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Abstract: The ability of ultrasound spectroscopy to characterise protein denaturation at relatively high concentrations and under conditions found in foods, is examined. Measurement of longitudinal sound velocity against concentration and frequency (20-160 MHz) for the bovine serum albumin monomer at pH 7.0 gave a frequency independent value for molecular compressibility of at 25 °C, corresponding to a sound velocity for the BSA molecule of 1920 ms⁻¹. At 160 MHz, the longitudinal sound attenuation in BSA molecules is ~5200 Npm⁻¹, a factor of 10 higher than in water. The excess attenuation of the solution over water was nearly 90 Npm⁻¹ at the highest measured volume fraction of 0.03 (or 3% v/v). Concentration-dependent ultrasound velocity (20 - 160 MHz) and attenuation (2 - 120 MHz) spectra were obtained over time for heated bovine serum albumin (BSA) solutions up to 40 mg/mL at neutral pH and at 25 °C. An acoustic scattering model was used which considered the solute molecules as scatterers of ultrasound, to determine the molecules’ sound velocity, compressibility, and attenuation properties. Mild heat treatment caused the molecule to organise into dimers and trimers, without change in sound velocity; implying that there is little or no change in secondary structure. Changes in attenuation spectra correlated with estimated molecular weight as determined through DLS and SEC measurements. During oligomerisation, the BSA molecules continue to behave acoustically as monomers.

Under severe heat treatment, BSA rapidly suffered irreversible denaturation and gelation occurred which affected both ultrasound attenuation spectra and the velocity of sound, consistent with significant molecular conformation changes and/or molecule-molecule interactions.
Ultrasound spectroscopy (velocity and attenuation 2-160 MHz) measurements can follow all stages in the denaturation of bovine serum albumin, from dimerisation through to severe denaturation. Velocity and attenuation relate to different features of the molecule. Velocity is sensitive to changes in conformation, secondary structure, and any significant changes in elastic or shear properties, attenuation is affected by relaxation processes and possibly aggregation.
Investigation of bovine serum albumin denaturation using ultrasonic spectroscopy

Malcolm J. W. Povey\textsuperscript{a*}, Jonathan D. Moore\textsuperscript{b}, Julian Braybrook\textsuperscript{c}, Howard Simons\textsuperscript{c}, Ron Belchamber\textsuperscript{d}, Meera Raganathan\textsuperscript{e}, Valerie Pinfield\textsuperscript{f}

* Corresponding author : Malcolm Povey, Phone +44 1133432963 Fax +44 1133432982, Email: m.j.w.povey@leeds.ac.uk.

\textsuperscript{a} School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT

\textsuperscript{b} National Physical Laboratory, Hampton Road, Teddington, Middlesex, TW11 0LW

\textsuperscript{c} LGC, Queens Road, Teddington, Middlesex TW11 0LY

\textsuperscript{d} Process Analysis and Automation Ltd, Fernhill Road, Farnborough, Hampshire, GU14 9RX

\textsuperscript{e} Department of Systems Engineering, Brunel University, Uxbridge, Middlesex UB8 3PH

\textsuperscript{f} Electrical Systems and Optics Division, Faculty of Engineering, University of Nottingham, Nottingham, NG7 2RD

Abstract

The ability of ultrasound spectroscopy to characterise protein denaturation at relatively high concentrations and under conditions found in foods, is examined. Measurement of longitudinal sound velocity against concentration and frequency (20-160 MHz) for the bovine serum albumin monomer at pH 7.0 gave a frequency independent value for molecular compressibility of $\kappa' = 2.05 \times 10^{-10}$ Pa$^{-1}$ at 25 °C, corresponding to a sound velocity for the BSA molecule of 1920 ms$^{-1}$. At 160 MHz, the longitudinal sound attenuation in BSA
molecules is ~5200 Npm$^{-1}$, a factor of 10 higher than in water. The excess attenuation of the solution over water was nearly 90 Npm$^{-1}$ at the highest measured volume fraction of 0.03 (or 3\% v/v). Concentration-dependent ultrasound velocity (20 - 160 MHz) and attenuation (2 - 120 MHz) spectra were obtained over time for heated bovine serum albumin (BSA) solutions up to 40 mg/mL at neutral pH and at 25 °C. An acoustic scattering model was used which considered the solute molecules as scatterers of ultrasound, to determine the molecules’ sound velocity, compressibility, and attenuation properties. Mild heat treatment caused the molecule to organise into dimers and trimers, without change in sound velocity; implying that there is little or no change in secondary structure. Changes in attenuation spectra correlated with estimated molecular weight as determined through DLS and SEC measurements. During oligomerisation, the BSA molecules continue to behave acoustically as monomers.

Under severe heat treatment, BSA rapidly suffered irreversible denaturation and gelation occurred which affected both ultrasound attenuation spectra and the velocity of sound, consistent with significant molecular conformation changes and/or molecule-molecule interactions.

**Keywords:** ultrasound spectroscopy; compressibility; bovine serum albumin; aggregation; gelation; protein-protein interactions; protein-solvent interactions.

1. **Introduction**

Ultrasound spectroscopy is increasingly used to investigate the properties of biomolecules, for example functional properties such as storage modulus arising from protein-protein interactions in high protein concentration solutions (Sahuja, Badkar, Zeng, Nema, & Kalonia, 2007) and for folding studies (El Kadi, et al., 2006). In this work we measure for the first time both velocity spectra (20 – 160 MHz) and attenuation spectra (2 – 160 MHz). Velocity
and attenuation relate to different features of the molecule. Velocity is sensitive to changes in conformation, secondary structure, and any significant changes in elastic or shear properties, attenuation is affected by relaxation processes and possibly aggregation. Ultrasound velocity has the unique property that it is affected by the adiabatic compressibility of the molecule and hence is sensitive to molecular transitions that alter compressibility. An example of such a transition is crystallization, to which ultrasound velocity is very sensitive, primarily due to adiabatic compressibility (or bulk modulus) change associated with the phase change (Povey, Awad, Huo, & Ding, 2007). Ultrasound has the further advantage that it can be used for bulk measurement in optically opaque, concentrated or multiple scattering media and is therefore a complementary technique to light scattering and circular dichroism. Unlike circular dichroism however, ultrasound does not provide specific information about secondary and tertiary structure, but does respond to changes in molar free energy (Chalikian, 2008). The key advantage of ultrasound in this work is its ability to provide information at all stages in the aggregation of the protein BSA, from dimerisation through to gelation, offering a method for characterising proteins under the conditions in which they are used in foods.

