Article

Probing Globular Proteins Self-Assembling Dynamics by Heterodyne Transient Grating Experiments

Sara Catalini[1,*], Andrea Taschin[1,2], Paolo Bartolini[1], Paolo Foggi[1,3] and Renato Torre[1,2]

[1] European Laboratory for Non-Linear Spectroscopy (LENS), Università di Firenze, Italy;
[2] Dip. di Fisica ed Astronomia; Università di Firenze, Italy;
[3] Dip. di Chimica, Biologia e Biotecnologie, Università di Perugia, Italy;
* Correspondence: catalini@lens.unifi.it ; LENS, via N.Carrara 1, 50019 Sesto Fiorentino, Firenze, Italy.

Abstract: In this work, we have studied the propagation of ultrasonic waves of lysozyme solutions characterized by different degrees of aggregation and networking. The experimental investigation has been performed by means of the Transient Grating (TG) spectroscopy as a function of temperature; this technique enables to measure the ultrasonic acoustic proprieties over a wide time window, ranging from nanoseconds to milliseconds. The fitting of the measured TG signal allows the extraction of several dynamic properties, here we focused on the speed and the damping rate of sound. The temperature variation induces in the lysozyme solutions a series of processes: protein folding-unfolding, aggregation and sol-gel transition. Our TG investigation shows how these self-assembling phenomena modulate the sound propagation, affecting both the velocity and the damping rate of the ultrasonic waves. In particular, the damping of ultrasonic acoustic waves proves to be a dynamic property very sensitive to the protein conformational rearrangements and aggregation processes.

Keywords: protein self-assembling; protein hydrogel; lysozyme; ultrasonic sound propagation; transient grating spectroscopy

1. Introduction

In order to fulfill protein functions the polypeptide chains must be organized in their native conformation. In fact, after protein biosynthesis the folding process occurs, giving the protein a three-dimensional structure that makes it biologically active. Sometimes, an erroneous proteins structuring (misfolding) may occur resulting in the onset of many diseases [1]. In general, the development of the protein structural organization is given by the tendency to minimize interactions between the hydrophobic residues and the polar solvent and depending on solvating conditions, folding or misfolding and aggregation can be favored. It is possible to modulate the proteins unfolding and aggregation processes changing the environmental conditions such as pH, ionic strength, solvent composition and temperature. These self-assembling phenomena are characterized by different steps in which the protein undergoes conformational rearrangements and intermolecular association to form stable structures of increasing complexity [2,3], such as amyloid fibrils and hydrogel networks [4,5]. Proteins based hydrogels have recently received great attention since they can be the key to develop novel biomaterials. In fact, hydrogels have the capability to retain inside their matrix a great amount of water (up to 97%), [6] which make these materials similar to biological tissues [7,8]. Protein hydrogels are therefore of great interest in many fields spanning from the medical to the nanotechnological ones [9-11]. Moreover, hydrogels hold the promise to help in the understanding of tissue formation and transformation; for example, the β-sheet intermolecular motifs, that are responsible of several diseases, form hydrogel structures self-organizing into three-dimensional networks. The hydrogel matrices have been utilized as scaffolds for stem cells [12], highlighting how the mechanical properties of gel can modulate the cells differentiation processes.

In the class of globular proteins, an excellent study case is Hen Egg White Lysozyme (HEWL). In fact, HEWL is a small globular protein easy to find, that is capable to produce a variety of aggregated structures, including the intermolecular β-sheet. The self-assembly of HEWL, and more in general of globular proteins, rise much interest in biomaterial science since they can originate biocompatible hydrogels whose properties can be modulated by changing the aggregation condition. Lysozyme hydrogels are promising candidates to produce successful cell scaffolds, thanks to their high cyto-compatibility [13]. With the aim of studying the protein aggregation processes and following the evolution of the system gelation, we choose to work with an acid (pH= 1.4) and highly concentrated (240 mg/mL) HEWL solution [14]. In particular; the low pH value favors the unfolding process and the high protein concentration facilitates the aggregation/gelation of the system [15].

The investigation of protein solutions and hydrogel by ultrasonic propagation has been performed by a limited number of research studies [16-18], showing the complexity of the elastic and structural proprieties. We investigate the propagation of ultrasonic acoustic wave in the lysozyme solution and hydrogel by Heterodyne Transient Grating (HD-TG) spectroscopy[19]. This is a powerful tool that provides valuable information about the elastic properties and their relationship with the relaxation and structural phenomena, both in homogeneous matter [20-24] and materials characterized by complex structures [25-30].

HD-TG experiments can be considered an important advance in the investigation of hydrogels, thanks to their ability to measure collective relaxation times in a wide time/frequency window, hardly accessible by other methods. HD-TG measurements as a function of the environmental conditions can support a more comprehensive understanding of the relationship between the elastic and structural properties with the self-assembling phenomena taking place in biologic samples.

