Hydrophobicity Alone can not Trigger Aggregation in Protonated Mamalian Serum Albumins

3.1. Introduction:

Amyloid fibril formation is a specific form of protein self oligomerization. Amyloid fibril formation process which is known to be associated with several neuronal as well as non neuronal diseases. Amyloid formation is the results of improper protein folding, therefore partially unfolded conformation of fragmented part of precursor proteins are considered to be more prone to form amyloid fibrils (Uversky and Fink, 2004, Mishra et al., 2007). Considerable progress has been made in understanding the molecular mechanism of amyloid formation, however the comprehensive details of this phenomenon are still not known. Under in-vitro conditions, the fibrils can be generated by using a variety of conditions such as extreme pH, high temperature, high pressure and use of additives etc. (Kumar et al., 1996, Malisauskas et al., 2003, Arora et al., 2004, Kumar and Udgaonkar, 2009). Surfactants are one of the common additives that are employed for inducing amyloid formation. Among various surfactants, SDS is mostly used for this purpose (Necula ct al., 2003, Barghorn et al., 2005). The in-vitro studies have provided several useful clues to explore the mechanism underlying amyloid fibril formation. In our previous report, we have demonstrated that SDS can induce aggregation in proteins of different origin at a pH below two units of their respective isoelectric point (pI) due to strong electrostatic interactions between a negatively charged head group of SDS and positively charged center of proteins but we did not explore the role of hydrophobic tail in details (Khan et al., 2012). It is well known that amyloid formation is the result of the complex interplay of both electrostatic as well as hydrophobic forces (Schmittschmitt and Scholtz, 2003, Izawa et al., 2012, Fukunaga et al., 2012, Ramakrishna et al., 2012). In order to emphasize the role of hydrophobic interaction in the formation of amyloid fibrils, we have sought to compare the amyloid inducing capability of three negatively charged surfactants viz sodium dodecyl sulphate (SDS), sodium dodecylbenzene sulfonate (SDBS) and sodium bis-(2-ethyl 1-hexyl) sulfosuccinate (AOT). All of the three surfactants contain single sulphate moiety on the head but differ in length of hydrocarbon tail. SDS contains 12 carbon long aliphatic tails while in case of SDDBS and AOT the hydrocarbon tail contain 18 and 20 carbons respectively.
micellar concentrations of these surfactants are 8.0 mM for SDS, 2.5-4.0 mM for SDBS and 0.8 mM for AOT in distilled water. The protein chosen for carrying out present study were serum albumins viz human serum albumin (HSA), bovine serum albumin (BSA), porcine serum albumin (PSA), sheep serum albumins (SSA) and rabbit serum albumin (RSA). Serum albumins are α-helical globular proteins found in the circulatory system, particularly most abundant in plasma (60%) with an average concentration of 50 g L⁻¹. Their important function is to bind and transport fatty acids, drugs, metabolites and also maintain the colloid of osmotic pressure (Carter and Ho, 1994). The mammalian serum albumins show great similarity in terms of sequence, structure and function (Peters, 1996). They contain single polypeptide chain arranged into three domains (I, II, III) and adopt a heart shaped structure at physiological pH (Sugio et al., 1999). HSA and RSA contain one Trp residues while BSA, SSA and PSA contain two Trp residues. Apart from performing various important functions as mentioned above, albums are known to form amyloid fibrils under different conditions and hence are well exploited for carrying out aggregation studies (Taboada et al., 2006, Vetri et al., 2007b, Xu et al., 2012).

In the present study, we have examined the effect of increasing concentration of SDS, SDBS and AOT, the surfactants having the same charge at the head but differing in the tail hydrophobicity. In this study we were also trying to know the role of charge and hydrophobicity in amyloid fibril formation of five serum albumins at pH below and above two units of their pl. For this we have employed various spectroscopic techniques as well as microscopy to check the fibril induction propensity of negatively charged surfactant with bearing different hydrophobic tail.

