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Effect of Sodium Salicylate and Sodium Deoxycholate on Fibrillation of Bovine Serum Albumin: Comparison of Fluorescence, SANS and DLS Techniques

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Abstract

The impact of biocompatible additives on fibrillation and defibrillation of proteins provide valuable insight into the development of suitable formulations for treatment of protein related diseases or storage of proteins in the laboratory. We have studied the effect of addition of sodium deoxycholate (NaDC) and sodium salicylate (NaSal) on fibrillation of bovine serum albumin (BSA) using fluorescence, circular dichroism, dynamic light scattering and small angle neutron scattering. Spectroscopic studies indicate that the additives are adsorbed on the surface of proteins and change the secondary structure, irrespective of the sequence of addition. DLS and SANS studies show that both NaSal and NaDC slow down or arrest the formation of fibrils, if added to native proteins. However, the additives do not defibrillate preformed fibrils, when added after fibril formation. Thus, NaSal and NaDC can act as a potential adjuvant for prevention of fibril formation in BSA solutions.

Introduction

Many proteins undergo aggregation and amyloid formation under specific biophysical conditions and is associated with certain neurodegenerative diseases such as Alzheimer's, Parkinson's etc. Protein fibrillation is influenced by various factors; for example, salt concentration, temperature, pH and other additives such as nanoparticles and various metal ions.¹⁻⁹ It is believed that the key step in amyloid fibril formation is the destabilization of the native state of proteins, initiated by the above mentioned factors which results in the formation of partially folded intermediates.¹⁰⁻¹¹ Because of the close association of these fibrils with vital diseases, the mechanism of formation of fibrils and its inhibition is of great relevance and has become a topic of current interest.¹¹⁻¹⁶ Recent research has focused in identifying potential additives which either inhibit or reverse the formation of fibrils.¹⁷⁻²³ Nanoparticles composed of N-isopropylacrylamide: N-tert-butylacrylamide copolymers have been reported to be good inhibitors of amyloid β protein, mainly by affecting the nucleation step of fibrillation.²⁴ Surfactants have been reported to alter the structure of proteins in different ways. For example, premicellar and micellar aggregates of a star-like tetrameric quaternary ammonium surfactant can disassemble mature β -amyloid fibrils.¹⁷ Cetylpyridinium bromide induces unfolding and refolding of Bovine Serum Albumin (BSA).²⁵ Cetyltrimethylammonium bromide (CTAB) and tetradecyltrimethylammonium bromide (TTAB) have been reported to unfold rabbit serum albumin (RSA).²⁶ Bieschke et al. reported that black tea theaflavins are good inhibitors of toxic amyloid- β and α -synuclein fibrils.²⁷ Therapeutic approaches for protein misfolding diseases have been suggested based on binding of chemical chaperons or small molecules to proteins leading to slowing down, arrest or reversal of protein misfolding.²⁸⁻²⁹ Identifying the impact of various hydrophobic additives and lipids on fibrillation of amyloid proteins have gained much attention in recent years.³⁰⁻³² Aromatic small

molecules are identified as important additives to hinder fibrillation of amyloid β peptides.³³⁻³⁴

In a recent study, Woods et al. showed that rifamycin SV inhibits the formation of fibrils in β_2 - microglobulin via binding to monomers and favoring the formation of spherical aggregates.³⁵

These studies prompted us to investigate inhibition of fibrillation in proteins using small molecules capable of binding with proteins via aromatic rings or through formation of micellar assemblies. In this context, we choose one bile salt and a hydrotrope to identify their ability to modulate protein fibrillation.

Bile salts are biological surfactants that help in the solubilisation of hydrophobic ingredients in water through micelle formation.³⁶⁻³⁸ Hydrotropes are another class of compounds that have ability to increase the solubility of various hydrophobic compounds in water and can also coassemble with surfactants by inserting its hydrophobic part in micelles. Most hydrotropes possess a small aromatic part that can bind with other hydrophobic parts of macromolecules. Sodium deoxycholate (NaDC) is a common bile salt and sodium salicylate (NaSal) is a hydrotrope both of which are widely used in biological systems as solubilisers or drugs. In spite of the recent investigations on effect of various additives in defibrillation of proteins, the effect of bile salts or hydrotropes on protein fibrillation has not yet been reported, to the best of our knowledge. Thus, in this manuscript we have investigated the effect of NaDC and NaSal on fibrillation and defibrillation of a model protein, Bovine Serum Albumin (BSA), using fluorescence, circular dichromism (CD), dynamic light scattering (DLS), and small-angle neutron scatterings (SANS). The combination of different techniques provides complementary information about the system.

