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Hydrophobicity Character of α-lactalbumin Nanoparticles: An Ultrasonic Study

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Abstract—Effect of pH and cosolvent on the stabilization of protein structure is a well established study in protein or food science. Among the various applications of proteins, the use of protein nanoparticle as drug or bioactive compound carriers is the one which is of most interest to many diverse researchers. The synthesis of such protein nanoparticles and their characterization is of prior requirement for the realization of these drug or bioactive carriers. On this basis, the present work deals with the ultrasonic analysis of hydrophobic interactions exhibited by the α-lactalbumin nanoparticle synthesized by heat treatment using acetone as desolvating agent. In order to enrich the variations in hydrophobicity, heat or temperature and cosolvent (glucose) are included in the study. The results are interpreted in terms of the interactions existing among the components and the evolved discussions reveal the bulk nature of the medium is controlled by the existing hydrophobicity interactions. The obtained results indicate that the dependency of protein denaturation on heat and the strengthening of non- covalent interactions by the cosolvent and/or the steric exclusion effect can be attributed to the structural modifications of protein.

Keywords: *α-lactalbumin nanoparticle, glucose, Ultrasonic velocity, Viscosity, heat, hydrophobic interactions*

INTRODUCTION

Proteins are the most abundant organic molecules of the living system and are, among others, the macromolecules that perform almost all important tasks in an organism, called as the machinery of life. Proteins are formed by joining amino acids by amide bonds into a stretched chain. They differ in length (from 30 to over 30,000 amino acids), and in the arrangement of the amino acids (Branden and Tooze 1991). It is a well known fact that the protein three-dimensional structure determines protein function. But the structure, otherwise called as 'the fold' is uniquely determined by the specificity of the sequence (Anfinsen 1973). As a class, globular proteins are more in conformation than fibrous proteins, have a far greater variety of biological functions and are dynamic rather than static in their activities (Anfinsen 1973). Lactalbumin, found in the whey of milk, is one of the significant simple globular proteins with a high degree of biocompatibility, specificity in sequence and solubility.

Solubility is an important criterion especially in food processing industry and for drug efficacy, independent of route of administration. Over the last 10 years, nanoparticle engineering processes have been developed and reported for enhancement of solubility of poorly aqueous soluble drugs and bioactive compounds. In this approach, poorly water soluble compounds are formulated as nanometre sized compound or drug particles (Kocbek *et al*. 2006). According to Muller *et al* (2001), nanoparticles are solid colloidal particles ranging in size from 1 to 1000 nm (1 μm).

Arroyo-Maya *et al* (2012) have analyzed, adopted various techniques for the preparation of bovine α-lactalbumin (α-LA) nanoparticles, successfully synthesized by various methods and critically commented the relative merits and demerits along with the particle size of the synthesized nanoparticles. They have also made an extensive study on the synthesized particles such as transmission electron microscopy, size and distribution, molecular weight and zeta potential, surface hydrophobicity, in-vitro digestibility, antioxidant capacity, etc and concluded that the controlling hydrophobic interactions are a means to control the size of α-LA nanoparticles. Further they commented on the two different pre-treatment processes, the heat and high hydrostatic pressure on the size of the nanoparticles.

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The objective of the present work is to study the effect of heat pre-treatment and cosolvent on the hydrophobic interaction existing in the said system by using ultrasonic techniques. This technique is highly specific as regards the bulk nature of the system and is a well-established approach for the study of molecular interactions (Swain and Priyadarshini 2010; Taulier *et al* 2005; Palaniappan and Nithyanandham 2019; Bahadur 2017). Further the effects of pre-treatment, pH and cosolvent are sharply reflected in the trend of observed ultrasonic parameters.

MATERIALS AND METHODS

All the chemicals used are AR grade. 0.2 M aqueous solutions of both monobasic and dibasic sodium phosphates were mixed in different proportions to prepare phosphate buffer of pH 9, marked as system B whereas the system B + G indicates the 1M solution of glucose prepared in same phosphate buffer of pH 9 (Velusamy and Palaniappan 2016) and both are used as solvent (re-dispersion agent) for the synthesized α-LA nanoparticle.