In this work we use a method previously reported in this journal (Apenten, Buttn er, Mignot, Pascal, & Povey, 2000) for obtaining molecular compressibility of BSA from ultrasound measurements of velocity and attenuation. Our previous study was carried out at a single frequency and only velocity was measured. As in the previous study, here it is assumed that the protein molecule is a particle that scatters ultrasound according to classical scattering theory.

A number of mechanisms, other than that of scattering, have been proposed for the features in the absorption spectra of protein solutions including BSA (Kessler & Dunn, 1969; Hussey & Edmonds, 1971; Holmes & Challis, 1996). These include changes in conformation, proton...
transfer reactions, solvation effects and other relaxation effects. Models have been developed to estimate the contribution of the proposed mechanisms to the attenuation. In particular, relaxation models are used to identify the relaxation frequencies (Holmes & Challis, 1996). Some workers have fitted a small number of relaxations in their experimental frequency range, whilst others fit a continuous range of relaxation frequencies.

(Bryant & McClements, 1999) proposed that the overall attenuation of ultrasound by a protein solution ($\alpha_{\text{soln}}$) be divided into molecular relaxation ($\alpha_{\text{MR}}$) and scattering contributions ($\alpha_s$). This presupposes that the relaxation contribution can be identified prior to separating out the scattering contribution. Holmes and Challis (1996) found that at a temperature of 37 °C, higher than that used in this study, a large frequency squared dependent term needed to be subtracted from their data, before a two frequency relaxation model could be fitted to their data. Whilst they suggest this subtracted term is due to another high frequency relaxation, our data suggest that the subtracted term could be, at least in part, due to scattering of sound by the BSA molecule. In this paper we analyse the attenuation data entirely in the context of the scattering model, without attempting to separate the relaxation contribution.

### 1.1 Scattering model

The straightforward classical model of sound propagation relates the velocity of sound $v$ to the elastic modulus $M$ and density of a material $\rho$ through

$$v = \sqrt{\frac{M}{\rho}}$$

The elastic modulus depends on the type of wave propagating and the material through which it propagates. In this work we measure only the longitudinal propagation mode (also called the compressional mode) which is a pressure wave. Other modes include shear and surface modes. The shear mode requires a significant shear modulus, $G$, in order to propagate.
solid materials the elastic modulus governing longitudinal wave propagation is related to the
bulk modulus $K_B$ given by

$$M = K_B + 4G/3$$

In fluids such as the protein solutions which form most of the subject of this paper, the elastic
modulus is given by the bulk modulus which is related to the adiabatic compressibility $\kappa$
through

$$M = K_B = 1/\kappa$$

The classical model is formally correct but cannot predict the effects of scattering by small
particles. In contrast, the scattering model approach considers each molecule or dispersed
phase particle as a scattering object, which has the effect of removing energy from the
forward transmitted sound wave (usually an incident plane wave) and scattering it in all
directions (Povey, 1997). Such scattering may affect both longitudinal wave attenuation
(through the wave amplitude) and velocity (through a change in phase of the wave). These
ultrasound propagation parameters depend on the properties of the continuous and dispersed
phase materials, and the concentration of the dispersed matter (Challis, Povey, Mather, &
Holmes, 2005; Povey, 1997).

In the present work, the systems were measured in the long wavelength region, in which the
wavelength of the sound wave is much larger than the scatterer (molecule) radius. In this
case, the scattering model shows that the wave number ($K_{\text{soln}}$, not to be confused with the
bulk modulus $K_B$) of the solution can be written in terms of the solvent (water) wave number
($K = \omega/\nu + i\alpha$, where $\omega$ is radial frequency, $\nu$ is sound velocity, $i = \sqrt{-1}$ and $\alpha$ is attenuation
coefficient). At the concentrations used in this study, the second order (in concentration)
multiple scattering term, is negligible, and the attenuation and velocity vary linearly with the
concentration (\(\phi\)) expressed as the product of partial molar volume (Table 1 and Table 2) and the mole fraction as a fraction of the mean molar volume of the solution (Povey, 1997):

\[
(\alpha_{\text{soln}} - \alpha_{\text{solv}} - \alpha_{\text{MR}}) = C_\alpha \phi + O(\phi^2)
\]

\[
\frac{1}{v_{\text{soln}}^2} - \frac{1}{v_{\text{solv}}^2} = C_\nu \phi + O(\phi^2)
\]

Here subscripts \(\text{soln}\) and \(\text{solv}\) refer to the properties of the protein solution and to the solvent containing buffer. The scattering model shows that the concentration dependence is obtained as a volume fraction, rather than other concentration measures often used such as weight fraction. This means that the partial molar volume (Table 1) has a significant impact on sound velocity. Attenuation and inverse square velocity show a linear dependence in volume fraction when measured relative to the same parameter in the pure solvent. The parameters \(C_\alpha\) and \(C_\nu\) define the respective gradients of the excess parameters with volume fraction.

Note in relation to those data analyses that extrapolate linearly to infinite dilution, scattering theory predicts the inverse square velocity of sound to depend linearly on volume concentration (Equation 5).

According to the scattering model, the gradient \(C_\nu\) obtained from the velocity measurements relates to the real parts of the coefficients given above, which are a fractional compressibility difference \(\Delta \kappa/\kappa\), a fractional density difference \(\Delta \rho/\rho\) and a thermal contribution \(Y\).

\[
C_\nu = \left\{ \frac{1}{\nu^2} \right\} \left[ (\Delta \kappa/\kappa) + (\Delta \rho/\rho) + Y \right]
\]

Each of the terms is independent of frequency in the long wavelength region. Thus it is expected that the velocity gradient parameter should be independent of frequency. For the thermal term, the frequency independence only applies when the thermal wavelength is also...
long, so that if the velocity gradient is indeed independent of frequency, the validity of this limit is confirmed. Rearranging the expression leads to the compressibility of the molecules.

\[ \kappa' = \kappa \left( 1 + v^2 C_v - \frac{\Delta \rho}{\rho} - Y \right) \]  

where the factor \( Y \) arises from thermal scattering (Povey, 1997).

The molar compressibility and hence the sound velocity will be affected by changes in partial molar volume, heat capacity, solvation and the pressure of the applied acoustic field. Because the acoustic field has frequency and duration, relaxation effects will certainly be present. The pressure may have an effect because an acoustic field is a pressure fluctuation with amplitude of approximately 20 kPa for a typical 100 W m\(^{-2}\), 1 MHz pulse in water at 25 °C (Povey & McClements, 1988; Povey, 1997). No attempt has been made in this work to unravel in detail the relative contributions of these effects during oligomerisation and denaturation of the studied protein, although we studied the frequency dependence of both sound velocity and attenuation. Determined compressibility includes both intrinsic and hydration terms.