2. Experimental Methods

2.1. Sample Preparation

The dialyzed and lyophilized powder of HEWL (Sigma Aldrich, 62970) was dissolved without further purification in deuterium oxide (99.9 atom % D, Sigma Aldrich), to prepare solutions with concentrations of 120 mg/mL and 240 mg/mL. The protein solubilisation was increased by leaving the sample at 50°C for an hour allowing the exchangeable hydrogens of the proteins to be replaced by deuterium. Deuterium oxide was chosen as a solvent because of will be interesting in the future to compare the TG measurements with those of infrared absorption. After the total dissolution, the pH was adjusted with a 2M deuterium chloride (DCl) solution, to have a final pH value of 1.4. The solution was filtered with Millex-HV sterilized syringe filter, 0.45 μm pore size, directly into a cuvette. The filtration is mandatory to remove the larger impurities that would disturb the signal acquisition. Regarding the preparation of the gel, we start from the solution with the highest concentration, which is filtered and placed in a cuvette. The solution is left in the thermostat at 55°C for two hours and then quickly cooled by placing the cuvette in water at room temperature. A very viscous solution is obtained and it is left at room temperature for about 10 hours, to obtain a transparent gel.

2.2. Transient Grating Experiments

The HD-TG experiment is a time-resolved optical technique, based on third-order non-linear optical response. The experimental details have been described in previous works[19,24,28,31] and here we summarize only the main experimental aspects. Two infrared (1064 nm) laser pulses, with a temporal duration of 20 ps, interfere and produce into the sample a temperature and density spatially periodic variation that generates a refractive index grating. The angle between the two pump pulses define the wave-vector **q**, which characterizes the spatial modulation of the sample optical properties. The modulus of **q** is defined through $\left(\frac{4\pi}{\lambda_{ex}}\right) sin(\vartheta_{ex})$ where λ_{ex} and ϑ_{ex} are the wavelength and the incidence angle of the exciting pulses, respectively. A third continuous-wave laser beam, at 532 nm, is used to probe the modulation of the material optical properties, induced by

the pump beams. The time-resolved intensity of the diffracted probe provide information on collective relaxation phenomena in a wide temporal range, from nanoseconds to milliseconds, in a single experiment. To properly measure the natural damping of the induced acoustic oscillations, the pump is focused with a cylindrical shape to have on the sample an excitation grating extended in the q-direction, while the probe beam is focused to a circular spot in order to have a probe area with smaller dimensions in q-direction. The pump beams produce two acoustic pressure waves (i.e. two longitudinal bulk ultrasonic waves), which counter-propagate in the q-direction, their superposition create a stationary wave. The probe beam is scattered by the standing wave, producing the oscillations in the acoustic signal. The induced standing wave have a life time related to the pump spatial extension in the q-direction (geometrical damping time). The natural damping time of acoustic wave is properly extracted if it is shorter compared to the geometrical one. Since in our samples the natural damping time is quite long (tens and hundreds of ns), a very precise alignment of the pump and the probe beams is required in order to measure the natural damping. Therefore, the alignment is done through a camera, which permits a micro meter control on the centring and on the spatial superposition of the beams.

2.3. Data Collection

We record the data using a fast time window (0-80 ns range with a 50 ps time step of sampling) and a long one (0-2 μs range with 800 ps time step). The measurements are merged in a single data file. Each data is the average of 1000 records, this procedure produces an excellent signal to noise ratio. The HD-TG signal can be defined according to the following equation [19,28]:

$$S(q,t) \propto \langle |E_d(q,t)|^2 \rangle + \langle |E_l|^2 \rangle + 2\langle |E_d(q,t)| \rangle \langle |E_l| \rangle \cos \Delta\varphi \ ,$$

where E_d is the electric field diffracted by the grating, E_l is the local field and $\Delta\varphi$ is the phase difference between the E_d and E_l. If the local field contribution, $\langle |E_l|^2 \rangle$, is higher than the diffracted one, $\langle |E_d|^2 \rangle$, the homodyne contribution becomes negligible and the time variation of the signal is dominated by the heterodyne term, $2\langle |E_d(q,t)| \rangle \langle |E_l| \rangle \cos \Delta\varphi$. In addition, the heterodyne detection enables the cancelling of eventual spurious contributions present in the signal; we recorded two HD-TG signals with different phases of the local field, the first signal S_+ with $\Delta\varphi_+ = 2n\pi$ and a second one S_- with $\Delta\varphi_+ = 2(n+1)\pi$; then we subtract S_- from S_+. This procedure extracts the pure HD signal from the not phase sensitive contributions, including the homodyne and local field ones:

$$S^{HD}(q,t) = [S_+ - S_-] \propto \langle |E_d(q,t)| \rangle \langle |E_l| \rangle .$$

We measure the relaxation processes of our samples at $q = 2.1\ \mu m^{-1}$ in the temperature range 20 – 80 °C. The samples are kept directly in the cuvette introduced in a copper cell holder, connected to a thermostat and a thermocouple to feedback the temperature control. During the data collection, the diluted 120 mg/mL HEWL solution remains liquid in all the investigated temperature range. Instead, both the 240 mg/mL HEWL solution and the hydrogel, at 60 °C becomes opaque, making impossible to extend the measurements at temperatures higher than 60 °C.