Powdered α-LA from bovine milk purchased from Sigma Aldrich is used for nanoparticle preparation. Nanoparticles of bovine α-lactalbumin (α-LA) were prepared by desolvation process using acetone as desolvating agent, heat as pre-treatment, cross- linkage by glutaraldehyde solution, followed by 5 cycles of centrifugation and then size verification by dynamic light scattering (DLS) method. Though the procedure (Arroyo-size of nanoparticle, the present work has been done for three different temperatures, *viz.,* 333 K and 333±5 K in order to understand the importance at/near that temperature. The obtained pellets were re-dispersed in the above said solvents B and B+G and are respectively called as B+L and B+G+L.

The DLS technique basically measures the apparent hydrodynamic radius (or diameter) of the particles. This technique is employed here as it can able to determine as well as to sense the changes in size due to processing (Alexander and Dalgleish 2006). The size measurements were carried out using Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK) at a scattering angle of 90° at 298 K after dilution of the nanoparticles with deionised water in the ratio of 1:400.

The pH of these solutions was measured by the digital pH meter. After preparation, the stock solution was kept stored at 293 K overnight. These solutions were then degassed and each measurement was made after 20 minutes of thermal equilibration (303±0.01 K).

The choice of glucose quantity to be 1 M is a safe concentration that ensures that it will not rupture any of the amino acid residues in the chain (Morrison and Boyd

1992) as well as apt for the term 'specific' in the last two parameters. Same way, protein concentration taken in the present work (5 mg/ml), though extremely quite high in terms of physiological values, it is worth to note that this is needed to have good reliable ultrasonic values and the same can be extended for biosensor application studies (Malhotra *et al* 2017). Thus the present study is well planned to analyze the situation in all possible dimensions with the available ultrasonic data.

MEASUREMENTS

Measured parameters in the present work include ultrasound velocity (u), density (ρ), viscosity (η) and surface tension (τ) whereas the calculated parameters are.

Adiabatic compressibility (β), intermolecular free length (Lf), acoustic impedance (z), relaxation time (t), relative association constant ((RA), the partial apparent specific volume (φv) and the partial apparent specific adiabatic compressibility (φk).

Experiment was carried out in four different temperatures, viz., the room temperature 303 K, the nanoparticle forming temperature 333 K, ± 5 degree of nanoparticle forming temperature i.e., 328 K and 338 K. In the entire study, the temperature was controlled to ± 0.01 K by water thermostatic bath provided by Ragaa Industries, Chennai, India. At least six repeated reliable observations were made for the measurement of each property and the reported values correspond to the average of these six independent measurements. The standard deviation of all the trials for each property was found to be satisfactory (not shown here).

The density (ρ) of all samples was measured at room temperature (303 K) using 5 ml specific gravity bottle. The accuracy in the measurement was about ±0.0001 kgm-3. The ultrasonic velocity (u) in all experimental solutions was measured by a single frequency (2 MHz) ultrasonic interferometer (Mittal's model F-81). The accuracy of sound velocity was ±0.1 ms-1. The viscosity measurements were done by relative method using Ostwald's viscometer of 10 ml capacity, accurate to ± 0.001mNsm–2.

Surface tension values are obtained at 303 K by drop weight method, using platinum- iridium Du Nouy ring, accurate to ±0.0001 kg. Details of measurements, instruments

and the procedures adopted are available in our earlier work (Palaniappan and Velusamy 2004; Velusamy and Palaniappan 2013).

CALCULATED PARAMETERS

The chosen thermo acoustical parameters are calculated using the following standard relations (Velusamy and Palaniappan 2013; Velusamy *et al* 2007; Vanathi *et al* 2019; Ravichandran and Ramanathan 2010; Kadi *et al* 2006).

$$\beta = \left[pu^2 \right]^{-1} \quad (1)$$

$$Lf = K_T \beta^{1/2} \quad (2)$$

$$z = up \quad (3)$$

$$t = \left[\frac{4\eta}{3u^2 p} \right] \quad (4)$$

$$T_A = \left[\frac{p}{p_0} \right] \left[u_0 / u \right]^{1/3} \quad (5)$$

$$\varphi_v = \frac{1}{p_0} + \left[p_0 - p \right] / \left[C_p p_0 \right] \quad (6)$$

$$\varphi_k = \beta_0 + \left[2\varphi_v - 2\left[u \right] - \frac{1}{p_0} \right] \quad (7)$$

where KT is the temperature-dependent constant having a value 199.53 x 10-8 in S.I system, ρo and ρ are the densities, uo and u are the ultrasonic velocities of the solvent and solution respectively, Cp is the protein concentration, βo is the adiabatic compressibility of the solvent and [u] is the relative specific sound velocity increment given as.

