various vibrational components. The challenge arises mainly from the arduous task of differentiating between hydration and bulk water vibrational contributions.

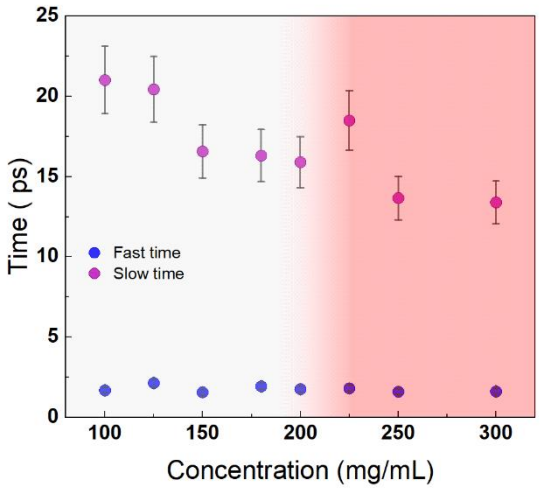

Figure 2. Relaxation times, τ_1 and τ_2, extracted from the bi-exponential fit function reported in Method. The data of 50 mg/mL lysozyme solution does not enable reliable extraction of relaxation dynamics of the hydration water component, so this has not been reported. The red area of the graph highlights a dynamic crossover point at the critical concentration of 225 mg/mL indicating the beginning of a different dynamic region influenced by the formation of dynamical cluster in solution.

The HB intermolecular vibrational bands of the hydration water occupy the same spectral region as the bulk water bands[37]. Alike, some protein modes are characterized by vibrational frequencies similar to water modes. As a result, the hydration water contribution to the vibrational spectrum was typically handled as a component of the global solvent spectrum[24] or was incorporated into the hydrated protein signal[19,22].

In our experimental investigation the OKE signal in the sub-picosecond time range, already subtracted from the bulk water contribution, is characterized by two main vibrational contributions of hydration water and the protein intramolecular modes, i.e. vibrations of protein structures and librations of lysozyme amino acid side chains[24]. As already described, the recorded OKE signal in this time interval was completely reproduced by four DHOs (panel D of figure 1). The DHO n.3 and n.4 can certainly be assigned to lysozyme librations, as proved by the comparison with previous Raman spectra recorded on protein crystals[38]. Differently, the DHO n.1 and n.2 contributions in the vibrational spectrum can confidently be attributed to the hydration water modes.

Figure 3A shows the hydration water spectra obtained by subtracting the bulk water and the lysozyme oscillators contributions from the full data spectrum of the 250 mg/mL protein solution. Also, the bulk water component is reported resembles the spectrum of bulk water (violet line), even if some differences are clearly present. Moreover, if we further subtract the structural relaxation component (grey shaded curve), we obtain the clear vibrational modes of the clear vibrational modes of hydration water (blue lines and circles); these can be reproduced by two oscillators at 55 and 166 cm^{-1}, the DHO n.1 and n.2 in the fitting function These oscillators are quite similar to the modes characterizing the hydration water layers of silica nanopores[37]. According to our spectrum analysis, these modes represent the intermolecular bending and stretching vibrations of the water molecules in the first layer around the proteins, respectively.

CONCLUSION

In this HD-OKE investigation, thanks to the high signal-to-noise ratio of our data, precise data analysis and fitting procedures, and an extensive concentration study, we successfully differentiate and quantify the various dynamic processes taking place in protein-water solution.

Our focus was on the dynamics of hydration water. We conducted measurements of structural dynamics over extended time windows, encompassing the sup-picosecond regime. Our findings revealed the existence of two relaxation processes: one associated with the water α-relaxation and another linked to structural reorganization of water coupled with protein fluctuations. This observation, for our knowledge, constitutes the first experimental validation of phenomena predicted by simulation studies[25,26] and provides crucial insights into the debated understanding of protein hydration water dynamics.