2. Materials and Methods

2.1 Protein solutions

The water used in all experiments is MilliQ water (Millipore, Ma, USA), prepared by ion exchange, used because it gives much more reproducible results than triple distilled water in ultrasound experiments.

Bovine serum albumin (BSA- Product number A2153, Fraction V, Sigma-Aldrich Company Ltd, Gillingham, UK) is a widely used globular protein with a molecular weight of 66500 Da (Murayama & Tomida, 2004). 40 g/l solutions of BSA were prepared by the slow dissolution
of the freeze-dried protein in 0.1 M potassium phosphate buffer (pH 9.6) and then stored at 4 °C, prior to use. Just before measurement, samples were buffered at pH 7.0.

Concentrations are quoted as mass of protein in volume of solution and later converted to volume fraction by using the solution density measurements. Solutions were degassed in an ultrasonic bath. For the cross platform and oligomerisation studies 5 g/l solutions of BSA were finally prepared in 20 mM potassium phosphate buffered to pH 7.0.

2.2 Oligomerisation experiments

Samples were prepared in 10 ml aliquots and heated in a water bath at 70 °C +/- 0.02 °C for 60, 120, and 180 min. After heating, the samples were allowed to cool to room temperature. The control sample was maintained at room temperature. Sub-samples were removed after heating and were analysed by dynamic light scattering (DLS) and size exclusion chromatography (SEC). The remaining sample was analysed by ultrasound spectrometry.

2.3 Gelation experiments

The BSA solutions were heated in a thermostat-controlled oil-bath (refrigerated and heating circulator from Jubalo, F25-MC, accuracy +/- 0.02 °C) so that the time-temperature profiles could be controlled and registered. The solutions were sealed in A4 cans and maintained at 25 °C during 10 min for the equilibrium time. They were then heated up to 80 °C over a period of 25 min, maintained at 80 °C during a few minutes (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 minutes), and finally cooled down to 25 °C during 25 minutes. In-can temperature was measured in two positions and logged together with the bath temperature using type T thermocouples. Measurements of the storage modulus (\(G'\)) and of the loss modulus (\(G''\)) were performed using a Bohlin C-VOR rheometer from Bohlin Instruments.
The measuring system used was C14, cup and vane tool (vane diameter of 14 mm and gap width of 0.15 mm), which has a stress range of 1–750 Pa, with a strain sensitivity of $\sim 10^{-4}$. After pre-shearing, 4 drops of silicone oil AS4 (Fluka, from Sigma-Aldrich) were added to avoid evaporation. The temperature was set to 80 °C, and $G'$ and $G''$ were measured during 2 hours. More severe heat treatments were applied using a computer controlled steam retort (Figure 1).

2.4 Instrumentation

2.4.1 Ultrasound velocity and attenuation

Ultrasound velocity and attenuation measurements were made using the ICHOS II instrument (PAA Ltd, Farnborough, UK). The system is based on the “pitch and catch” method, with a longitudinal transmitted pulse passed through the sample to a receiver; in the frequency range 1-200 MHz, operating in the near field. In practice, this operating frequency range is significantly reduced by signal to noise considerations. The sample cell is made of stainless steel and requires a sample volume of 7-11 ml, with a path length for the ultrasound signal of 11.2 mm at 25 °C. The cell temperature is maintained by a water jacket and homogeneity is assisted by a magnetic stirrer. The sample temperature in all experiments was 25 °C ± 0.2 °C.

The instrument measures the intensity of the ultrasound signal received after transmission through the sample, and the time of flight of that signal in the sample. For instrument details, please refer to the manufacturer’s documentation. Measurements are calibrated against a reference sample, in this case, pure water because of the high accuracy data available. The velocity of longitudinal sound in pure water is obtained by the “148-point” equation of Bilaniuk & Wong, (1993, 1996), which defines its temperature dependence up to fifth order.
The measured temperature was 24.8 °C, and the velocity in water is therefore 1496.2 ms\(^{-1}\) and independent of frequency.

The velocity in the sample is determined by calibrating the time of flight against that measured in pure water at each frequency. Similarly, for attenuation, the intensity measured in the sample is calibrated against that measured in water.

The measurements were found to be accurate over the range 20-160 MHz. Outside this range, the intensity in water (and sample) was too low. Velocity measurements were carried out in the ICHOS II spectrometer with an accuracy of ±0.2 m s\(^{-1}\). The main determinant of the accuracy of ultrasound velocity measurements is temperature control (±200 mK) because the temperature coefficient of the velocity of sound in water at ambient temperature and pressure is 3 ms\(^{-1}\)°C\(^{-1}\). Relative to velocity, ultrasound attenuation is much less sensitive to temperature variation.

Data were also obtained using a Malvern Ultrasizer ultrasound spectrometer. This operates by producing a quasi-static longitudinal standing wave in a chamber holding at least 500 ml of sample, within which transducers, operating between 2 MHz and 120 MHz, move. The transducers are moved to obtain optimal signal to noise ratio and hence accuracy, within given frequency bands. This contrasts with the PAA instrument whose transducers operate at a fixed path length and which operate in pulsed mode. The Ultrasizer produces absolute attenuation spectra, whereas the PAA system operates in a comparative mode with water.

### 2.4.2 Dynamic light scattering

A Malvern Zetasizer Nano was used at both NPL and Leeds to follow the size of the BSA and its aggregated states. There are some issues with interpretation of data for the highly aggregated forms of BSA as the light scattering technique relies on particles undergoing
Brownian motion. In particular, once a gel network forms in which diffusion is arrested, sizing can no longer be carried out. The refractive index used for the BSA is 1.45 (the protein refractive index) and a value of 0.00 for the absorption (at the laser wavelength of 633 nm).

For the phosphate buffer (the dispersant), the viscosity measured was 1.04 cP with 1.3357 for the refractive index.

### 2.4.3 Density and partial molar volume

The method to obtain the volume fraction for the ultrasound measurements was as described in Apenten et al. (2000) with partial molar volume determined according to the method of Atkins (Atkins & de Paula, 2002). Measurements of density (Table 1) were made using a vibrating tube densitometer (Anton Paar, Gratz, Austria) for solutions of various concentrations.