$$\left[u \right] = \left[u - u_0 \right] / u_0 C_p \quad (8)$$

RESULTS AND DISCUSSION

Some of the salient characters of the synthesized nanoparticles obtained from Zetasizer Nano ZS90 using DLS are presented in Table 1. The measured parameters for all four systems, viz., buffer (B), buffer + glucose (B+G), buffer + lactalbumin (B+L) and buffer + glucose + lactalbumin (B+G+L) at various temperatures are summarized in Table 2, whereas Table 3 shows the calculated values of first four thermo-acoustical parameters. Other calculated parameters against temperatures are depicted in Fig 1 A to 1 C respectively.

Inspection of Table 1 reveals that the particles obtained are well in nano region and are well distributed. Nanoparticles at specified pre-treatment temperature, i.e. 333 K and without pre-treatment are found to be almost of same size. However, pre-treatment offers a narrow dispersion of particles, as shown by polydispersity index.

Table 1: Characters of Nano Particles using DLS Study

Pre-treatment	Hydrodynamic Diameter in nm	Polydispersity Index
None	184.7	0.172
333 K; 30 minutes	189.2	0.122
(333 + 5) K; 30 minutes	210.8	0.134
(333 – 5) K; 30 minutes	206.4	0.119

The perusal of Table 2 shows that in general, the effect of temperature is found to lessen the magnitude of almost all measured parameters. However there is a deviation of this trend in viscosity values of B+G+L system at nanoparticle forming temperature.

Density as well as sound velocity is the immediate measure of the compactness of the substance. They basically depend on the number of particles in the medium. Of the four systems considered, phosphate buffer solution shows minimum density whereas the B+G+L systems records maximum values, that shows the availability of more particles in the latter system. However, this is not true for other two parameters. Apart from mass, the surface morphology, surface area and shape are important (Waris 2003) for the other two measured parameters.

Hydrophobicity is an important factor of nanoparticles as regards their applications, especially if designed for oral delivery systems as revealed by Jun *et al*. (2011). Further they suggested that these interactions are fundamental to control the size of nanoparticles and are sensitive to the changes in additive/cosolvent, concentration, pH, etc. In the present work, cosolvent is fixed, concentration is fixed and pH is alkaline. This pH 9 for the present system is interesting in the sense it has no or negligible reversal of trend in many physical parameters including density and sound velocity (Velusamy and Palaniappan 2016). Thus the observed variations reveal the peculiarity of pH 9 and clearly reflect its unanimous behaviour.

As regards viscosity, the system of B+L shows least magnitude whereas the B+G has the higher compared with any other systems, irrespective of temperature This shows that the lactalbumin nanoparticles are finely re-dispersed in the buffer that leads to aid the flow mechanism. But the suppression of the observed flow dynamics due to the addition of cosolvent may be attributed to the aggregation of nanoparticles (Samuel Ebinezer and Palaniappan 2007). It is peculiar to note that the η value at 333 K in B+G+L system is lesser than at 333 ±5 K. A lesser viscosity is a sign of greater fluidity and is an indication of fine dispersion of suspended particles.

In the case of surface tension also the B+L system at 333 K, has a higher surface energy and in B+G+L system the same becomes the least. Energy minimum is an excellent indication of stability (Burkhard Rost, 2010) and it is again a sign of fine re-dispersion of nanoparticles. The two factors the viscosity and the surface tension suggests that the addition of cosolvent supports the highly stable particle suspension and as regards temperature, a value other than 333 K seems to be unfavourable.

Table 2: Measured Parameters at pH 9 for the systems of Buffer (B), Buffer + Glucose (B+G), Buffer + Lactalbumin (B+L) and Buffer + Glucose + Lactalbumin (B+G+L)