3. Results and Discussion

In the HD-TG experiments the pump fields interact with the material inducing into the sample a density grating; this is build up by both a weak absorption of the laser pulses and the electrostriction effects. The absorption is due to the presence in the sample of harmonic/combination vibrational bands having frequencies resonant with the laser wavelength, 1064 nm; the electrostriction is due to the migration of the induced dipole in the regions of the maximum of the electric field gradient. These mentioned effects produce two standing acoustic waves characterized by a phase difference of π/2. Both the standing waves decay in time with an exponential law, $e^{-\Gamma_A t}$. The laser absorption phenomena induces in the sample a third interaction effect: the thermal grating. This is static grating that decays through the heat diffusion processes; represented by an exponential

law, $e^{-\Gamma_H t}$. The thermal diffusion time constant, $\tau_H \propto {}^1/_{\Gamma_H}$, is typically much longer than the damping time of the acoustic oscillation, $\tau_A \propto {}^1/_{\Gamma_A}$.

The S^{HD} can be found using the generalized hydrodynamic equations and it can be expressed as follows [19,21,32]:

$$S^{HD} = A\, e^{-\Gamma_A t}\, cos(\omega_A t) + B\, e^{-\Gamma_A t}\, sin(\omega_A t) + C\, e^{-\Gamma_H t}\,.$$

This expression has been utilized to perform the fitting of the measured HD-TG signals. The fitting parameters are: the acoustic angular frequency, ω_A, the acoustic damping rate, Γ_A, the thermal decay rate, Γ_H, and the corresponding amplitude constants, A, B and C. In this work we focus our attention on the speed of sound, $C_s = \omega_A/q$, and the acoustic damping rate, Γ_A; in particular on the trend of C_s and Γ_A as a function of temperature. The relative uncertainties associated with these values are those related to the fitting procedure, which are $\Delta\omega_A/\omega_A \leq 0.1\%$ and $\Delta\Gamma_A/\Gamma_A \leq 1\%$, respectively.

In the HD-TG measures performe in this work, the angle between the pump laser beams is 10.4° and this condition defines a wave-vector of 2.1 μm^{-1} to which correspond a sound wavelength of 3 μm. Figure 1 shows the linear-log plot of the HD-TG signal and the relative fit of the 120 mg/mL lysozyme solution at room temperature.

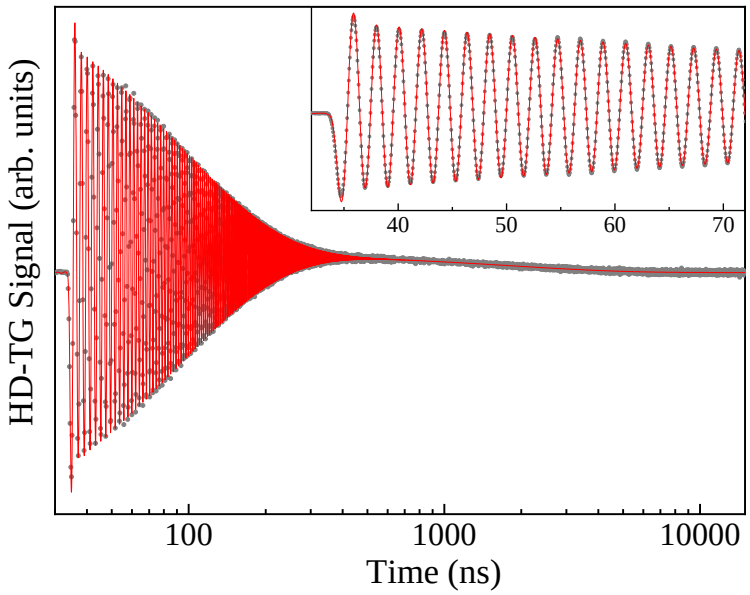

Figure 1: HD-TG decay signal (gray dots) and its relative fit (red line) of 120 mg/mL lysozyme aqueous solution at T= 23 °C and wave-vector q = 2.1 μm^{-1}.

Figure 2 shows the linear-log plot of the HD-TG decay signals and their relative fit of both lysozyme solutions and of the gel system, at room temperature and at 60°C. Figure 2, shows that at room temperature (left panel of Fig. 2) the damping rate of the acoustic oscillation is higher in the solution with the highest protein concentration (Γ_A = 14.7 MHz), and even higher in the gel system (Γ_A = 20.0 MHz). Such behaviour is related to the increase of the sample viscosity going from the diluted solution to the gel matrix, and to the amount of the relaxation channels that an acoustic wave comes across propagating in the sample. Furthemore, scattering processes can start to play a role during the propagation of ultrasonic waves in these complex materials. The increase of damping rates reflects the increase of the system complexity.