3.2. Material and Methods:

3.2.1. Materials:

Human serum albumin (068K7538V), Bovine serum albumin (110M1661V), Porcine serum albumin (094K7636), Sheep serum albumin (117K7540), Rabbit serum albumin (117K7565), sodium dodecyl sulphate (SDS), sodium dodecylbenzene sulfonate (SDBS) and sodium bis-(2-ethyl 1-hexyl) sulfo succinate (AOT), Thioflavin T (ThT) and Congo Red (CR) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were used of analytical grade.
3.2.2. Methods:

3.2.3. Protein concentration determination:

The stock solutions of proteins were prepared in 20 mM sodium phosphate buffer pH 7.4. The protein concentrations were determined spectrophotometrically using molar extinction coefficients, $e_m = 35700 \, \text{M}^{-1} \, \text{cm}^{-1}$ (HSA), $43827 \, \text{M}^{-1} \, \text{cm}^{-1}$ (BSA), $43385 \, \text{M}^{-1} \, \text{cm}^{-1}$ (PSA and RSA) and $42925 \, \text{M}^{-1} \, \text{cm}^{-1}$ (SSA) at 280 nm on a Perkin Elmer (Lambda 25) double beam spectrophotometer. The mother stocks of every albumin were prepared in 20 mM sodium phosphate buffer with 200 µM concentrations and which is further diluted for experiments.

3.2.4. pH measurements:

pH measurements were carried out on Metler Tolado pH meter (seven easy S 20-K) using Exper “Pro3 in 1” type electrode. The least count of the pH meter was 0.01 pH unit. The protein samples were prepared in 20.0 mM sodium phosphate buffer pH 7.4. The buffers were filtered through PVDF 0.45 µm syringe filter (Millipore Milex-HV) before using experimental purpose.

3.2.5. Turbidity measurements:

The turbidity was measured in the absence and presence of varying concentration of surfactants (SDS, SDBS and AOT) at pH 3.5 and 7.4 by monitoring the change in turbidity at 350 nm on Perkin Elmer (Lambda 25) in cuvette of 1 cm path length. Before the turbidity measurements albumins were incubated with varying concentration of surfactants for 12 hrs at both pH. The protein concentrations of albumins were kept 5 µM in all the samples.

3.2.6. Rayleigh light Scattering measurements:

Rayleigh light Scattering experiments were performed on Hitachi spectrofluorometer (F-4500) at 25 °C in a cuvette of 1 cm path length. The samples were exited at 350 nm and spectra were recorded in the range of 300-400 nm. Plots between fluorescence intensity (Fl) at 350 nm versus varying concentration of surfactants were plotted. The protein concentration in the entire sample was kept 5.0 µM. Both the excitation and emission slit width were set at 5 nm. Before performing the experiments, the albumins were incubated with surfactants for 12 hrs at pH 3.5 and 7.4.
3.2.7. **Kinetic measurements:**

Time kinetics studies were performed on a Shimadzu fluorescence spectrophotometer at 25 °C with a 1 cm pathlength cell. Light Scattering was measured after excitation the sample at 350 nm and emission was taken at 350 nm versus time in seconds. In time kinetics study albumins concentration was kept 0.1 µM and surfactant were taken 40 µM. The data were plotted between fluorescence intensity versus time in second and the data were fitted by origin 7. The albumin concentration was taken very low in kinetics study because limitation of instruments it can not detect the FI beyond 1000. For this purpose we maintained the protein surfactant ratio (1:400).

43.2.8. **Thioflavin T (ThT) binding assay:**

A stock solution of the ThT was prepared in double distilled water and its concentration was determined using a molar extinction coefficient 36000 M⁻¹cm⁻¹ at 412 nm. The protein samples (5.0 µM), in the absence as well as presence of varying concentration of surfactants (SDS, SDBS and AOT) were incubated for 12 hrs after that we added equimolar concentrations of ThT (5.0 µM) for 30 min in the dark. The ThT fluorescence spectra were recorded on Hitachi F-4500 fluorescence spectrofluorometer by exciting the samples at 440 nm and monitoring the emission in the range of 450-600 nm. Both excitation and emission slit width were fixed at 10 nm.

3.2.9. **Congo Red (CR) binding:**

The stock solution of CR was prepared in double distilled water and concentration was determined using a molar extinction coefficient 45000 M⁻¹cm⁻¹ at 498 nm. Albumins (5.0 µM) in the absence and presence of 2000.0 µM surfactants were incubated for 12 hrs. Then aggregated and non-aggregated sample was further incubated with Congo Red (5 µM) for 30 min in the dark. The absorbance spectra (200-900 nm) of the samples were recorded on Perkin Elmer (Lambda 25) UV-Visible spectrophotometer in a 1 cm path length cuvette.