Materials and Methods

Materials: BSA (Sigma), Thioflavin-T (Sigma), NaDC (Fluka), NaSal (Fluka) and NaCl (Merck) were used as obtained without further purification. All solutions were prepared in Mill-Q water. di-sodium hydrogen orthophosphate anhydrous (S. D. Fine Chemicals Limited), potassium chloride (Fluka), sodium chloride (Qualigens), potassium di-hydrogen orthophosphate (Qualigens) and HCl (Merck) were used for preparing phosphate buffer of pH 7.3. The pH of the buffer was checked using a pH meter from Toshnival instruments.

Preparation of BSA Fibrils: A 1 % BSA solution in 5 mM phosphate buffer containing 0.2 M NaCl was prepared and incubated at 65 ± 1 °C in a water bath without stirring for two hours, which resulted in the formation of BSA fibrils. This was treated as a stock solution for BSA fibrils and for making different measurements such as fluorescence, CD spectroscopy, DLS and SANS, a known volume from this stock was taken, to which the additives were added. The resultant solutions were equilibrated to room temperature before making the different measurements. To study the concentration variation of the two additives, a known volume of the pure BSA/ fibril solutions was taken separately, to which different amounts of additives were added by weighing to obtain the required concentrations. In order to study the effect of additives prior to fibril formation, the appropriate amounts of additives by weighing were added to the BSA solution prior to incubation.

Fluorescence Measurements: Steady-state fluorescence measurements were performed using a Hitachi F4500 FL spectrophotometer. For the fluorescence measurements, the prepared BSA solution (in native form or as fibrils) were mixed with 6.25 μ M Thioflavin-T (ThT) prepared in 5 mM phosphate buffer and spectra recorded at an excitation wavelength of 450nm.

Circular Dichroism Spectroscopy (CD): The CD spectra of the protein samples were recorded on a Jasco J-810 CD spectrometer equipped with a peltier-type thermostatic cell holder. The far uv CD spectra in the range 200-250 nm were recorded using 1 mm path length cuvette at 25°C. The thermal denaturation curves were obtained by recording changes in the dichroic intensity at 222 nm as a function of temperature in the range 20 to 90 °C at a heating rate of 2 °C /min. For both experiments the protein concentration was 1mg/ml (i.e 0.1 %w/w).

Dynamic Light Scattering (DLS): DLS measurements were performed using a Malvern 4800 Autosizer employing 7132 digital correlator. The light source was He-Ne laser operated at 632.8 nm with a maximum output power of 15 mW. The average decay rate was obtained by analyzing the electric field autocorrelation function, $g^1(\tau)$ vs. time data recorded at 90° using the cumulants method.

Small Angle Neutron Scattering (SANS): SANS measurements were carried out on the samples prepared in D₂O at DHRUVA reactor, Bhabha Atomic Research Centre, Mumbai, India.³⁹ The mean incident wavelength was 5.2 Å with $\Delta\lambda/\lambda = 15\%$. The scattering data were measured in the wave vector transfer ($Q = 4\pi\sin(\theta/2)/\lambda$, where θ is scattering angle) range of 0.017 to 0.35 Å⁻¹. Samples were held in Hellma quartz cells. The measured SANS data were corrected for the background, the empty cell contributions and the transmission, and normalized to absolute cross-sectional unit using standard protocols.

SANS Analysis

In SANS, one measures the differential scattering cross-section per unit volume ($d\Sigma/d\Omega$), and for a system of monodisperse particles it can be expressed as

$$\frac{d\Sigma}{d\Omega}(Q) = nP(Q)S(Q) + B \quad (1)$$

where n is the number density of the micelles. $P(Q)$ is the form factor characteristic of specific size and shape of the scatterers and $S(Q)$ is the structure factor that accounts for the inter-particle interaction. B is a constant term that represents the incoherent background scattering mainly from the hydrogen atoms present in the sample.³⁹⁻⁴¹

The form factor for prolate ellipsoidal shape having semi major and minor axes a and $b=c$ can be given by

$$F(Q) = \frac{16\pi^2}{9} (\rho_p - \rho_s)^2 (ab^2)^2 \int_0^1 [F(Q, \mu)]^2 d\mu \quad (2)$$

where

$$F(Q, \mu) = \frac{3(\sin x - x \cos x)}{x^3} \quad \text{and} \quad x = Q[a^2 \mu^2 + b^2(1 - \mu^2)]^{1/2} \quad (3)$$

The variable μ is the cosine of the angle between the directions of a and Q . For oblate ellipsoid ($b=c > a$), a and b are interchanged in above equations. ρ_p and ρ_s are scattering length densities of particles and solvent, respectively.