### 2.4.2 Size Exclusion Chromatography

For all SEC measurements the HPLC (Jasco) was operated using Borwin (Version 1) software. Control and heat-treated BSA measurement samples were injected (10 μl) onto a BioSep-SEC-S2000 (Phenomenex) column equilibrated in 1 x PBS [10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4] running buffer at room temperature. The injector was washed twice between injections. The column method run time was 10 minutes at room temperature, under isocratic conditions where the mobile phase was 1 x PBS pH 7.4 at a flow rate of 1 ml min⁻¹. Eluted protein was monitored at multiple wavelengths using a Multiwavelength detector (Jasco MD-2010 Plus). The column was calibrated using a set of protein standards covering a range of known molecular weights (Blue Dextran (2000 kDa), Thyroglobulin (Bovine) (669 kDa), IgG (Bovine) (150 kDa), Serum Albumin (Bovine) 66kDa, Ovalbumin (Chicken) 44 kDa, Lysozyme (Chicken) 14.5 kDa).
The procedure followed was a modification of the methods of Whitaker (1963) and Andrews (1964). Void volume ($V_o$) was determined by recording the elution volume (retention time) of Blue Dextran at 280 nm. The elution volume ($V_e$) of each protein in the $M_w$ marker mixture was determined at 280 nm by observing the retention time recorded by the instrument software.

A standard curve was created by plotting the $\log_{10}$ of the $M_w$ vs. $V_e/V_o$ for each protein in the mixture. $V_e/V_o$ values were calculated for the unknown samples and the standard curve used to determine the estimated $M_w$.

3 Results

3.1 Determination of Acoustic Parameters

As indicated by Equation 3 above, it is necessary to measure the dependence of the velocity of longitudinal sound on the volume fraction of the protein in solution, in order to determine the molecular compressibility. Studies of concentration dependence were carried out on BSA solutions and density measurements as previously reported in Apenten et al (2000) were used. The partial molar volume, effective molecular density, and effective molecular radius for BSA were obtained from these data using a molecular weight of 66500 Da (Murayama & Tomida, 2004) and are given in Table 2. These data were then used to calculate volume fraction.

Velocity measurements taken in the ICHOS II, over the range 20-160 MHz and a concentration range up to 40 mg ml$^{-1}$ showed no frequency dependence. The concentrations were converted to volume fraction using the partial molar volume, and a single mean value of
velocity taken for each concentration. Figure 2 shows the inverse of the velocity relative to water plotted against volume fraction, following the method of Equation 5.

A linear relationship is clearly suitable for this experimental data. Hence, it is appropriate to neglect the multiple scattering contribution to velocity at these concentrations. The gradient of the straight-line fit is the parameter $C_v$ (Equation 6). If thermal contributions are neglected initially and we assume that molecular relaxation is insignificant, the velocity gradient $C_v$ relates only to the compressibility and density difference between water containing buffer and the BSA molecules in solution. Since the density of the BSA molecules has been determined from the solution density measurements (Table 2), the compressibility of the BSA molecules can be found (Equation 7): this is the molar adiabatic compressibility for BSA and lumps together the intrinsic compressibility of the protein chain, the compressibility of any cavities within the protein structure arising from its tertiary structure and solvent interactions with the protein which are also a function of solvent accessible surface (– see discussion below). Its value of $\kappa' = 2.05 \times 10^{-10} \text{Pa}^{-1}$ (corresponding to a longitudinal sound velocity for the hydrated BSA molecule of 1920 ms$^{-1}$) and our previously obtained value for BSA of $1.2 \times 10^{-10} \text{Pa}^{-1}$ compare with the value for water of $4.48 \times 10^{-10} \text{Pa}^{-1}$. Our previous work indicated that the compressibility was very sensitive to pH and to the thermal history of the protein; the experiments reported here were carried out isothermally, whereas our earlier experiments involved scanning the temperature at 20 °C h$^{-1}$. Our value ($\kappa' = 2.05 \times 10^{-10} \text{Pa}^{-1}$) for the adiabatic compressibility of the hydrated molecule between 0 mg ml$^{-1}$ and 40 mg ml$^{-1}$ can be compared with the value of $0.89 \times 10^{-10} \text{Pa}^{-1}$ given by Chalikian (1996) for the ‘apparent specific adiabatic compressibility’ (The hydrated value, not the intrinsic value) for BSA at a single concentration of 3 mg ml$^{-1}$. The Chalikian (1996) method involved subtracting the velocity of sound in the solvent from that in the 3 mg ml$^{-1}$ solution, this is called the velocity
increment. On the other hand, our method plots the inverse square velocity against concentration over the entire concentration range (Figure 2), a much more accurate procedure. The scattering theory calculation estimates the contribution of thermal scattering to the compressibility of 4%, much less than the estimate made in our earlier work by the frequency independent, temperature scanning method. The compressibility value determined by this method is the property of the scatterer of sound, which is the BSA molecule and any modified water surrounding it (hydration layer). Scattering theory does not independently provide a method for identifying the contribution of hydration and intrinsic compressibility terms, but does identify the likely effect of thermal scattering in the compressibility calculation. Given the nature of the temperature dependence of the compressibility (Figure 3 in Apenten et al. (2000)), the thermal scattering contribution may have a big relative impact on compressibility close to the melting temperatures (T_{m1} and T_{m2}) because here the compressibility is small and the thermal contribution may therefore be a large proportion of the total compressibility. Comparison of the two sets of the experiments indicates that there may be a significant time dependent and hysteresis component in the compressibility. As discussed below, we can also expect the entropic contribution from the hydrophobic groups coming in contact with water as the molecule unfolds to have an impact on compressibility.

Holmes and Challis’ (1996) data indicate that at the relaxation peak of 20 MHz; approximately half of the attenuation relative to water is due to relaxation processes at 37 °C. The attenuation data for the BSA solution at 25 °C is shown in Figure 3. We have not attempted to separate out the contributions of relaxation and scattering, due to the complexity of the problem. The attenuation for the molecules is determined from Equation 4, through a plot of attenuation against concentration of BSA, where we have combined together relaxation contribution and scattering contributions. It is notable that the attenuation of the
molecules is very large compared with water. At 160 MHz, the attenuation in water is
\( \sim 560 \text{ Npm}^{-1} \), but for the BSA molecules, it is \( \sim 5200 \text{ Npm}^{-1} \), almost a factor of 10 higher. The
excess attenuation of the solution over water was nearly 90 Npm\(^{-1}\) at the highest
concentration, which corresponded to a volume fraction of 0.03 (or 3% v/v).