3.2.10. **Dynamic light scattering (DLS):**

DLS measurements were done at 830 nm by using DynaPro–TC–04, equipped with a temperature controlled microsampler. Before performing experiments, all the solution was spun at 5000 rpm for 10 minutes and filtered through 0.22 µm pore size micro filter (Whatman International, Maidstone, UK) directly into 12 µl black quartz cell. Albumin concentration was 15.0 µM throughout the DLS measurements.
were incubated with 6000.0 µM of surfactants at pH 3.5 for 12 hrs. Protein surfactant ratio (1:400) was fixed throughout the DLS measurements. Measured size was presented as an average of 50 scans taken at 25 °C. The data were analyzed by Dynamics 610.0.10 software at optimized resolution. The mean hydrodynamic radius \( R_h \) and polydispersity \( P_d \) were estimated on the basis of an autocorrelation analysis of scattered light intensity based on the translational diffusion coefficient, by Stokes–Einstein equation.

\[
R_h = \frac{kT}{6\pi \eta D_{25^\circ C}}
\]

where \( R_h \) is the hydrodynamic radius, \( k \) is the Boltzman’s constant, \( T \) is the absolute temperature, \( \eta \) is the viscosity of water and \( D_{25^\circ C} \) is the translational diffusion coefficient.

3.2.11. Circular dichroism measurements:

Circular dichroism measurements were carried out on JASCO spectropolarimeter (J-815). The instrument was calibrated with D-10-camphorsulfonic acid. All measurements were carried out at 25 °C with a speed of 100 nm min\(^{-1}\) and response time of 1 second. Far-UV CD spectra were collected in the wavelength range 200-250 nm in a cell of 0.1 cm path length with a protein concentration of 5.0 µM in the absence as well as presence of varying concentrations of surfactants. Spectra were smoothed by the Savitzky-Golay method with 25 convolution width. The results were expressed as mean residual ellipticity defined as

\[
\text{MRE} = \frac{\theta_{\text{obs}} \text{(mdeg)}}{10 \times n \times C_p \times l}
\]

where \( \theta_{\text{obs}} \) is the CD in millidegree, \( n \) is the number of amino acid residues \( l \) is the path length of the cell in centimeters and \( C_p \) is the molar fraction of proteins. The percent secondary structure proteins was calculated by K2d method.

3.2.12. Isothermal titration calorimetry (ITC):

ITC measurements were performed on VP-ITC from microcal (Northampton, MA) U.S.A at 25 °C in 20.0 mM sodium acetate buffer at pH 3.5. The sample cell was filled with 15.0 µM serum albumins at pH 3.5 and reference cell contained respective pH 3.5 buffer. Titrations were carried out using desired surfactant at pH 3.5. Duration
of each injection was 20 seconds and time delay to allow equilibration between successive injections was 180 seconds. Stirring speed was 307 rpm and reference power was a 15 μ call s⁻¹. Control experiments were done to correct the data for that of dilution of ligand and buffer mixing. The heat signals from ITC was integrated using the origin 7.0 software supplied by microcal Inc.

3.3. Results:

3.3.1. Turbidity Measurements:

The turbidity of proteins was monitored at 350 nm in order to determine the aggregation behavior (Tian et al., 2012). The turbidity of five albumins in the presence of increasing concentration of SDS, SDBS and AOT concentration range 0.0-11.0 mM at pH 3.5 was examined. As shown in Figure 3.1, all the albumins gave maximum turbidity in the presence of 0.5-2.5 mM SDS and SDBS beyond which there were sharp decline in turbidity was noticed. On the other hand, in case of AOT, the turbidity of the sample began to increase from 0.5 mM concentration and reached a plateau around 1.0-11.0 mM, without any noticeable reduction in turbidity as observed form SDS and SDBS. This shows that aggregation increase from 0.5 mM to 2.5 mM of SDS and SDBS, while for AOT, aggregation pattern does not change up to 11 mM concentration. From the above observation two surfactants SDS and SDBS are showing dual behavior at lower concentration promoting aggregation because of electrostatic interaction between negatively charged head of SDS and SDBS but at higher concentration aggregation were not found due to increase in hydrophobic interaction. In case of AOT only aggregation induction behavior was found. The turbidity of all albumins were also measured in the presence of all three (SDS, SDBS and AOT) surfactants at pH above two units of pl, pH 7.4 but no turbidity was noticed (data not shown). The surfactants unable to induce amyloid at pH 7.4 due to strong electrostatic repulsion between proteins and surfactants because both have a same charge.
Figure 3.1. Turbidity of the sample was measured to detect the aggregation. Turbidity measurements of human (A), bovine (B), porcine (C), sheep (D) and rabbit (E) serum albumins at 350 nm were obtained in the absence and presence of varying concentration of SDS (-■-), SDBS (-○-) and AOT (-▲-) in 20.0 mM sodium acetate buffer at pH 3.5 at 25 °C. Albumins concentration was kept constant 5.0 µM ml⁻¹ in all conditions in 20.0 mM sodium acetate buffer at pH 3.5 and experiments were performed after 12 hrs incubation.