The inter-particle structure factor $S(Q)$ describes the interaction between the particles present in the system and is calculated by Hayter and Penfold analysis under Rescaled Mean Spherical Approximation for charged macroions.⁴²

For polydispersed systems, $d\Sigma/d\Omega$ in equation (1) is modified as

$$\frac{d\Sigma}{d\Omega}(Q) = \int \frac{d\Sigma}{d\Omega}(Q, R) f(R) dR + B \quad (4)$$

where $f(R)$ is the size distribution and usually accounted by Schultz distribution as given by

$$f(R) = \left(\frac{Z+1}{R}\right)^{Z+1} R^Z \exp\left[-\left(\frac{Z+1}{R}\right)R\right] \frac{1}{\Gamma(Z+1)} \quad (5)$$

where \bar{R} and Z are the mean value and width of distribution, respectively. The polydispersity of this distribution is given by $\sigma = \frac{1}{\sqrt{Z+1}}$. For simplification, the integration in equation (4) is carried out over $P(Q)$ whereas $S(Q)$ is calculated for the mean size of the particle.

The data have been analyzed by comparing the scattering from different models to the experimental data. Throughout the data analysis corrections were also made for instrumental smearing. The modeled scattering profiles were smeared by the appropriate resolution function to compare with the measured data. The fitted parameters in the analysis were optimized by means of nonlinear least-square fitting program.⁴³⁻⁴⁴

Results and discussion

Effect of NaSal and NaDC on BSA Fibrils: To understand the effect of NaSal and NaDC on BSA fibrils, we first monitored the fluorescence from a well known protein fibril probe; ThT. ThT has gained widespread use for the detection of amyloid fibres due to its ability to enhance the fluorescence intensity by binding to the amyloid fibrils. The fluorescence spectra of ThT in pure BSA (in its native form) and in fibrils produced by incubating at 65° C, show a marked difference, as reported for various proteins. The ThT fluorescence intensity increases due to fibrillation⁴⁵ and is consistent with the increase in the content of β -sheet structure upon aggregation. It is noticed that addition of NaSal to the preformed BSA fibrils leads to a marked difference in the fluorescence intensity of ThT, indicating its interaction with BSA fibrils. The ThT fluorescence spectra of BSA in presence of different concentrations of NaSal are shown in Fig.1(A) and compared with spectra observed for fibrils. ThT fluorescence intensity is often used as a measure of the extent of fibril formation in many proteins. Close examination of the intensity of fluorescence at the λ_{\max} reveals that it increases initially, at very small concentrations

of NaSal ($< 0.05\text{mM}$) (Fig.1 (B)). This is an unexpected behavior, as the protein has already been subjected to heat treatment to facilitate fibril formation. However, on further addition of NaSal the intensity decreases and reaches a value close to that of unfibrillised proteins. The initial increase in the fluorescence intensity is attributed to the adsorption of the additives on the protein⁴⁶. Khan et al. investigated the effect of various anionic surfactants like SDS, SDBS and AOT on fibrillogenesis of different mammalian serum albumins.⁴⁶ and found that with the addition of surfactants the fluorescence intensity increases initially, at submicellar concentrations, and then falls off at higher surfactant concentrations. At high additive concentrations, the observed decrease in ThT fluorescence intensity can be attributed to defibrillation of the proteins. Such defibrillation is expected to alter the secondary structure of the protein, as revealed from the far uv CD spectral changes in such proteins. Isothermal titration calorimetric data also suggest the association of surfactant with albumins. From pH dependant fibrillogenesis studies it is concluded that both electrostatic and hydrophobic forces are responsible for the binding of surfactants to the protein.