As regards the 120 mg/mL HEWL solution, the damping rate of the acoustic oscillation at 60 °C (rigth panel of Fig. 2) is lower compared to that at 23°C (left panel of Fig. 2). In fact, at high temperature a decrease of solution viscosity occurs, due to the increase of the thermal motions of the molecules. Concerning the concentrated solution and the gel matrix at 60 °C, their HD-TG decay signals are comparable. In fact, the parameters that describe the propagation of the acoustic wave

into the materials are Γ_A = 13.2 MHz, C_s=1.52 Km/s for the concentrated solution and Γ_A = 14.3 MHz, C_s=1.53 Km/s for the gel system.

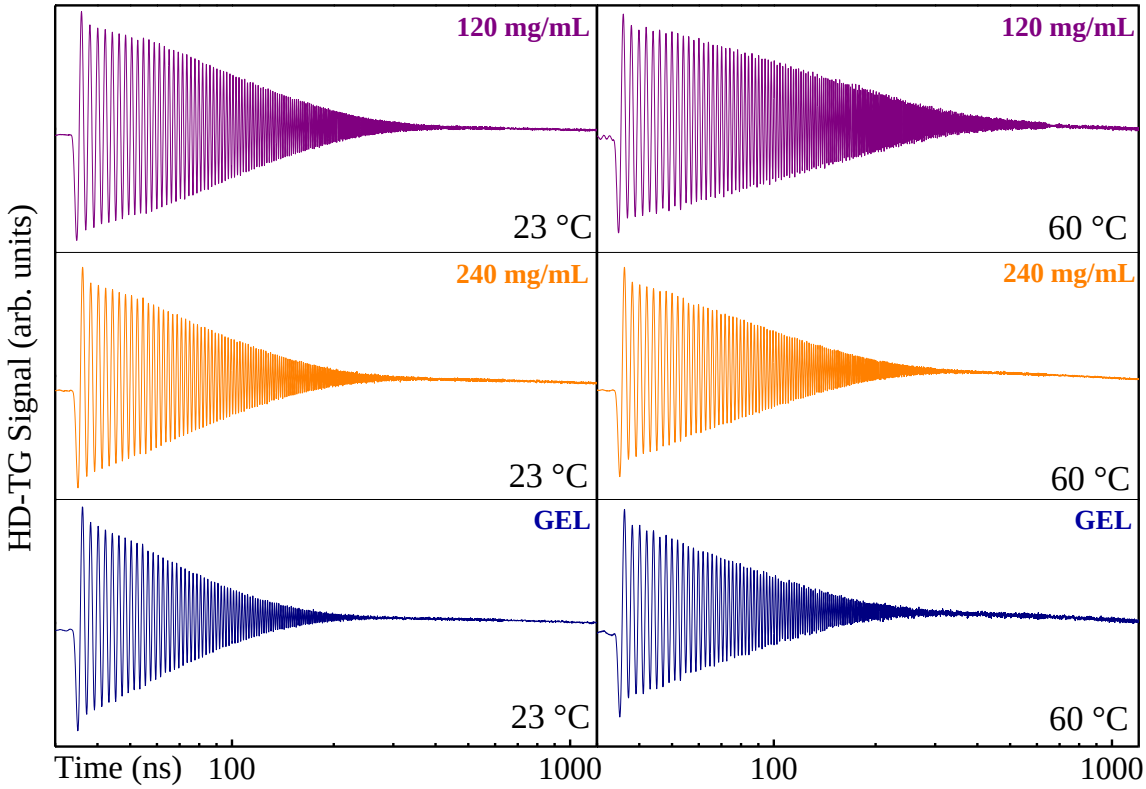

Figure 2: HD-TG decay signals for the wave-vector q= 2.1 μm^{-1} of: HEWL 120 mg/mL solution (purple line), 240 mg/mL solution (orange line) and gel (blue line) at 23°C (left panel) and 60°C (rigth panel).

Figure 3 plots the variation of the speed of sound in the studied samples. In all conditions the speed of sound curves have a similar trend as a function of temperature. This is related to the sound velocity temperature behaviour of deuterium oxide (D_2O), in fact the solvent is the most abundant component in the samples. Fig. 3 panel (a) shows the temperature dependence of the speed of sound of the diluted HEWL solution, which is very close to that of the pure solvent. In fact, C_s increase rapidly with temperature until 50 °C, and less rapidly between 50-75 °C and then decreases after 75°C, as experimentally observed in D_2O [33] and H_2O [20]. The C_s data of the concentrated (240 mg/mL) HEWL solution, reported in Fig. 3 panel (b), shows an overall increase of the velocities with a monotonic temperature dependence similar to the diluted solution dependence up to 55 °C. At this temperature value there is a small but clear deviation. Also the gel material shows the solvent trend but again a deep in the sound velocity shows up at a lower temperature of about 45 °C.

We must remember that the present HD-TG experiment measures the propogation of longitudinal acoustic bulk waves, whereas the gel/aggregation formations are expected to modulate mainly the transverse components of the sound velocities. Nevertheless, the self-assembling phenomena modulate the sound velocities increasing its overall value and producing the osberved deeps in correspondence to the folding-unfolding processes for the concentrated solution and to the gel-sol transition for the gel. This is also in agreement with the damping rate trends that we will considered in the following.