3.3.2. Rayleigh light Scattering measurements (RLS):

The aggregation propensity of SDS, SDBS and AOT was further determined by RLS at 350 nm of albumin samples in the presence of increasing concentration of surfactants. The aggregation of proteins was measured by RLS is also reported earlier (Vetri and Militello, 2005; Mir et al., 2010). The data thus obtained showing good agreement with that obtained from turbidity measurements. As shown in Figure 3.2, light scattering at 350 nm for albumin samples containing SDS and SDBS showed maximum FI in between 0.5-2.5 mM concentration, while above 2.5 mM the intensity of scattered light decreased significantly and become almost negligible. On other hand
sample containing AOT exhibited an enhanced scattering which remained persistent up to 11.0 mM of surfactant. These results again demonstrated that aggregation were promoted in the concentration range 0.5-2.5 of SDS and SDBS respectively beyond which aggregation was suppressed. In the presence of AOT the aggregation was not suppressed even up to 11.0 mM concentration. The Turbidity and RLS results were demonstrated showed different patterns of aggregation in the presence of SDS, SDBS and AOT but in the presence of SDBS, AOT the turbidity and extent of light scattering is more in comparison to SDS because SDBS and AOT has a long hydrophobic chain.

Figure 3.2. Aggregation of albumins was measured by Rayleigh light scattering (RLS). Rayleigh scattering measurements of human (A), bovine (B), porcine (C), sheep (D) and rabbit (E) serum albumins at 350 nm in the presence of increasing concentration of SDS (–•–), SDBS (–○–) and AOT (–▲–) in 20.0 mM sodium acetate buffer at pH 3.5 for 12 hrs incubation. The albumin concentrations were kept 5.0 μM ml⁻¹ in all the samples.
3.3.4. Kinetics of surfactant induced aggregation by RLS:

The time dependent changes in aggregation of albumins at pH 3.5 in the presence of 40.0 µM SDS, SDBS and AOT were studied through light scattering measurements. The molar ratio of albumins and surfactant was fixed for 1:400. It can be observed that aggregation was induced in the protein by SDS, SDBS and AOT at a very fast rate and no lag phase could be detected shown in Figure 3.3. So, it suggested that aggregation reaction was not nucleus dependent. Similar kinetic behavior was also reported in human apolipoprotein (apo) C-II and Tau proteins (Ryan et al., 2011, Ramachandran and Udgaonkar, 2012). Further in case of AOT, saturation was achieved very fast while in case of SDBS the aggregation occurred at a relatively slower rate than AOT. In case of SDS the rate of aggregation was least among all surfactant as the log phase was prolonged. Also the extent of aggregation induced by the surfactant followed the order AOT>SDBS>SDS. The result shows that rate as well as extent of aggregation was greatly influenced by the chain length of the surfactants.

Figure 3.3. Kinetics of aggregation in albumins were monitored by RLS measurements. Time dependent change in aggregation was monitored by Rayleigh scattering measurements in human (A), bovine (B), porcine (C), sheep (D) and rabbit (E) serum albumins (0.1 µM ml⁻¹) in the presence 40.0 µM ml⁻¹ SDS (- -), SDBS (- •) and AOT (- ▲ -) at pH 3.5. The protein surfactant ratio was taken 1:400.
3.2.5. ThT binding assay:

ThT is an amyloid specific dye which is used to detect the presence of fibrillar aggregate in both tissues as well as in vitro samples. An enhanced ThT fluorescence is a characteristic of the amyloid fibrils (Touchette et al., 2010, Luo et al., 2012). ThT binding assays were performed to study the nature of aggregates induced by all the three surfactants in serum albumins at pH 3.5. A plot of F1 at 485 nm versus varying surfactant concentration is shown in Figure 3.4. From the figure it can be seen that at lower concentration 0.5-2.5 mM of SDS and SDBS the ThT florescence of five albumins were enhanced significantly. Beyond 2.5 mM a marked reduction in FI occurred. The ThT fluorescence was negligible for sample containing 4-5 mM of SDS and SDBS. It is interesting to note that the FI was much higher in the presence of SDBS (18C) than that of SDS (12C), which suggests that SDBS is more efficient in induction of order fibrillar structure than SDS. A very unique behavior was shown by AOT, in the presence of 0.5-2.5 mM AOT the ThT fluorescence intensity was lesser than that obtained for SDBS. However the FI did not decline as in case of SDBS and a considerable higher fluorescence could be observed at much higher concentration of AOT. The ThT binding data shows that aggregates formed have fibrillar structure and that chain length of the surfactant plays an important role in fibrillation of the serum albumins.
Figure 3.4. Amyloid fibrils formation in five albumins were measured by ThT fluorescence. The effect of hydrophilic head and hydrophobic tail of SDS (●), SDBS (○) and AOT (▲) on amyloid fibrillogenesis of human (A), bovine (B), porcine (C), sheep (D) and rabbit (E) serum albumins was measured by ThT fluorescence Intensity at 485 nm after excitation at 440 nm at pH 3.5. Prior to measurements the aggregated sample was incubated with ThT for 30 min in a dark. The protein concentration was taken in all cases 5.0 µM ml\(^{-1}\). The protein ThT ratio 1:1 was fixed.

3.3.6. Congo Red (CR) binding assay:

The fibril formation of five albumins in the presence of 2000.0 µM ml\(^{-1}\) SDS, SDBS and AOT was further assessed by Congo Red (CR) binding assay at pH 3.5. CR is known to intercalate between β-sheets of fibril and produces a red-shift in the absorbance spectrum (Kim et al., 2003, Srinivasan et al., 2003). The CR absorbance spectra of five albumins with and without SDS, SDBS and AOT are shown in Figure 3.5. The spectra obtained in the absence of surfactant gave maximum absorbance at 503 nm. In the presence of surfactants i.e. SDS, SDBS and AOT, a prominent red shift in the spectra was observed. The shift in the absorbance maxima further indicates
that surfactant induced aggregates of albumins have β-sheet structure and are arranged into well defined fibrils.

![Figure 3.5. CR binding was performed to detect the amyloid fibril. Interaction of CR with aggregated and non aggregated human (A), bovine (B), porcine (C), sheep (D) and rabbit (E) serum albumins were seen. The spectrums of non-aggregated albumins are (black) and in the presence of 2000.0 μM ml⁻¹ SDS, SDBS and AOT are red, green and blue (aggregated). The aggregated and non aggregated samples were incubated with CR for 30 min in a dark prior to measurements. Final concentrations of albumins were taken 5.0 μM.

3.3.7. Dynamic light scattering analysis (DLS):

DLS is a technique used to characterize the size of particle as well as to detect protein aggregates in a solution (Ahrer et al., 2003) (Kumar and Udgaonkar, 2009). The change in hydrodynamic radii of five albumins in their native state (pH 7.4), acid induced state at pH 3.5 and in the presence of 6 mM SDS, SDBS and AOT were studied by DLS (Figure 3.6). The $R_h$ of albumins in their native state (pH 7.4) was found to be 3.8 nm (HSA, SSA) 3.4 nm (BSA, RSA), 3.6 (PSA). At pH 3.5, a slight increase in $R_h$ was observed 4.0 nm (HSA, PSA and RSA), 3.8 nm (BSA) and 4.2
Chapter 3

(SSA) owing to the loosening of protein structure under acidic condition. Upon incubation with surfactant a significant increased in $R_h$ occurred which suggest the formation aggregated species. Table 4.1. summarizes the results obtained from DLS study.