A similar feature has been observed with the addition of bile salt, NaDC to BSA as shown in Fig. S1 (supporting information). This suggests that both NaSal and NaDC is capable of associating with the protein and alter its structure, once the additive concentration reaches above a critical value. This critical concentration is, however, different for NaSal and NaDC. For NaSal, this critical concentration has been found to be 330 mM and for NaDC, it is ~ 35 mM. Thus it appears that NaDC is about 10 times more effective than NaSal in inducing conformational changes in BSA. Such conformational changes in protein are expected to be highly conducive for defibrillation, as it is reported that fibril formation is associated with specific secondary structure. The higher efficacy of NaDC as compared to NaSal indicates the

involvement of hydrophobic interaction as the major factor for defibrillation.

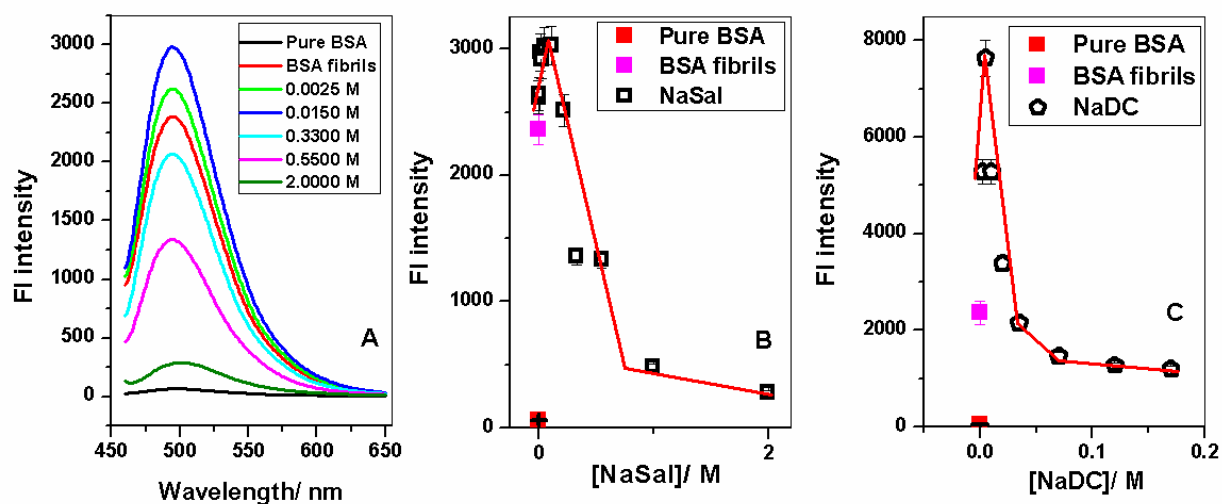


Fig.1. (A) Fluorescence spectral changes of Thioflavin T (ThT) as a function of [NaSal]. The concentrations of NaSal are shown in the inset. (B) and (C) Plots of variation of Th-T intensity as a function of [NaSal] and [NaDC] respectively.

It may be noted that though there is a decrease in intensity at high additive concentration, it does not completely return to the level that is observed in the native state of pure BSA. Though ThT fluorescence has been widely used as a marker for assessing amyloid beta-sheet content, recent studies using 2D IR spectroscopy and other complementary tools suggest that ThT fluorescence alone cannot be taken as a concluding evidence for aggregation.⁴⁷⁻⁴⁹ Thus, the reversal of the role of additives with changes in concentration possibly arises from changes in the nature of association of the additives. It is likely that, at low additive concentrations, the electrostatic binding of additives to the oppositely charged functional groups on BSA destabilizes the structure and thus favors β -sheet formation. However, at high concentrations the hydrophobic binding of the additive dominates thereby increasing the charges on protein and prevents the β -sheet structure. Such concentration mediated reversal in the function of additive has been