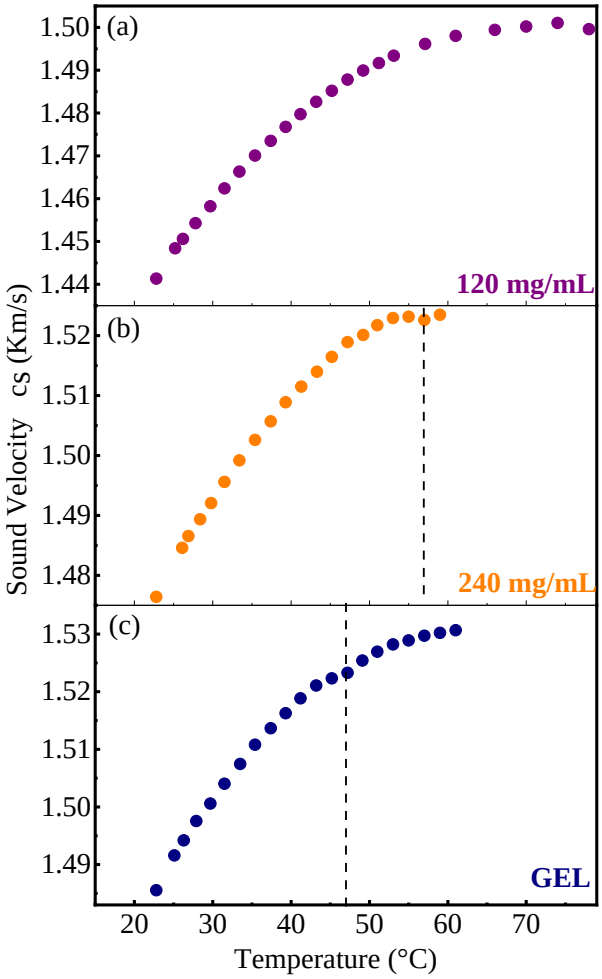

Figure 3: Temperature dependence of the sound velocity on: (a) the diluted (120 mg/mL) lysozyme solution, (b) The concentrated (240 mg/mL) lysozyme solution and (c) The gel system. The vertical lines point out the temperatures where the speed sound variations take place.

Figure 4 shows the temperature dependence of the acoustic damping rate of the studied samples. For the 120 mg/mL HEWL solution (Panel (a) of Fig. 4), starting from room temperature to ~ 45 °C, a decrease from 11.1 MHz to 7.3 MHz of the damping rate occurs, similarly to what is observed for a pure liquid [21]. In the range 45-55 °C the damping rate remains constant, this behaviour can be connected to the transition of proteins from folded to unfolded state. The literature reports a temperature of about 75 °C [4,34] for the folding-unfolding transition of the lysozyma in pure aqueous solution. Nevertheless, in our sample the acidification of the solution destabilizes the protein's tertiary and secondary structure; so that the unfolding process occurs at lower temperature [35], in agreement with the damping rate observed variation. During the folding-unfolding transition the damping rate does not change, this could be due to a stabilization of the solution viscosity induced by the increasing concentration of denatured proteins. After 55 °C the trend of the damping rate becames almost linear with the temperature, recovering the expected behaviour of the solvent, D_2O. Another slight deviation from the quasi-linear T-dependence occurs around 70 °C; this is could related again to the unfolding of a residual fraction of HEWL proteins less affected by the acid addition [6]. Panel (b) of Fig. 4 reports the temperature trend of the damping rate for the 240 mg/mL HEWL solution. In this experimental condition the damping rate behaviour is totally different with respect to that of the diluted solution. In particular, in the 23-45 °C temperature range, Γ_A decreases due to the reduction of solution viscosity. Above 45 °C the damping of the acoustic wave oscillation increases strongly with temperature; this could be due to the formation of protein aggregates. Indeed, from about 45 °C proteins starts to unfold, since this is higly concentrated solution they also start to aggregate. From literature it is well know that even a relatively low percentage of unfolded

proteins promote the aggregation processes [36]. Panel (c) of Fig. 4 reports the temperature behaviour of the gel system. The damping rate shows a linear temperature dependence from 23 °C to 45 °C, and above 45 °C remains almost constant. We interpret this trend with the breaking of the gel network that implies the transition to the liquid state, in other words according to our results the gel-sol trasition take place at 45 °C.