Figure 3.6. Hydrodynamic radii were measured to know the size of aggregates. The hydrodynamic radii of native human serum albumins 15.0 µM ml$^{-1}$ (A), at pH 3.5 (B), in the presence of 30000.0 µM ml$^{-1}$ SDS (C). SDBS (D) and AOT(E) and bovine serum albumin 15.0 µM ml$^{-1}$ at pH 7.4 (F), at pH 3.5 (G) and in the presence of 30000.0 µM ml$^{-1}$ SDS (H), SDBS (I) and AOT (J). Prior to measurements all the samples were incubated for 12 hrs.

Table 3.1. Hydrodynamic radii of all albumins at different conditions.

<table>
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<th>S.No.</th>
<th>Protein</th>
<th>$R_h$ (nm)</th>
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<th>pH 3.5</th>
<th>SDS</th>
<th>SDBS</th>
<th>AOT</th>
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<td>4.4</td>
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<td>2</td>
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<td>4.7</td>
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<td>4.1</td>
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<td>3.8</td>
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<td>4.3</td>
<td>89.0</td>
<td>4.9</td>
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3.3.8. Far-UV CD measurements:

Far-UV CD studies were carried out in order to study the effect of surfactant on the secondary structure of albumins at pH 3.5. As shown in Figure 3.7, far-UV CD spectra of five albumins at pH 7.4 and 3.5 exhibited two minima, one at 208 nm and other at 222 nm which is characteristics in the presence of α-helical structure, similar spectral behavior were also reported in other study (Mir et al., 2010a, Johnston et al., 2010, Ahmad et al., 2011). At 2.0 mM concentration of SDS, SDBS and AOT, the spectra of the albumins showed only a single negative peak which indicates the acquisition of prominence β-sheet structure in all the cases studied. The existence of β-sheet structure is a characteristic features of amyloid fibrils. The % α-helix to % β-sheet transition of all albumins at different concentration of surfactants were calculated by K2d software are shown in Table 3.2. The spectra of albumins in the presence of SDS and SDBS obtained beyond 2.5 mM was similar to the spectra of albumins at pH 3.5, which indicates the higher concentration of these surfactants inhibited conformational transition from α-helix to β-sheet structure. The AOT persists β-conformation even at higher concentration (11 mM) in all albumins. From CD results it can be concluded that SDS and SDBS are showing dual behavior in five albumins. But at higher concentration (beyond 3 mM) of SDBS the CD measurements will not be performed because it’s showing high HT noise.
Figure 3.7. The secondary structure transition was measured by Far-UV CD. Far-UV CD spectra of human (A), bovine (B), porcine (C), sheep (D) and rabbit (E) serum albumins were recorded in the presence of SDS (red spectra), SDBS (green spectra) and AOT (blue spectra) and absence (black spectra) at pH 3.5. In (F) Human serum albumin at pH 7.4 (cyan spectra), at pH 3.5 (black spectra), presence of 3.0 mM SDS and AOT at pH 3.5 (yellow and magenta spectra).
Table 4.2. % secondary structure calculated by K2d method at different conditions.

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<tr>
<th>S.No.</th>
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<th>β %</th>
<th>Random Coil</th>
<th>α %</th>
<th>β %</th>
<th>Random Coil</th>
<th>α %</th>
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3.3.9. Transmission Electron Microscopy (TEM):

The morphology of surfactant induced aggregation of albumins was further examined by TEM. The TEM was used to check the morphology of aggregates whether fibrillar or amorphous (Pallares et al., 2004, Hettiarachchi et al., 2012). The protein sample at pH 7.4 and at pH 3.5 did not show any fibrillar structure, while in the presence of 2.0 mM SDS, SDBS and AOT at pH 3.5 mature fibrils were formed which is shown in Figure 3.5. From the TEM image it can be concluded that the aggregates are formed by SDS, SDBS and AOT is having fibrilar morphology which is also supported by ThT, CR and CD results.
Figure 3.8. Morphology of aggregates was detected by TEM. TEM images of human serum albumins in the presence of 2000 µM SDS (A), SDBS (B) and AOT (C), bovine serum albumins in the presence of 2000 µM SDS (D), SDBS (E) and AOT (F). Before taking the image sample were incubated for 12 hrs.