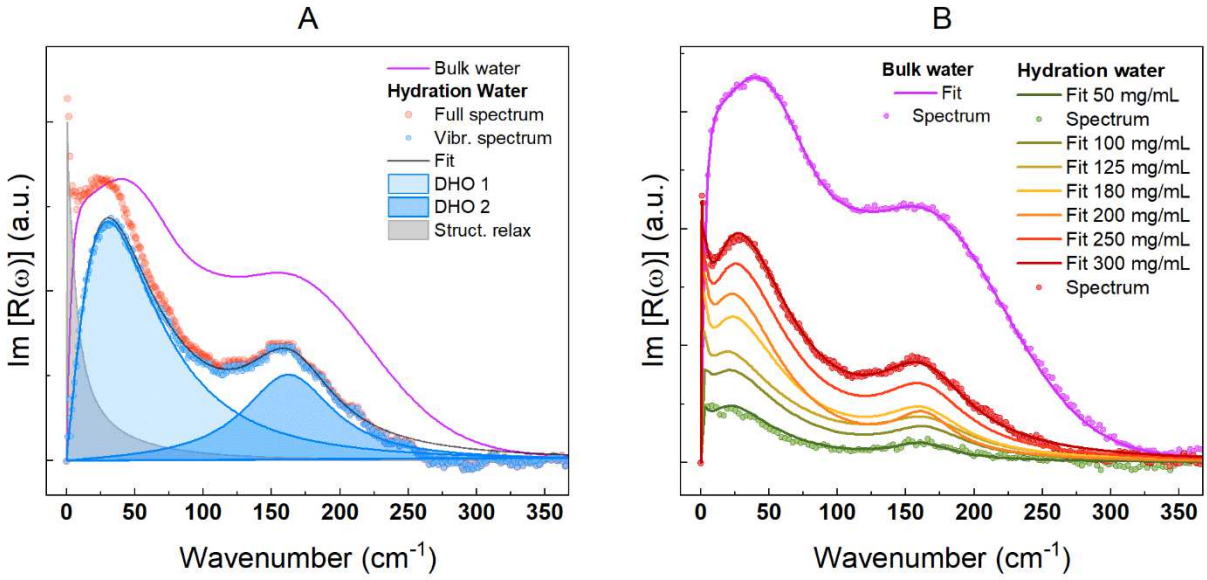

Figure 3. (A) Comparison between the full spectrum (red circles) and the vibrational spectrum (blue circles) of the hydration water dynamics, both extracted from data of 250 mg/mL lysozyme-water sample; for completeness, we also show the spectrum of the bulk water component weighted by the bulk water volume fraction (violet line). The fitting result for the structural component (grey shaded line) and the two hydration water oscillators (blue shaded lines, DHO1 and DHO2) are also reported. (B) Fitting functions and data spectrum of hydration water extrapolated from lysozyme-water data from 50 mg/mL (green line and circles) to 300 mg/mL (red line and circles), and results from a bulk water sample (violet line and circles). We do not report the data of intermediate concentrations to avoid crowded figure.

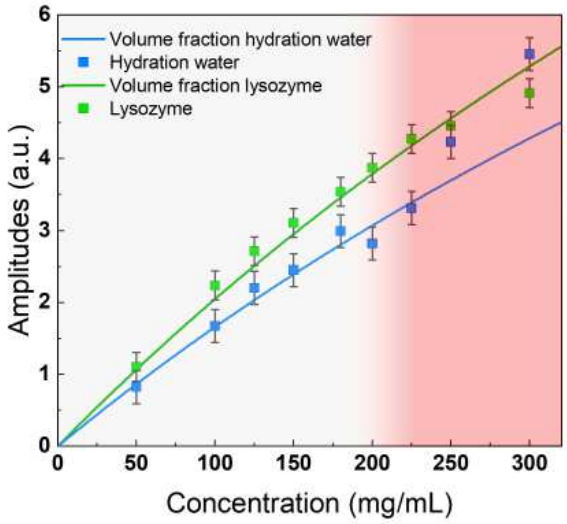

Figure 4. We report the amplitudes of vibrational modes and the volume fractions of both hydration water and lysozyme protein. The amplitudes are obtained from the integral areas of the DHO spectra: the sum of DHO n.1 and n.2 for hydration water (blue squares) and the sum of DHO n.3 and n.4 for lysozyme protein (green squares). The volume fraction values of hydration water (blue line) are calculated assuming a monolayer of water surrounding the protein, while those for lysozyme protein (green line) are determined using an ellipsoidal geometric approximation of the globular protein in solution (further details provided in the Supporting Information). The red shaded region on the graph highlights concentrations at which the amplitudes of hydration water deviate from the volume fraction trend.

Additionally, our measurement methodology facilitates direct examination of the vibrational dynamics within the protein solution, allowing for the isolation of hydration water components from overarching signals. Our findings reveal a resemblance between the vibrational spectrum of hydration water and that of bulk water, both exhibiting bending and stretching intermolecular modes. However, distinctive features are observed in hydration water modes, differentiating them from bulk water vibrational modes.

The remarkable capability of the HD-OKE experiment to discern the impacts of exceedingly subtle biomolecular interactions within a solution is noteworthy. Such interactions are notably challenging to detect experimentally using alternative modes. These findings underscore the potency of the HD-OKE technique as a formidable tool for identifying weak interactions among proteins through the analysis of hydration water properties.