### 3.2 Oligomerisation (Protein-protein interaction)

The BSA samples were heated in such a way that all stages of the
oligomerisation/aggregation process of the protein could be studied. The ICHOS II
instrument had the advantage that it requires only a few millilitres of sample. Figure 4
contains data from three replicated experiments and shows that aggregation can be
reproducibly induced in BSA at 60 °C increasing the particle size from approximately 8 nm
to approximately 21 nm within one hour, according to DLS measurements (insert in Figure
4). Further treatment up to 3 h appears to have no further effect on particle size, although the
higher resolution SEC data measured at 70 °C (Figure 5, Figure 6) indicates that the amount
of monomer and dimer continue to decrease with an increase of the trimer; nevertheless, only
the monomer, dimer and trimer are present in significant quantities. It is unlikely that
significant denaturation is occurring in these experiments because no change in the velocity
of sound was seen, consistent with studies in HSA (Mitra, Sinha, & Pal, 2007) and an
unchanging molecular conformation.

### 3.3 Heat denaturation of BSA

This part of the study presented a challenge due to the large quantities of protein (500 ml)
required by the Malvern Ultrasizer. The Ultrasizer was nevertheless included because it
gives absolute attenuation values, useable as a reference. Thus, it was necessary to heat large
quantities and this was achieved using canning techniques for which a typical heating curve is
shown in Figure 1. The resultant attenuation spectra and DLS data are shown in Figure 7, Figure 8, together with an estimate of the relative proportions of monomer, dimer and trimer in the 20 nm peak (Table 4). It is clear that aggregation of the BSA is manifested in these data, showing an increase in attenuation with heating. Little change in the main peak in the DLS data is seen, in comparison with the control sample for the first three minutes. After four minutes the size of the main peak begins to increase and becomes more scattered with a distinct increasing trend in size. The secondary peak appears after ten minutes then rapidly rises for the rest of the experiment. Associated changes in the velocity of sound and density (not shown) are too small to explain the changes in the attenuation spectra. However, significant changes in the velocity of sound occurred at the point where it became impossible to carry out attenuation spectroscopy and DLS measurements, probably due to the introduction of a frame modulus to the elastic modulus, in addition to compressibility, arising from gelation (see later discussion). On heating at 80 °C for 100 s, the initial BSA solution becomes yellow and translucent, then more cloudy. After 1000 s at 80 °C, the sample becomes gel like, and becomes stiffer with heating time.

The storage modulus \( G' \) increases with heating time and approaches \( G'' \) at roughly two minutes (Figure 9). The DLS data (Figure 8) indicates the appearance of large aggregates after 10 minutes. Between 100 and 300 seconds, \( G' \) increased rapidly signifying that aggregates were forming in solution, then after ten minutes gelation occurred, coinciding with the rapid rise in the secondary DLS peak in Figure 8. Then \( G' \) kept on increasing with the time of cooking, indicating that gelation continued to develop (Baier, Decker, & McClements, 2004). The fact that \( G' \) continues to increase throughout 2 hours shows that the gel formation was not completed and that the structure of the gel was still evolving. Neither DLS nor attenuation spectroscopy could be performed after roughly ten minutes at 80 °C, in
the case of the Ultrasizer; this was because the transducer movement broke up the gel structure. Whilst this problem does not present in the case of the ICHOS II instrument, measurements were only carried out on samples that did not gel. No measurements were possible on the BSA sample heated at 105 °C because it was not possible to insert the solid sample into the ultrasound measurement cells, which are designed to take liquids.

The DLS data (Figure 8) indicates that dimerisation and trimerisation (Table 4) dominates the first 4 minutes of ‘cooking’, followed by the rapid increase in large aggregates. However, it was not possible to resolve dimers from trimers based on the DLS data. Attenuation shows an increasing trend with cooking time, presumably as it responds (Figure 10) to the aggregation process.

4 Discussion

4.1 Relaxation

BSA has been the subject of significant attention in ultrasound studies (Kessler & Dunn, 1969; Zana & Lang, 1970; Hussey & Edmonds, 1971; Lang, Tondre, & Zana, 1971; Kremkau & Cowgill, 1985; Barnes, Evans & Lewis, 1985; Kremkau, 1988; Bryant & McClements, 1999), presumably because the quantities required to make accurate attenuation measurements necessitated a readily available protein. At lower frequencies than our range (Barnes et al. (1985, 1986, 1988), some absorption peaks indicate conformational changes and proton transfer at amino groups.

In the frequency range of the experiments reported in this work, most absorption was previously attributed to proton transfer equilibria (Kessler and Dunn (1969), Dunn & Kessler, (1970), Barnes et al.(1985) Choi, Bae & Takagi (1990), Holmes and Challis (1996)) These
workers carried out a variety of relaxation frequency fitting. In contrast, in our work no absorption peaks were observed. We analyse the attenuation spectra as a scattering phenomenon, where relaxation effects are lumped together in the apparent attenuation properties of the solute (i.e. in the frequency squared dependence of the attenuation coefficient).

Mitra, Sinha & Pal (2007), recently reported measurements in the temperature range 20 °C to 70 °C, on human serum albumin (HSA) using a range of techniques including ultrasound, which suggest a rapid loss in α-helicity beyond 55 °C but no observable change in secondary and tertiary structure below this temperature.

It is apparent from these studies, that relaxation processes make a significant contribution to the molecular attenuation of sound, in addition to scattering effects. The separation of the contributions of relaxation and scattering is non-trivial and is the subject of current studies in our laboratory. Whilst, in the case of ultrasound attenuation, we have not separated out the relaxation contribution and have studied the ultrasonic data in the context of scattering by the molecules, we have shown above that in the case of BSA, the dependence of the velocity of sound is accurately explained by a scattering model (Figure 2).

### 4.1 Denaturation

When the BSA solution is heated, the protein molecules unfold, the compact structural form of the molecule is disrupted. The globular proteins undergo conformational changes that allow hydrophobic groups or groups containing sulphydryls, to be exposed. Protein aggregation and gel formation occur when the protein surface activity is sufficient. According to Clark, Kavanagh & Ross-Murphy (2001), heat-induced gelation of BSA is considered a multistage process that involves three main stages. The first step is the initial protein
unfolding under heating which leads to a more reactive structural form. Sometimes dimerisation happens. The second step is the formation of linear fibril aggregates via disulfide and hydrophobic bonding. The aggregates form by nucleation and growth. The third step is a random cross-linking of the fibrils via non-covalent interaction. The association of the fibrils follow a random poly-condensation process that generates many species. The BSA gels show a strong inter-fibril connection. The visual observation of the samples after heating gives an idea of the nucleation and growth of the aggregates, because the more the samples were heated, the more it was possible to see bigger particles. The random poly-condensation of the fibrils can also explain a big variation in the size distribution of particles even if globally the size of the BSA particles increases with heating.