reported in the self assembly of certain amphiphiles. For example, urea (or n-butylurea) stabilizes sodium dodecyl sulphate (SDS) up to around 0.25 mol kg^{-1} by lowering the cmc of SDS, while beyond this concentration it destabilizes SDS. NaSal also shows a similar kind of variation in the cmc of sodium dodecyl sulphate (SDS)⁵⁰⁻⁵² and sodium dioctylsulfo- succinate (AOT).⁵³ It is very likely that a balance of electrostatic and hydrophobic interaction is responsible for the observed trend in fluorescence intensity. NaSal, being a hydrotrope, is less hydrophobic than the bile salt, NaDC and thus it alters the protein conformation at higher concentration than that of NaDC. The role of electrostatic interactions in stabilizing the protein structure is verified from the effect of NaCl on BSA fibrils. As expected, we observed that the ThT intensity keeps on increasing with increasing concentration of NaCl (Fig. S2) and no reduction in the intensity are observed. Thus, it appears that there are two regions of interactions of proteins with ionic surfactants, bile salts or hydrotropes. The first region corresponds to electrostatic binding of the additive to reduce the surface charge and hence promotes β -sheet structure. In the second regime, hydrophobic association of the additive occurs with the hydrophilic part providing charge stabilization to the protein.

To further identify the conformational changes in BSA with the addition of NaSal and NaDC, we performed far UV circular dichroism (CD) measurements in the presence of these additives. Fig. 2 shows the far UV-CD spectra of BSA in the native form and its fibrils in presence of different concentrations of NaDC. The α helical structure of many proteins show characteristic CD spectra with two minima located at 208 nm and 222 nm. The pure BSA solution clearly possesses the two minima at 208 and 222 nm, which indicates that BSA has a predominant helical conformation in its native state. When BSA is incubated with 0.2M NaCl at 65 °C, a drastic reduction in the α -helical content is noted, as observed from a decrease in the ellipticity.⁵⁴ It is

worth mentioning that due to the high concentration of protein and the presence of NaCl needed to form fibrils, the conversion of measured ellipticity to mean residue ellipticity is prone to error. In view of this, the data are reported as measured ellipticity only and is restricted to wavelengths longer than 200 nm. Concentration dependence of the measured ellipticity of BSA shows that the nature of the curve is independent of concentration. Comparing the measured ellipticity of BSA at constant concentration but with different concentration of additives indicate an increase in the unordered structure of protein and formation of β -sheet content. This observation is in good agreement with the reported behavior of BSA and other proteins.^{6,15,17,25,55} It is also noted that on addition of NaDC to the BSA fibrils, the intensity of the helical structure increases slightly indicating its ability to return to its native state. However, the fibrils do not completely return to the native state, as is evident from Fig.2, which is in accordance with our fluorescence measurements.

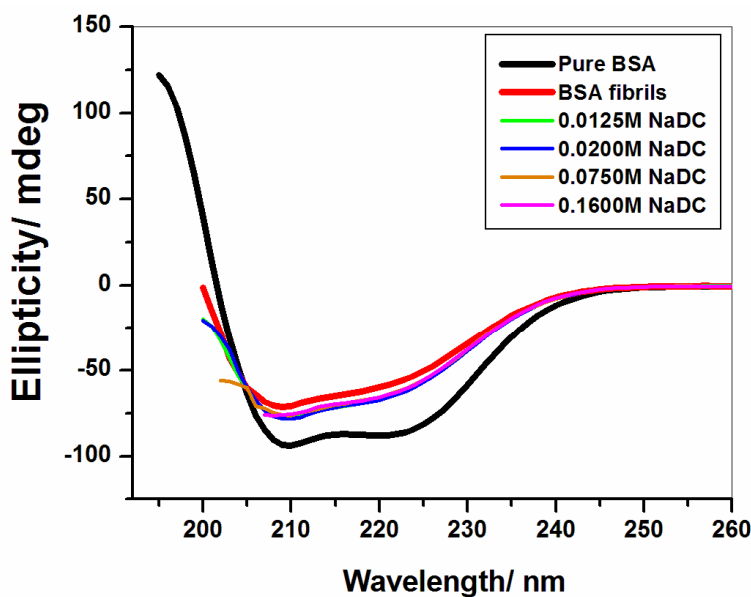


Fig.2 CD spectra (at 25 °C) of BSA in the far-uv region showing secondary structural changes as a function of [NaDC]. The concentrations of NaDC (in M) are shown in the inset. The [BSA] = 0.1 wt. % and [NaCl] = 0.1M.

It may be noted that the CD signal of the BSA fibrils completely disappeared with the addition of NaSal (Fig.S3). Similar effect was also observed when NaSal is added to pure BSA. This is due to the high absorption of NaSal in the wavelength range studied. This is confirmed from the changes in CD spectra at different concentrations of NaSal (figure not shown). It was observed that above 5mM NaSal concentration, the spectra changes drastically and the signature of BSA could not be seen. Due to this limitation, no attempts were made to elucidate the conformational changes of BSA using CD spectra, in the presence of NaSal.