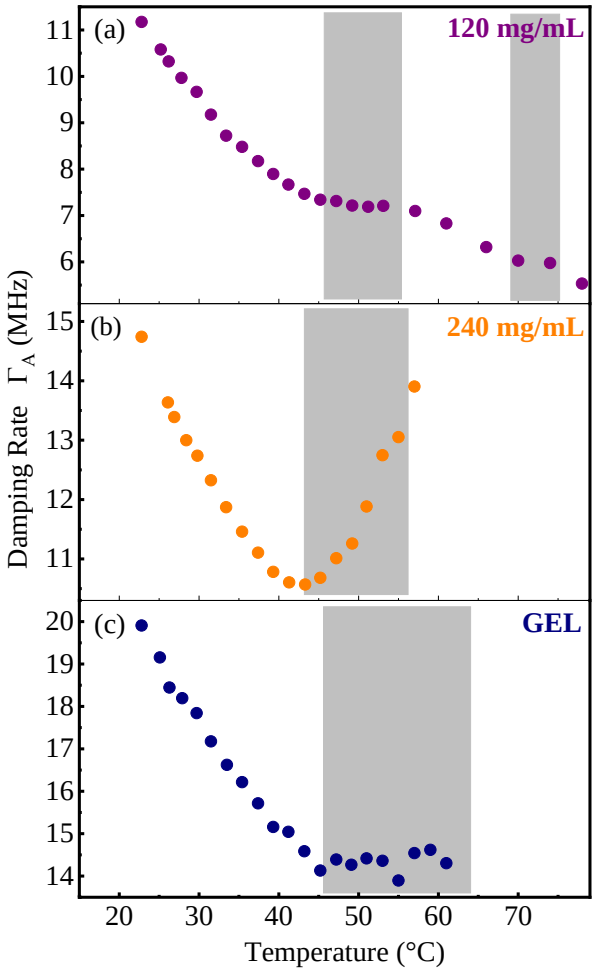

Figure 4: Temperature dependence of the acoustic damping rate measured on the samples for the 2.1 μm^{-1} q-vector. (a) The diluted (120 mg/mL) lysozyme solution. (b) The concentrated (240 mg/mL) lysozyme solution. (c) The gel system. The shaded band underline the temperature zones characterized by different dyanmic regimes.

According to our investigations, the ultrasonic velocities and attenuation processes are both sensitive to the aggregation phenomena; nevertheless, it seems that the attenuation processes are more sensitive to the self-assembling even from its earlier stages of the process, in agreement with other investigations [17]. For both the solution samples, the deviation in the damping rates occur around 45 °C, where the aggregates start to form without necessarily modify in the overall elasticity of the sample. Afterward, increasing the temperature the self-assembling processes will proceed forming an high content of lysozyme aggregates [17,37]. For the low concetration solution the unfolding process stabilizes the damping rate, whereas in the high concentrated solution the aggregates produce a strong increase of the attenuation phenomena; this could be due to a different level of coordination between the lysozyme aggregates or to different structure/dimensions of the clusters. The sound speed seems to be sensitive to these phenomena only at 55 °C, where the aggregation extent is high enough to largely affect the sample elasticity.

For the gel system we must consider a different scenario; in fact, both the ultrasonic parameters, speed and damping, are affected by the gel-solution transition of the system taking place at 45 °C. According to our results, the speed of longitudinal ultrasonic acoustic waves of gel system is

dominated by the solvent proprieties, see Fig. 3 panel (c), whereas the damping rate shows clear differences from solvent features. We must note that the gel liquid phase does not have the same acoustic proprieties of the high concentrated solution, see Fig. 4 panels (b)and (c), showing a different structural nature of the lysozyme cluster/aggregates, which depends only by the thermal treatment.

4. Conclusions

We have studied the acoustic properties of lysozyme aqueous systems at a wave vector of 2.1 μm^{-1}, corresponding to an ultrasonic frequency $\nu_A = {C_s q}/{2\pi} \cong 500$ MHz. Our results shows that both the speed and the attenuation of sound show articulate temperature dependences clearly affected by the concentration and phase nature of the system. In this work we demonstrate that HD-TG provides a versatile approach to study the dynamical behaviour of proteins such as unfolding, aggregation and gelation processes. This technique offers a fast data acquisition and the possibility to probe at the same time the viscoelastic properties and its relationship with the self-assembling phenomena. In particular, the acoustic wave damping shows a great sensibility to the cluster formation and phase transition.

The damping of the HD-TG signal is clearly due to the presence of lysozyme proteins and its temperature dependence is very likely due to the transition from folded to unfolded state. In fact, the two different protein conformations (folded and unfolded) lead to a markedly different environment, due to the hydrophobic sites exposition in the unfolded structure compared to the folded one and the following local arrangement and aggregation phenomena. In particular, in the lysozyme solutions the variation of Γ_A with temperature occurs for the double effect of viscosity and aggregation processes, whereas in the lysozyme gel the damping regimes reflect the matter phases. Moreover, some scattering processes could contribute to the damping effects [16] when the cluster structures reach an adequate stiffness contrast and a dimension comparable to the sound wavelength, $\lambda \cong 3$ μm. The lysozyme solution presents a hydrodynamic behaviour that is more complex than a pure viscoelastic liquid, this is related with the continuous protein conformational rearrangements due to the temperature increase. Following the simple viscoelastic model the maximum damping rate will correspond to $\omega_A \tau_s \cong 1$ condition, where ω_A is the acoustic frequency and τ_s is the structural time [32]. In our experiment this condition will be met when τ_s is about some hundreds of picoseconds. The unfolding process modify the interactions between the proteins modulating the structural relaxation channels; it is meaningful to note, that the unfolding is supported by the breaking of the protein H-bonds that takes place in hundreds of ps. This could explain the new dynamic regime appearing in all the investigated samples around 45 °C, where the unfolding processes begin to be active.