Tem pera ture K	Density (ρ) kgm-3		Sound velocity (u) m		Viscosity (η) mNsm-2		Surface tension (τ) Nm-1	
	System B	System B+G	System B	System B+G	System B	System B+G	System B	System B+G
303	1023.2	1075.4	1551.0	1593.8	0.8135	1.1178	0.2573	0.2684
328	1011.7	1060.4	1515.6	1588.6	0.5124	0.9925	0.1851	0.1842
333	1008.2	1058.2	1510.8	1575.5	0.4221	0.9851	0.1782	0.1775
338	1006.7	1056.4	1506.4	1565.2	0.3345	0.9652	0.1704	0.1707
	System B+L	System B+G+L	System B+L	System B+G+L	System B+L	System B+G+L	System B+L	System B+G+L
303	1021.0	1079.3	1532.0	1585.2	0.6730	0.8538	0.2791	0.2592
328	1010.4	1063.2	1501.8	1580.4	0.4724	0.9314	0.1932	0.1762
333	1007.1	1059.0	1493.7	1571.2	0.4110	0.8992	0.1828	0.1721
338	1005.2	1057.2	1486.2	1561.3	0.3125	0.9522	0.1792	0.1684

Table 3: Calculated Parameters of Adiabatic compressibility (β), Intermolecular free length (Lf), Acoustic impedance (Z) and Relaxation time (t) at pH 9

Tempe rature K	β × 1010 Pa-1		Lf × 1011m		Z × 10-6 kgm-2s-1		t × 1010 s	
	System B	System B+G	System B	System B+G	System B	System B+G	System B	System B+G
303	4.0627	3.6606	4.1835	3.9711	1.5869	1.7139	4.4066	5.4558
328	4.3030	3.7368	4.4999	4.1930	1.5333	1.6845	2.9398	4.9487
333	4.3454	3.8071	4.5612	4.2693	1.5231	1.6671	2.4456	5.0005
338	4.3774	3.8639	4.6171	4.3379	1.5164	1.6934	1.9523	4.9726
	System B+L	System B+G+L	System B+L	System B+G+L	System B+L	System B+G+L	System B+L	System B+G+L
303	4.1730	3.6871	4.2399	3.9854	1.5641	1.7109	3.7446	4.1974
328	4.3881	3.7657	4.5442	4.2096	1.5174	1.6802	2.7622	4.6765
333	4.4504	3.8250	4.6159	4.2793	1.5043	1.6639	2.4388	4.5860
338	4.5039	3.8803	4.6834	4.3471	1.4939	1.6506	1.8766	4.9264

In Table 3 also, in general, the effect of temperature on the calculated parameters reflects the same tendency as in Table 2. Though the magnitudes are becoming opposite to Table 2, this Table 3 also supports the weakening of interactions with rise in temperature. Compressibility and free length are in increasing trend with temperature indicates that the existing interactions are weakened (Mahendran and Palaniappan 2011).

Acoustic impedance is a factor governed by the inertial and elastic properties of the medium, or simply, the reluctance of the medium for any change. This reluctance seems to be least for B+L system and is appreciable for B+G+L system, irrespective of temperature. This may be the indication of the weak interactions. Presence of weak interactions with a high degree of fluidity is somewhat peculiar and needs more reasoning.

The magnitudes of interactions are found to be becoming stronger with addition of cosolvent that ultimately reduces the aqueous solvent. A similar trend as regards relaxation time is noticed and it reveals that the addition of glucose stabilizes the nanoparticles at all temperatures. But in system B+G+L, in which glucose on hydrolysis produces both types of charges and are available due to the temporary or induced dipoles (Velusamy and Palaniappan 2013). The aqueous solutions of glucose have lower dielectric constant than pure water. It indicates that the electrostatic interactions are stronger in these solutions than in pure water as reported in literature (Akerlof 1932) and thereby restrict the uncoiling of α-LA or improves the stability. Always the electrostatic interactions are not weak in nature, but manifest as weak. Their inherited dominancy is suppressed by the selective exclusion effect of water molecules by the involved protein, termed as steric exclusion effect (Miyawaki 2007) and hence the interactions are said to be hydrophobic in nature.

Comparing the relaxation time (t) of the systems of B+L and B+G+L, the protein with cosolvent takes larger duration than without cosolvent. It is a general expectation that rise in temperature will reduce the relaxation time as found in B+L system. This is total contrary in B+G+L system, indicating the significance of cosolvent molecules. Rise in temperature make the cosolvent to generate large number of ions in the medium, which can attach with protein molecules, said steric effect expels water, aids in further increase of ion production, ultimately leads to higher t values.

It is to be noted that even in the presence of cosolvent, at 333 K, t is not as high as in 333±5 K. This reveals the importance of particular temperature as nanoparticle forming temperature. It is only at 333 K, the system is in minimum energy level and shows a very good re-dispersion with a low relaxation time.