Having noticed that NaDC and NaSal are able to induce changes in preformed fibrils of BSA, we also studied the effect of these additives on fibrillogenesis by adding the additives before fibrillation. This will provide insight into the role of these additives in preventing the formation of fibrils, once bound to the proteins in the native form. Fig.3 compares the evolution of ThT fluorescence spectra during BSA fibrillation in the presence of 10 mM NaDC and NaSal. The drastic decrease in fluorescence intensity in the presence of NaSal and NaDC clearly indicates that these additives alter the conformation of the protein, if added prior to fibrillation and thus can retard the formation of fibrils. The decrease in fluorescence intensity is much more than that is observed when added after fibril formation. This reveals that NaSal and NaDC are more effective in inhibiting the fibril formation, if added during the fibrillation process rather than disassembling the fibril by post addition.

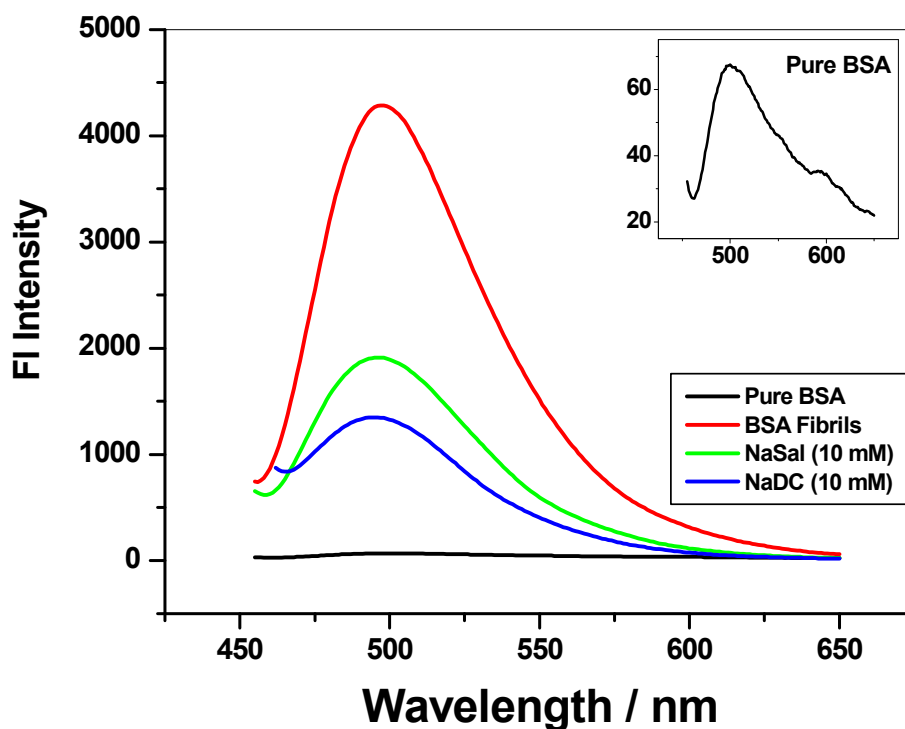


Fig.3. Fluorescence spectral changes of ThT in the presence of 10 mM NaSal and NaDC. Inset shows the spectra of pure BSA for clarity. Spectra recorded after 240 minutes of incubation.

The above observed effect of these two additives on suppression of the fibrillation process was monitored using CD spectra after addition of NaSal / NaDC and incubating the mixture for 240 minutes (Fig. 4). In pure BSA, two minima at 209 and 222 nm characteristic of helical structure are observed. The % α -helix in pure BSA was found to be $\sim 66\%$, which is in good agreement with those reported.²⁵⁻²⁶ In pure BSA, upon formation of fibrils, the % of α -helix was found to be ~ 36.1 , thus showing a reduction in the α -helix content by about 30 %, which indicates the formation of β -sheet or disordered conformation. Such losses of the helical content has been reported for BSA as well as for other proteins during fibril formation.^{6, 10,11}