Author Contributions: Experimental Investigations, S.C., A.T. and P.B; writing—original draft preparation, S.C. and R.T.; writing—review and editing, all the authors; supervision, P.F. and R.T.

Funding: This research was funded by Ente Cassa di Risparmio Firenze, 2016-0866 and European Community by Laserlab-Europe, H2020 EC-GA - 654148.

Acknowledgments: We thank M. Paolantoni, P. Sassi and L. Comez for the idea of studying the thermal behavior of lysozyme in conditions of high concentration and low pH; B. Rossi for providing the lysozyme sample. The technical staff of LENS for support our mechanical and electronic experimental needs. The student C. Rediti for attending to some experimental investigations.

Conflicts of Interest: The authors declare no conflict of interest

References

(1) Dobson, C. M. Principles of protein folding, misfolding and aggregation. *Semin Cell Dev Biol* **2004,** *15* (1), 3.

(2) Stradner, A.; Sedgwick, H.; Cardinaux, F.; Poon, W. C.; Egelhaaf, S. U.; Schurtenberger, P. Equilibrium cluster formation in concentrated protein solutions and colloids. *Nature* **2004,** *432* (7016), 492.

(3) Porcar, L.; Falus, P.; Chen, W. R.; Faraone, A.; Fratini, E.; Hong, K. L.; Baglioni, P.; Liu, Y. Formation of the Dynamic Clusters in Concentrated Lysozyme Protein Solutions. *J Phys Chem Lett* **2010,** *1* (1), 126.

(4) Sassi, P.; Onori, G.; Giugliarelli, A.; Paolantoni, M.; Cinelli, S.; Morresi, A. Conformational changes in the unfolding process of lysozyme in water and ethanol/water solutions. *Journal of Molecular Liquids* **2011,** *159* (1), 112.

(5) Giugliarelli, A.; Tarpani, L.; Latterini, L.; Morresi, A.; Paolantoni, M.; Sassi, P. Spectroscopic and Microscopic Studies of Aggregation and Fibrillation of Lysozyme in Water/Ethanol Solutions. *The journal of physical chemistry. B* **2015,** *119* (41), 13009.

(6) Yan, H.; Frielinghaus, H.; Nykanen, A.; Ruokolainen, J.; Saiani, A.; Miller, A. F. Thermoreversible lysozyme hydrogels: properties and an insight into the gelation pathway. *Soft Matter* **2008,** *4* (6), 1313.

(7) Sassi, P.; Giugliarelli, A.; Paolantoni, M.; Morresi, A.; Onori, G. Unfolding and aggregation of lysozyme: a thermodynamic and kinetic study by FTIR spectroscopy. *Biophys Chem* **2011,** *158* (1), 46.

(8) Yan, C.; Pochan, D. J. Rheological properties of peptide-based hydrogels for biomedical and other applications. *Chem Soc Rev* **2010,** *39* (9), 3528.

(9) Jonker, A. M.; Lowik, D. W. P. M.; van Hest, J. C. M. Peptide- and Protein-Based Hydrogels. *Chemistry of Materials* **2012,** *24* (5), 759.

(10) Yan, H.; Saiani, A.; Gough, J. E.; Miller, A. F. Thermoreversible protein hydrogel as cell scaffold. *Biomacromolecules* **2006,** *7* (10), 2776.

(11) Khoury, L. R.; Nowitzke, J.; Shmilovich, K.; Popa, I. Study of Biomechanical Properties of Protein-Based Hydrogels Using Force-Clamp Rheometry. *Macromolecules* **2018,** *51* (4), 1441.

(12) Sahoo, S.; Ouyang, H.; Goh, J. C.; Tay, T. E.; Toh, S. L. Characterization of a novel polymeric scaffold for potential application in tendon/ligament tissue engineering. *Tissue Eng* **2006,** *12* (1), 91.

(13) Yan, H.; Nykanen, A.; Ruokolainen, J.; Farrar, D.; Gough, J. E.; Saiani, A.; Miller, A. F. Thermo-reversible protein fibrillar hydrogels as cell scaffolds. *Faraday Discussions* **2008,** *139*, 71.

(14) Catalini, S., Università di Perugia, 2016.

(15) Raccosta, S.; Manno, M.; Bulone, D.; Giacomazza, D.; Militello, V.; Martorana, V.; San Biagio, P. L. Irreversible gelation of thermally unfolded proteins: structural and mechanical properties of lysozyme aggregates. *Eur Biophys J* **2010,** *39* (6), 1007.

(16) Bryant, C. M.; McClements, D. J. Ultrasonic spectroscopy study of relaxation and scattering in whey protein solutions. *J Sci Food Agr* **1999,** *79* (12), 1754.

(17) Corredig, M.; Alexander, M.; Dalgleish, D. G. The application of ultrasonic spectroscopy to the study of the gelation of milk components. *Food Res Int* **2004,** *37* (6), 557.