3.3.10. Isothermal titration calorimetry (ITC) measurements:

Isothermal titration calorimetry (ITC) is routinely used to allocate the stoichiometry of the association between ligand and protein. ITC also gives information about heat released or absorbed when ligand is bound to macromolecules and reveal the nature of interactions whether electrostatic or hydrophobic. We studied the interaction of negatively charge surfactants (SDS, SDBS and AOT) with human serum albumins at pH 3.5. In Figure 3.9, the enthalpogram shows that heat changes accompanying binding of HSA with SDS, SDBS and AOT were exothermic as also evident from enthalpic values while higher concentrations of SDS was required for complete saturation, SDBS and AOT showed complete saturation at a far lesser concentration owing to their increased hydrophobicity. The heat released (enthalpy change ΔH) during binding of SDS is -2.179±0.083, SDBS -2.339 ±0.061 and AOT -4.14±0.499 Kcal.mol\(^{-1}\) respectively with the human serum albumins.
Figure 3.9. The heat released was measured by ITC. Enthalpograms of SDS, SDBS and AOT titration into human serum albumins solutions with concentration of (15.0 μM) at pH 3.5. The enthalpy change for each injection has been plotted versus the molar ratio scale [surfactant] : [albumins] of SDS (-■-), SDBS (-●-) and AOT (-▲-) at pH 3.5.

3.4. Discussion:

The negatively charged surfactants and lipids have a strong propensity to induce the amyloid fibrils in both amyloidogenic as well as non-amyloidogenic proteins (Butterfield and Lashuel, 2010, Sambasivam et al., 2011, Friedman and Cafisch, 2011, Sasahara et al., 2012). However the detail mechanism of surfactant/lipid induced aggregation still remain unclear. In our previous publication, 25 diverse proteins were subjected to pH below two units of their respective pI which led to the development of positive charges on the protein (Khan et al., 2012). This positive charge was neutralized by negatively charged SDS which resulted in aggregation of all the 25 proteins. From this study it was concluded that the electrostatic interaction is playing very important role. The role of ionic interaction was discussed in our previous communication. In the present study, our aim is to explore the role played by hydrophobic tail of the surfactant in the formation of aggregates in serum albumins by taking three negatively charged surfactant having variable chain length viz SDS (12C), SDBS (18C) and AOT (20C). The turbidity and light scattering measurements showed that the extent of aggregation increased with increasing chain length of the surfactant and followed the order: AOT>SDBS>SDS. We also showed the rate of
aggregation is time dependent change on RLS for all the albumins. It was observed that the rate of aggregation was dependent upon the negative charge of the head and length of hydrophobic tail of surfactants. Maximum aggregation was obtained in the presence of AOT followed by SDBS and then SDS. Also, the reaction is very fast such that no lag phase could be detected which suggests that fibrillation of albumins by their anionic surfactants was nucleus independent. Similar type of reaction kinetics was also reported in many proteins (Kad et al., 2001, Ookoshi et al., 2008, Motamedi-Shad et al., 2009b, Motamedi-Shad et al., 2012, Ruzafa et al., 2012). The change in hydrodynamic radii of the aggregated species was monitored by DLS as it is well known that during amyloid formation, the size of protein molecules increased due to complex formation (Liu et al., 2010). In our study, it was observed that both hydrodynamic radii of protein sample (pH 3.5) increased dramatically when incubated with SDS, SDBS and AOT upto certain concentration. After that aggregation was suppressed and hydrodynamic radii were more or less same as that of hydrodynamic radii of native albumins (data not shown). Interestingly the hydrodynamic radii of the aggregated species were more in the presence of AOT than that of SDBS and AOT which could be due to the fact that AOT is more hydrophobic as compared to SDBS and SDS. The tobacco mosaic virus coat protein also formed bigger size aggregates in the presence of cationic surfactant i.e. CTAB in comparison to its thermally induced aggregate (Panyukov et al., 2006). In order to confirm the nature of aggregates whether fibrillar or amorphous, we performed ThT as well as a CR dye binding assay. Both ThT and CR are specifically used to detect the presence of amyloid fibrils (Klunk et al., 1999, Carrotta et al., 2001, Hoyer et al., 2002). ThT binding assay revealed that all the five albumins yielded significantly enhanced ThT fluorescence intensity in the presence of (0.5-3.5 mM) SDBS and SDS at pH 3.5, whereas the ThT fluorescence of native albumin as well as control sample (containing only surfactant and ThT) was quite insignificant. Further the ThT fluorescence of the AOT containing protein sample did not decline up to 11 mM of surfactant. In case of SDBS, ThT fluorescence intensity is high compared to SDS and AOT because SDBS induced to mature fibril than AOT and SDS. The Congo Red binding assay showed a prominent red shift in the absorbance of albumins in the presence of AOT, SDBS and SDS as compared to control. From these two dyes binding experiments, it was confirmed that aggregate formed by the anionic surfactants in albumins at pH 3.5 were fibrillar in structure. The change in secondary structure of the five albumins upon fibrillation was
checked via far-UV CD. Vast literatures are available showing the conversion of α-helical protein into β-sheet structure upon fibrillation (Sen et al., 2009, Juarez et al., 2009). Similar conformational transitions were observed in our case also. The α-helical structure of all the five albumins were converted into β-sheets in the presence of AOT, SDBS and SDS at pH 3.5 which suggested that the fibrils have well defined β-sheet structures. The morphology of fibrils was further examined by TEM which revealed the presence of thick unbranched fibrils formed by albumins upon incubation with all the three anionic surfactants. After that we further explored ITC to know the nature of interaction between surfactants and albumins. ITC data revealed that binding of surfactants to albumin involved large enthalpic change which suggests the involvement of electrostatic interaction. The binding of SDS with α-synuclein (αSN), was also investigated by other group and they found similar type of enthalpic change as we got in our case (Giehm et al., 2010).