This investigation into the structural and vibrational dynamics of hydration water components across varying protein concentrations has unveiled several intriguing phenomena. The HD-OKE data revealed a specific concentration value, about 225 mg/mL, marking a crossover between two distinct dynamics regimes. Our interpretation suggests that this crossover point arises from the formation of more stable protein clusters as the system approaches a self-crowded region. These clusters enhance protein-protein interactions, thereby influencing hydration water dynamics. In essence, the decrease in protein-protein distance amplifies the electrostatic potential effect experienced by the mean polarizability of water surrounding proteins.

Even more surprising is the abrupt nature of these dynamic variations, which manifest at a specific concentration. A comparison with the phase diagram of the lysozyme-water solution hints at a correlation between the observed crossover point and the critical point of the LLPS[14,15]. This connection is further substantiated by the close relationship between the formation of protein clusters and LLPS. Despite the ambient temperature, at which our measurements are being performed, considerably distant from the critical region of LLPS, the observed crossover point suggests a profound influence of this critical phenomenon on cluster formation within the equilibrium phase of the protein solution.

METHODS

HD-OKE SET-UP

The apparatus used for the HD-OKE measurements is based on a self-mode-locked Ti:sapphire laser (Femtolasers, model Fusion) producing pulses of 15 fs duration, 80 MHz repetition rate and 790 nm wavelength. The experimental set-up details are reported in *A. Taschin et al.*[31].

SAMPLE PREPARATION

The HD-OKE measurements are performed on lysozyme water solutions at nine different concentrations (50, 100, 125, 150, 180, 200, 225, 250 and 300 mg/mL) at a controlled temperature of 298.0±0.1 K. The samples were prepared by dissolving the specified amount of dry powder of Hen Egg-White lysozyme (HEWL) obtained from Sigma Aldrich (L6876) in 1 mL of pure water (S.A.L.F. sterile and non-pyrogenic water for injections). We achieved the complete dissolution using Vortex mixing. All the samples were then filtered with a syringe filter (membrane 0.22 μm pore size) to avoid the introduction of dust. We checked the pH value of each sample with a pH Meter (istek 730P) to be around 4.8 as expected by the absence of any added acidic solution. The features of our samples ensure that the globular native shape of the protein is preserved, avoiding the formation of stable aggregates during measurements [39].

For the data normalization procedure, we prepared three solutions of lysozyme (100, 200 and 300 mg/mL) dispersed in a specific mixture of pure water and Glycerol (Sigma Aldrich G7893). The mixture of the two solvents was at 10.74 % of glycerol molar fraction.

All the samples are analysed inside a fused silica cuvette with a dimension of 2x10x30 mm^3.

INSTRUMENTAL FUNCTION

A very reliable technique is to perform a measurement on a reference sample of calcium fluoride plate (CaF_2) placed inside the sample cuvette. The very simple nuclear response of calcium fluoride allows an accurate extraction of the instrumental function[31]. Unfortunately, this approach is not feasible due to the limited size of our cuvette. In [32] it's demonstrated that it's possible to build an artificial instrumental function by fitting the first part of the electronic peak (the rise of the signal) with an analytic function that, together with its symmetric image, gives a simulated instrumental function. The result of this method is not accurate as that one obtained with the CaF_2 procedure but at the same time it's the better compromise when the best option it's not applicable. So, we decided to extract $G(t)$ using this method.

FITTING PROCEDURE

The fitting functions appearing in the eq.(3) are simulated by few analytic functions [32]. The structural relaxation, $R_{struct}(t)$, is defined by two exponential decays combined with a very fast rising contribution with a fixed parameter is $\tau_R = 50$ fs. This parameter ensures that the response function has its origin at zero.

$$R_{struct}(t) = \frac{A_{exp1}}{\tau_1}\left(1 - e^{-\frac{t}{\tau_R}}\right)e^{-\frac{t}{\tau_1}} + \frac{A_{exp2}}{\tau_2}\left(1 - e^{-\frac{t}{\tau_R}}\right)e^{-\frac{t}{\tau_2}}$$

The vibrational response, $R_{osc}(t)$, is described by the time derivative of a series of Damped Harmonic Oscillators (DHO):

$$R_{osc}(t) = \sum_{n=1}^{4} \frac{A_n}{\sqrt{\omega_n^2 - \frac{\gamma_n^2}{4}}} e^{-\frac{(\gamma_n t)}{2}} sin\left(\sqrt{\omega_n^2 - \frac{\gamma_n^2}{4}}\, t\right)$$

The errors associated with the fitting parameter are primarily influenced by the exact reproducibility of the experimental conditions and measurements. As a result, these errors are estimated by comparing the dispersion of values from numerous measurements of the same sample.

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