Boye, Kalab, Alli, & C.Y. (2000) on the other hand showed that a BSA gel is made of a network of small BSA aggregates of different shapes and sizes in the nanometre scale. These aggregates form through both disulfide and hydrophobic bonding. When native BSA molecules are dispersed in phosphate buffer at neutral pH, they do not form gels because the attractive interactions, which are principally van der Waals, hydrogen bonding, and hydrophobic interactions, are weaker than the repulsive interactions, which are mostly electrostatic, hydration and configurational entropy (Baier, Decker & McClements, 2004). This point of view is more in accord with our results than the linear fibril view of Clark, Kavanagh and Ross-Murphy, although we cannot exclude the formation of linear fibrils in the final stages of gel formation.

The heat- or pressure-induced unfolding (denaturation) process is commonly treated as a two-state model or transition comprising a native (N) and unfolded (U) state $N \leftrightarrow U$. This model particularly applies to the unfolding of small monomeric globular proteins (Privalov, 1979), for which intermediate states are neglected since they are considered so sparsely populated.
The main driving forces for folding are the entropic contributions of free water and changes in entropy due to release/binding of water molecules, affecting the hydrophobic core of globular proteins. At ambient and physiological temperatures, unfolding carries a significant entropic cost, particularly due to the presentation of hydrophobic side chains to the solvent.

In order to understand how denaturation of the BSA molecule may impact its acoustic properties it is necessary to locate them in the preceding model of oligomerisation and denaturation.

Sound velocity is determined for the solution by the molar compressibility, which includes contributions from the intrinsic compressibility of the molecule and the modified compressibility of the water surrounding the molecule (commonly known as the hydration layer). Thermodynamically, the compressibility is related to free energy since isothermal compressibility is the pressure gradient of the chemical potential (Chalikian, 2008). Whilst the intrinsic compressibility is affected primarily by secondary structure, the hydration contribution is determined largely by the solvent-accessible area of the molecule. Since we measure adiabatic compressibility in acoustic experiments, an additional thermal term will be present.

A number of workers have reported studies of protein denaturation processes using ultrasound velocity and absorption techniques (Kremkau, 1987; Wang, Tolkach & Kulozik, 2006). A detailed discussion of the relationship between solvent accessible area, intrinsic compressibility, and hydration compressibility is to be found in Apenten et al (2000) and Chalikian (2008). Molecular compressibility is likely to change in step with structural changes associated with denaturation of the molecule, although the free energy difference between folded and unfolded states of BSA is relatively small, since enthalpic and entropic terms are approximately equal but opposite in sign, so folding in itself is unlikely to
contribute significantly to changes in compressibility. Our observation that the velocity of sound in the BSA molecule hardly changes during the initial stages of denaturation is consistent with small changes in free energy, solvent accessible area and secondary structure.

On the other hand, the attenuation of the molecule is not so directly related to free energy and solvent accessible area. Rather it is controlled by scattering and relaxation, which appears much more sensitive to denaturation. The ultrasound scattering model developed from our data indicates that particle size changes associated with oligomerisation (dimers, trimers, etc) are well below the size range where changes will impact on the ultrasound attenuation. We expect therefore, but cannot prove since we were unable to separate the effects of scattering and relaxation, that the attenuation changes we observe during oligomerisation arise due to relaxation effects.

Time- and frequency-independence of the sound velocity and density indicates that the compressibility of the weakly heated treated molecules is constant, consistent with an absence of significant changes to the primary, secondary and tertiary structure of the molecule.

Significant changes in the velocity of sound (not shown) were observed once gelation occurred, together with significant changes in elastic modulus (Figure 9). This may be expected since gelation arising from denaturation will add an additional term to the material compressibility due to the introduction of shear \( G \) and frame (an additional compressional modulus arising from molecule-molecule interactions in the solid state) moduli to elastic modulus governing the propagation of compressional ultrasound waves. Referring to Equations 1 and 2, Figure 9 and the determined value for the bulk modulus of water of 2.23 \( \times 10^9 \) Pa, the shear modulus in the most denatured samples does not exceed 100 Pa, far too small a value to be measureable through the longitudinal wave velocity. This leaves...
contributions from the frame modulus and/or changes in molecular structure as possible explanations for the changing velocity of sound under conditions of severe denaturation.

5 Conclusions

In the experiments described above, conditions were adjusted so that the initial stages of dimerisation and trimerisation could be studied. Less severe heat treatment caused the molecule to form dimers and trimers, detected by the ICHOS II system through changes in attenuation spectra consistent with DLS and SEC observed particle size increase. In this case, no velocity changes were observed; this is consistent with little or no molecular conformation change.

BSA was then ‘cooked’, the protein underwent all the stages of oligomerisation, aggregation, denaturation and gelation described above. Irreversible denaturation and gelation of the protein affected both ultrasound attenuation spectra and the velocity of sound, consistent with significant molecular conformation change and/or molecule-molecule interaction.

Ultrasound measurements are sensitive to all stages in the aggregation and denaturation of BSA, responding to changes in the tertiary and quaternary structure. Aggregation led to measurable changes in ultrasound spectra correlating with DLS sizes. During oligomerisation, the BSA molecules continue to behave acoustically as individual particles, exhibiting little change in their compressibility. It is only when significant denaturation occurs that molecular compressibility may change significantly. The burst-rf, pulsed-wave ICHOS II system was much more sensitive to protein aggregation than the quasi-continuous-wave Ultrasizer system for reasons which are still unclear.

In this study we set out to elucidate a theory which allowed quantitative interpretation of ultrasound data in terms of particle properties such as size and compressibility. This proved
difficult because (a) proteins both scatter sound and exhibit relaxation and (b) the interaction between the protein and water is a complicated one involving solvent accessible area, cavities and entropic hydrophobic interactions during folding, unfolding and oligomerisation. A significant question is why ultrasound attenuation detects dimers and trimers in a region where scattering predicts that it is insensitive to particle size? This is likely to be because oligomerisation changes molecular relaxation, without altering the acoustic scattering cross-section significantly.

In summary, concentration dependent ultrasound velocity spectroscopy, together with densitometry uniquely measures a key parameter which is characteristic of protein structure, the molecular adiabatic compressibility. This can be related to solvent accessible surface area, hence providing insight into protein-solvent interactions. A novel aspect of our study is the demonstration that ultrasound attenuation spectroscopy is sensitive to protein-protein interactions such as dimerisation, trimerisation and stages of denaturation including gelation. Together, velocity and attenuation spectroscopy reveal details of protein-protein and protein-solvent interactions at the relatively high protein concentrations found in foods. This can give a unique insight into the relationship between protein structure and function in foods and many other protein containing systems, including biological systems. Future work in this area requires the separate identification of relaxation and scattering contributions to acoustic propagation and comparison between the measured molecular adiabatic compressibility and that determined from molecular modelling.
Acknowledgements

The project was funded by the UK DIUS (formerly DTI) as part of the National Measurement System (NMS) Measurement for Biotechnology (MfB) 2004 – 2007 programme. Thanks are due to Dr Caroline Orfila for carefully reviewing the paper and providing helpful suggestions.