3.5. Mechanism of aggregation by negatively charged surfactants:

In this study, we studied the effect of negative charged surfactant (SDS, SDBS and AOT) with varying hydrophobic chain length on serum albumins at pH 3.5. At pH 3.5, native conformation of serum albumins undergo a transition into ‘F’ form which is a fast moving isomer of the protein. Further all the selected albumins exhibit a net positive charge at pH 3.5 (pI--5.5). The estimated charge on each albumin at pH 3.5 is given in Table 3.3. Negatively charged surfactants and lipids are used for amyloid induction in many proteins and it is reported that SDS interacts with Arg & Lys residues for inducing amyloid fibril formation. Serum albumins contain several basic and acidic residues. Below pI, the Arg, Lys & His which are positively charged residues become protonated while negatively charged residues were uncharged or vice versa. We incubated the serum albumins with negatively charged surfactant both below (pH 3.5) and above (pH 7.4) two units of pI of the proteins. It is believed that negatively charged head group of surfactant interacts with positive charge centers of the proteins (at pH 3.5) while the hydrophobic tail strongly repel water molecules leading to disruption of solute-solvent interactions. This causes enhanced solute-solute interaction. However, lack of fibril formation above pI of proteins suggest that strong electrostatic repulsion among the interacting molecules. This indicates that negatively charged surfactant have strong propensity to induce amyloid formation in proteins with basic residues (Arg, Lys & His) is ionized form i.e at pH below two
units of pl. Whereas hydrophobicity alone may not induce fibril formation, confirmed by studies performed above two units of pl. In this study, SDS and SDBS are showing dual behavior i.e fibril formation at lower concentrations and solubilization of aggregates at higher concentration. This is due to the fact that at the low concentration monomer of negatively charged surfactant interacted to the oppositely charged center of serum albumins and lead to amyloid fibril formation but at higher concentration the only hydrophobic part of surfactants playing important role in solubilization of aggregates. In contrast AOT was found to form fibril in micellar concentration also. It seems that one micelle interacts with more than one protein molecules and form amyloid. A proposed mechanism of SDS, SDBS and AOT is illustrated in Figure 3.10.

Table 4.3. Estimated charge of all albumins at pH 3.5 by "PROTEIN CALCULATOR v3.3" software.

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Figure 3.10. The proposed mechanism of aggregation by SDS, SDBS and AOT are illustrated.
3.6. Conclusion:

From this study it can be concluded that SDS, SDBS and AOT induces aggregation in all mammalian serum albumins at pH 3.5. At pH 3.5 albumins acquire net positive charge on the surface and the negative charge head of SDS, SDBS and AOT interacts electrostatically with the oppositely charged surface of serum albumins and leads to assembly of protein into characteristic amyloid fibrils. But the fibril formation propensity is also dependent on the chain length of these surfactants as evident from the results. The AOT has a very strong propensity to induce amyloid than SDBS and SDS because the overall hydrophobicity is higher in the AOT. Similarly we also have concluded that the hydrophobicity of surfactants is not playing a prominent role in amyloid fibril formation independently because charge neutralization is the most important factors. In simple word charge neutralization works first then hydrophobicity.