The other three calculated parameters, called as relative parameters, viz., the relative association (RA) in Fig 1 A, the partial apparent specific volume (φv) in Fig 1 B and the partial apparent specific adiabatic compressibility (φk) in Fig. 1 C are the relative and realistic parameters that can ascertain the effect of the extra component in the systems, i.e., these parameters directly link system B+L with B+G+L.

It is a simple logic that, for the stability improvement by glucose, the inclusion of glucose to the protein should make the system to behave in such a way that it has to oppose the trend of heat. In the system with glucose, the trend just enlarges and reveals that thermal denaturation is nullified by the glucose. The temperature 333 K is highly specific in this trend. In the same way, the other two parameters are also conveying that glucose largely supports for the stability improvement of the protein α-Lactalbumin.

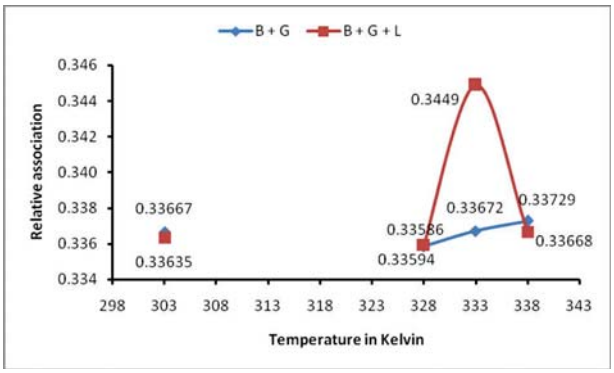

Fig. 1: A. Trend of Relative Association

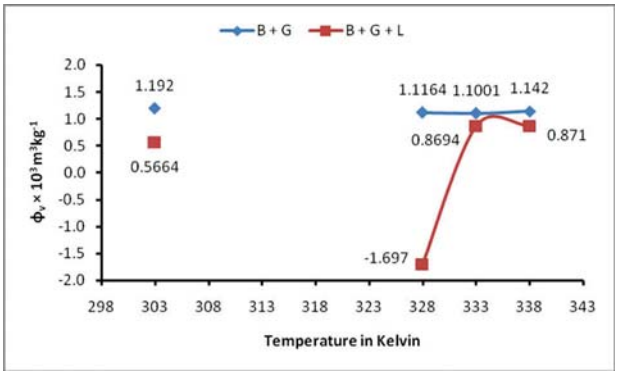

Fig. 1: B. Trend of Partial Apparent Specific Volume

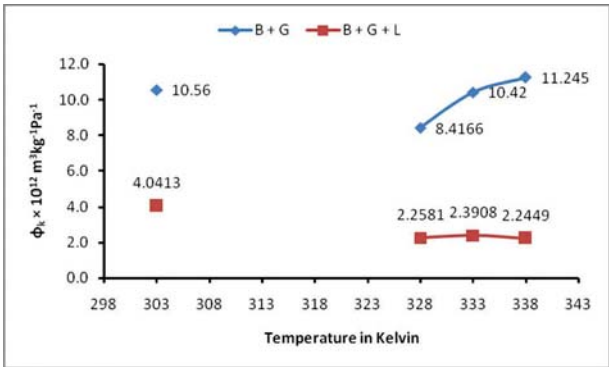

Fig. 1: C. Trend of Partial Apparent Specific Adiabatic Compressibility

Further the trend of φk offers another excellent confirmation that the specific compressibility with glucose is lower than that without glucose. A relatively less compressibility is the indication of higher compactness and thus glucose provides larger renaturation to the protein molecules. Further, the magnitude of it is extremely low and almost same at all temperatures indicating the role of cosolvent in the nullifying effects of heat. The extremely low magnitude of φk and the steric exclusion effect confirms that the existing interactions are hydrophobic (Shukla 2018) and are significant.

CONCLUSIONS

Significant findings of the present work are summarized as follows:

- The existing interactions in the chosen protein nanoparticle systems are found to be hydrophobic in nature and are sensitive to heat pre-treatment, pH and cosolvent.
- The specification of 333 K as nanoparticle forming temperature for the α- lactalbumin is highly valid as regards lactalbumin nanoparticles.
- Heat pre-treatment is found to be highly specific as regards the distribution rather than the size of the nanoparticles.
- Control of hydrophobic interactions by the alkaline pH, especially pH 9, is very evident.
- The addition of cosolvent, glucose in this case, largely aids in the reversal of denaturation due to heat pre-treatment.

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