References


For Food Hydrocolloids: Investigation of bovine serum albumin denaturation


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Tables

Table 1 Calculation of partial molar volume for BSA.

Table 2 Partial molar volume, density and radius of BSA molecules in solution, derived from the solution density measurements in this work and by Chalikian (1996), including the used relationships between molecule radius, partial molar volume, molecular density and molecular weight.

Table 3 Properties of water and BSA solute molecules used in calculations

Table 4 Monomer and oligomer data for BSA (Figure 8). Gravimetric data in the first row is taken from Table 2 and is computed on the assumption that only monomers exist. These data were obtained before heating. The DLS diameters are taken from the measured peaks in Figure 8. In the final row, the composition of material in the 20 nm peak is then estimated assuming it is composed of monomer, dimer and trimer.

Figure Captions

Figure 1 Representative temperature-time plot for the BSA denaturation studies. Solid line – retort temperature; dashed line – in-can temperature.

Figure 2 Inverse square velocity of sound plotted against partial molar volume fraction of BSA.

Figure 3 Attenuation relative to water, of BSA solution plotted versus frequency measured in the ICHOS instrument, of BSA solution (dotted line) heated at 60 °C for 1h and control (solid line) for 10 g/L at pH 7

Figure 4 Attenuation relative to water plotted against frequency for BSA in aggregated and monomer forms. Inset is the DLS plot of volume % versus size. The standard error of the attenuation measurements is ±0.2 Np m⁻¹ and data are plotted for three replicated experiments.

Figure 5 SEC retention time versus absorbance for BSA heated to 70 °C for 60 min (closed circles), 120 min (plus signs) and 180 min (inverted triangles), together with the unheated control sample (squares).

Figure 6 % total peak area plotted against heating time for the data of Figure 5. Peak 1 represents the trimer species. Peak 2 represents the dimer species and peak 3 represents the monomer species.

Figure 7 Total attenuation spectra for 40 g/l, pH 7 BSA solutions for ‘cooking times’ of 0 min ■, 2 min □, 4 min▲, 6 min X, 8 min ○ and 10 min ●.

Figure 8 DLS particle size distributions for 0 min (solid line), 4 min (dashed line), 8 min (dotted line) and 12 min (dotted-dashed line); for the system of Figure 7.

Figure 9 Plot of G’ and G” for BSA heated at 80 °C.

Figure 10 Total attenuation of BSA solution plotted versus cooking time at 80 °C for 40g/l at pH 7 and 115MHz.
Figure 1

Temperature (°C)

Time (s)

'Cooking time'
Figure 2

$10^9 \left( \frac{1}{v_{\text{soln}}} - \frac{1}{v^2} \right)$ vs. Volume fraction
Figure 3

![Graph showing frequency vs. attenuation with two lines]

- **Frequency (MHz)**
- **Attenuation (Npm⁻¹)**
Figure 4

Relative attenuation /Np m⁻¹

Size /nm

aggregated

control

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Frequency /MHz
Figure 5
Figure 6

Abscissa values are the heating periods in minutes

% Total Peak Area

Peaks

Peak 1
Peak 2
Peak 3
Figure 10
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\[
\frac{4}{3} \pi r^3 (6.022 \times 10^{23}) = \tilde{v}_{BSA}
\]

\[
\frac{4}{3} \pi r^3 (6.022 \times 10^{23}) \times \rho_{BSA} = MW \times 1.660 \times 10^{-24}
\]
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ᵃ Bryant & McClements (1999)
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Dear Editor,

I am pleased to submit an extensively revised version of our paper entitled “Investigation of bovine serum albumin denaturation using ultrasonic spectroscopy” for consideration for publication in Food Hydrocolloids.

Best wishes,

Malcolm Povey

Professor Malcolm J. W. Povey BA, PhD, FInstP, CEng
President, University of Leeds University and College Union
Professor of Food Physics
University of Leeds
LEEDS LS2 9JT UK
<http://www.leeds.ac.uk/ucu>
leedsucu.wordpress.com
<http://www.food.leeds.ac.uk/mp.htm>
m.j.w.povey@food.leeds.ac.uk
t: 44 (0) 113 343 2963
m: 44 (0) 797 055 8157
Author’s reply to reviewers comments.

We are very grateful to the reviewers for their careful reading of our manuscript and their valuable comments. Our replies (in blue italics) follow each comment below. We have provided a word manuscript using ‘track changes’ to mark our changes.

Reviewers' comments:

Reviewer #1: In this work, the authors report ultrasonic properties of BSA solutions (pH 7) before and after various heating steps. They characterize the resultant aggregation by dynamic light scattering and chromatography and the properties of gels forms by small deformation rheological measurements. As the authors describe, various authors have used ultrasonic methods to characterize the effects of thermal treatments on proteins. My biggest difficulty in this work was deciphering the important and novel findings. In general a shorter and more focused paper would be helpful.

What is the major distinction between the current work and the Apenten paper by some of the main authors? Are there any numbers to compare between papers? In general wherever possible provide direct numerical comparisons with data from other studies. What types of figures have been reported for protein density, ultrasonic velocity and compressibility?

The major distinctions between the current work and the Apenten work are that we measured (a) over a wide frequency range (2-160 MHz) rather than at one frequency (2.25 MHz); (b) isothermally rather than by scanning temperature at 20 C hr-1; (c) both velocity and attenuation.

We have added a detailed comparison of data from our previous work and other studies, quoting data where available. (Table 2 and )

P14, last sentence: I am not clear why protein folding thermodynamics would lead to differences between the analytical methods. Also there really hasn't been much discussion of protein denaturation to this point.

We have omitted this since it overly complicates the argument.

P15, first sentence: I don't see anything in Figure 4 that could support a statement on the reproducibility of the experiment nor on the kinetics of aggregation. Further down that page the authors state that the ICHOS technique is sensitive to oligomerization. Is it not equally possible that the changes seen are due to denaturation?