(18) Parker, N. G.; Povey, M. J. W. Ultrasonic study of the gelation of gelatin: Phase diagram, hysteresis and kinetics. *Food Hydrocolloids* **2012,** *26* (1), 99.

(19) Taschin, A.; Eramo, R.; Bartolini, P.; Torre, R. In *Time-Resolved Spectroscopy in Complex Liquids*; Torre, R., Ed.; Springer US: Boston, MA, 2008, DOI:10.1007/978-0-387-25558-3 10.1007/978-0-387-25558-3.

(20) Taschin, A.; Cucini, R.; Bartolini, P.; Torre, R. Does there exist an anomalous sound dispersion in supercooled water? *Philosophical Magazine* **2011,** *91* (13-15), 1796.

(21) Taschin, A.; Bartolini, P.; Eramo, R.; Torre, R. Supercooled water relaxation dynamics probed with heterodyne transient grating experiments. *Physical Review E* **2006,** *74* (3).

(22) Pratesi, G.; Bartolini, P.; Senatra, D.; Ricci, M.; Righini, R.; Barocchi, F.; Torre, R. Experimental studies of the ortho-toluidine glass transition. *Physical Review E* **2003,** *67* (2), 021505.

(23) Pick, R. M.; Dreyfus, C.; Azzimani, a.; Gupta, R.; Torre, R.; Taschin, a.; Franosch, T. Heterodyne detected transient gratings in supercooled molecular liquids. *The European Physical Journal B* **2004,** *39* (2), 169.

(24) Amirkhani, M.; Taschin, A.; Cucini, R.; Bartolini, P.; Leporini, D.; Torre, R. Polymer thermal and acoustic properties using heterodyne detected transient grating technique. *Journal of Polymer Science Part B: Polymer Physics* **2011,** *49* (9), 685.

(25) Malfanti, I.; Taschin, a.; Bartolini, P.; Bonello, B.; Torre, R. Propagation of acoustic surface waves on a phononic surface investigated by transient reflecting grating spectroscopy. *Journal of the Mechanics and Physics of Solids* **2011,** *59* (11), 2370.

(26) Taschin, A.; Cucini, R.; Bartolini, P.; Torre, R. Temperature of maximum density of water in hydrophilic confinement measured by transient grating spectroscopy. *EPL (Europhysics Letters)* **2010,** *92* (2), 26005.

(27) Plazanet, M.; Bartolini, P.; Torre, R.; Petrillo, C.; Sacchetti, F. Structure and acoustic properties of hydrated nafion membranes. *Journal of Physical Chemistry B* **2009,** *113* (30), 10121.

(28) Cucini, R.; Taschin, a.; Bartolini, P.; Torre, R. Acoustic, thermal and flow processes in a water filled nanoporous glass by time-resolved optical spectroscopy. *Journal of the Mechanics and Physics of Solids* **2010,** *58* (9), 1302.

(29) Taschin, A.; Cucini, R.; Bartolini, P.; Torre, R. Acoustic phenomena in porous media studied by transient grating spectroscopy: A critical test of the Biot theory. *EPL (Europhysics Letters)* **2008,** *81* (5), 58003.

(30) Taschin, A.; Bartolini, P.; Sánchez-Ferrer, A.; Mezzenga, R.; Mrzel, A.; Torre, R. Investigation of Relaxation Processes in Nanocomposites by Transient Grating Experiments. *Materials Science Forum* **2012,** *714*, 79.

(31) Cucini, R.; Taschin, a.; Bartolini, P.; Torre, R. Acoustic phenomena and hydrodynamic flow in a water filled nano-porous glass studied by transient grating spectroscopy. *Journal of Physics: Conference Series* **2010,** *214*, 012032.

(32) Azzimani, A.; Dreyfus, C.; Pick, R.; Bartolini, P.; Taschin, a.; Torre, R. Analysis of a heterodyne-detected transient-grating experiment on a molecular supercooled liquid. I. Basic formulation of the problem. *Physical Review E* **2007,** *76* (1), 011509.

(33) Wegge, R.; Richter, M.; Span, R. Speed of sound measurements in deuterium oxide (D2O) over the temperature range from (278.2 to 353.2) K at pressures up to 20 MPa. *Fluid Phase Equilibria* **2016,** *418*, 175.

(34) Matheus, S.; Friess, W.; Mahler, H. C. FTIR and nDSC as analytical tools for high-concentration protein formulations. *Pharm Res* **2006,** *23* (6), 1350.

(35) Zou, Y.; Li, Y.; Hao, W.; Hu, X.; Ma, G. Parallel beta-sheet fibril and antiparallel beta-sheet oligomer: new insights into amyloid formation of hen egg white lysozyme under heat and acidic condition from FTIR spectroscopy. *The journal of physical chemistry. B* **2013,** *117* (15), 4003.

(36) Giugliarelli, A.; Paolantoni, M.; Morresi, A.; Sassi, P. Denaturation and preservation of globular proteins: the role of DMSO. *The journal of physical chemistry. B* **2012,** *116* (45), 13361.

(37) Corredig, M.; Dalgleish, D. G. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. *Food Res Int* **1996,** *29* (1), 49.