We have added the standard error and the number of experimental replicates to Figure 4 to indicate the reproducibility of our experiments. No statements are made regarding the kinetics of aggregation, except to note that aggregation appears to be largely complete after 1h heat treatment. This is based on the fact that little measureable change in SEC or ultrasound data can be seen after that time, up to a further two hours of measurement. This has been clarified in the paragraph. It is possible that denaturation contributes to the measured changes, however, there is
no accompanying change in the velocity of sound which would be expected if denaturation was occurring.

Again on P14 Table 4 is referenced for the first time to state that the ICHOS method cannot distinguish the proportions of the oligomers. I don't follow what is being listed in the table, how the numbers were calculated, or how it supports the conclusion.

We agree that the presence here of Table 4 is misleading. It is an estimate of the proportions of oligomers obtained for the experiment conducted at 70°C and is based solely on DLS data.

Overall the first paragraph on P15 took me a long time to read and understand and would probably benefit from a rewrite.

This paragraph has been rewritten.

I found Figure 9 hard to follow and unhelpful. Surely the position of the median size within each peak is less important than the population of the peak.

This Figure has been removed from the paper.

In the chromatography data, how were the peaks deconvoluted? Is it certain that the three peaks correspond to monomers, dimmers and trimers?

The column was calibrated using a set of protein standards covering a range of known molecular weights (Blue Dextran (2000 kDa), Thyroglobulin (Bovine) (669 kDa), IgG (Bovine) (150 kDa), Serum Albumin (Bovine) 66kDa, Ovalbumin (Chicken) 44 kDa, Lysozyme (Chicken) 14.5 kDa. The procedure followed was a modification of the methods of Whitaker [19] and Andrews [20].


Void volume (Vo) was determined by recording the elution volume (retention time) of Blue Dextran at 280 nm. The elution volume (Ve) of each protein in the Mw marker mixture was determined at 280 nm by observing the retention time recorded by the instrument software. A standard curve was created by plotting the Log10 of the Mw vs. Ve/Vo for each protein in the mixture. Ve/Vo values were calculated for the unknown samples and the standard curve used to determine the estimated Mw. This detail has been added to the manuscript.

Was there any effort made to degas the gels and viscous solutions?

No, however, the headspace was degassed using steam during the canning process, and prior to heating.

I found Figure 10 unnecessary and the relevant observations could easily be conveyed in the text.

This Figure has been removed from the paper.
P19, Does the Clark, Kavanaugh and Ross-Murphy paper state that linear fibril formation is a general phenomenon in protein gelation? (I don’t have a copy in front of me but I don’t remember that). Seems to contradict the Boye ref in the next paragraph.

Clark, Kavanaugh and Ross-Murphy indeed say as quoted “network building appears to involve three main stages: initial protein unfolding, linear fibrillar aggregation, and random cross-linking of the fibrils” It does indeed appear to contradict Boye et al whose point of view is more consistent with our data. However, we were unable to make detailed measurements on the final stiff gel. We have added a comment to this effect.

The first complete sentence on P20 would seem to be a better fit with the start of the denaturation section before the discussion of aggregation mechanisms. The second complete sentence on that page introduces surface activity. This is the first mention of surface activity and appears to make no sense in context. The whole protein denaturation section seems to contain a lot of detail not really germane to the manuscript.

We have shortened the discussion according to this referees comments.

The first sentence on P21 seems to suggest that ultrasonic attenuation changes depends on free energy differences. Is ultrasonic velocity directly related to free energy?

Isothermal compressibility is given by the pressure gradient of the chemical potential which is related to the Gibbs Free Energy. The ultrasound velocity depends on the adiabatic compressibility which contains thermal terms as well and upon density. So it is not simply related to the free energy, although it can be argued that it is ‘directly’ related because there is a relationship. A comment along these lines has been incorporated into the paper.

In many cases the axis labels in the figures reproduces incorrectly.

The publisher’s pdf converter inaccurately converts our word documents to pdf. We have attended to this problem.

Check the abbreviation for cooking time in Figure 11 and 12

Corrected to min.

Is the oscillation in the G" data real (Figure 11).

The wiggles in the data are well within instrumental error. However, we do not have an explanation for these.

Reviewer #2: The manuscript was presented in a clear and organised manner. However, there are several points that needed to be clarified.

General comments and corrections
1. Please number each line of the manuscript for the ease of reviewing

We have done this.

2. Please elaborate the novelty of present investigation, with the aid of suitable citations. It was not clearly reveal in the introduction sections.
Please explain clearly the key advantages of using ultrasound spectroscopy in characterizing BSA denaturation. Indicate clearly in the introduction and / or results and discussion

Points 2 to 4 above have been addressed in a modified introduction.

Specific corrections
1. Figure 1, 3, 7, 9, 11: Please provide a better quality figure to indicate the y-axis clearly.

   This was caused by a fault in the word to pdf conversion during document submission and has now been corrected.

2. Section 2.4.2: Please explain the reason of using multiple wavelength detector in Size Exclusion Chromatography. Please provide valid citation, if this is a validated method. Usually, SEC was detected using RI or MALS detector.

   2. This has been done in the reply to the first referee.

Reviewer #3: The work by Povey et al. reported ultrasonic spectroscopic study on the denaturation and gelation of bovine serum albumin. The study provided a new insight into the structure change, molecular interaction, and molecule-solvent interaction during denaturation and gelation of proteins. The reviewer would recommend the publication of the paper after the following points have been considered during revision:

   1) The structure of the paper can be better balanced. For example, the theoretical background and instrumentation of ultrasonic spectroscopy should be shortened, and only the essential equations necessary for the discussion of data presented. More significantly, the authors reviewed extensively and in details the previous works on contribution of molecular relaxation to ultrasonic attenuation. This was just for the purpose of demonstrating that even without separating out the relaxation contribution their data could be described by scattering model. Such a detailed review of molecular relaxation therefore seems not absolutely relevant, and can be briefed.

   The discussion has been extensively revised.

   2) In page 16, the authors stated G' and G'' crossed at roughly two minutes. There however seemed to be no a real crossover between G' and G'' from Figure 11; they just approached each other. Considering this and the DLS data that showed no large scale aggregation before 10 mins, I would prefer to think that gelation did not occurred until 10 mins. The author however argued that the gel started to form between 100s and 300s.

   The text has been altered in accordance with the above comment.

   3) Most of the equations were not displayed correctly in the pdf file.

   This was due to a problem in the pdf converter which has been addressed. The original was a word document.

   4) The axes and axis labels of most of the figures, particularly in Figure 1, 3, 7, 9 and 11, need to be made clear.
This was also a problem caused by the pdf converter, which has been addressed.

5) In Figure 5, Please give the wavelength at which the absorption was measured.

The wavelength was 280 nm, more detail is given in the reply to the first referee.