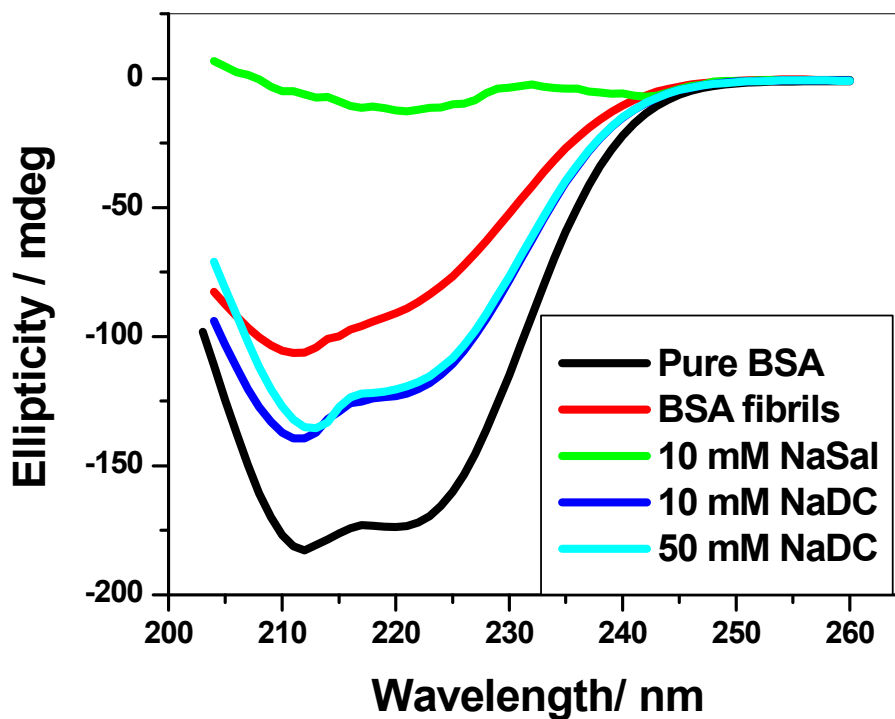


Fig.4. CD spectra (at 25 °C) of BSA in far-uv region showing secondary structural changes in the presence of NaSal and NaDC. The concentration of BSA is 0.15 % and that for NaSal and NaDC are mentioned on the inset. The spectra in presence of the additives and for BSA fibrils were taken after 240 minutes of incubation with 0.2 M NaCl and additives as mentioned in the inset.

On addition of 10 mM NaSal, as observed previously, no signature for BSA is obtained and is due to the high absorption of NaSal in this wavelength range. However, in the presence of NaDC, the α -helix content has reduced considerably and is independent of the concentration of NaDC, as is evident from the CD spectrum (Fig. 4). The % α -helix in BSA containing NaDC is found to be around 46 %. To further understand the role of NaSal on the secondary structure of BSA, we carried out the CD measurements as a function of temperature, as well. The thermal

denaturation was followed by monitoring changes in dichroic intensities at 222 nm from 20 to 105 °C and is shown in Fig.S4.

Pure BSA shows the characteristics transition of the protein from the native to the unfolded state. In the case of BSA fibrils, the ellipticity increases and the transition is not sharp. At high temperature all curves merge indicating the loss of conformation. The thermal denaturing behavior of the protein in the presence of NaDC is intermediate between native BSA and fibrils. This suggests that NaDC is inhibiting the fiber formation.

As noted earlier, though fluorescence and CD measurements provide insight into the conformational changes associated with fibril formation, this cannot be taken as a quantitative measure of the extent of fibril formation. Recent studies indicate that complementary scattering techniques such as DLS and SANS provide valuable information about conversion of β -lactoglobulin monomer into semiflexible protein fibrils upon heating to 90°C.⁵⁶ Thus, we employed DLS and SANS measurements to evaluate the efficacy of additives towards defibrillation of BSA. Fig. 5 shows the variation of scattered light electric field correlation function, measured at a scattering angle of 90°, obtained from the protein, with and without NaSal or NaDC. As it was observed that addition of NaSal or NaDC prior to incubation is more effective than adding after fibril formation, these measurements were made using samples after addition of NaDC or NaSal and incubated for 30 minutes to induce fibrillation. The solid lines are fit to the data using the modified cumulants method, to calculate the mean relaxation time and polydispersity index (PDI). The mean relaxation time and PDI are tabulated in Table 1 as obtained from the spectra.

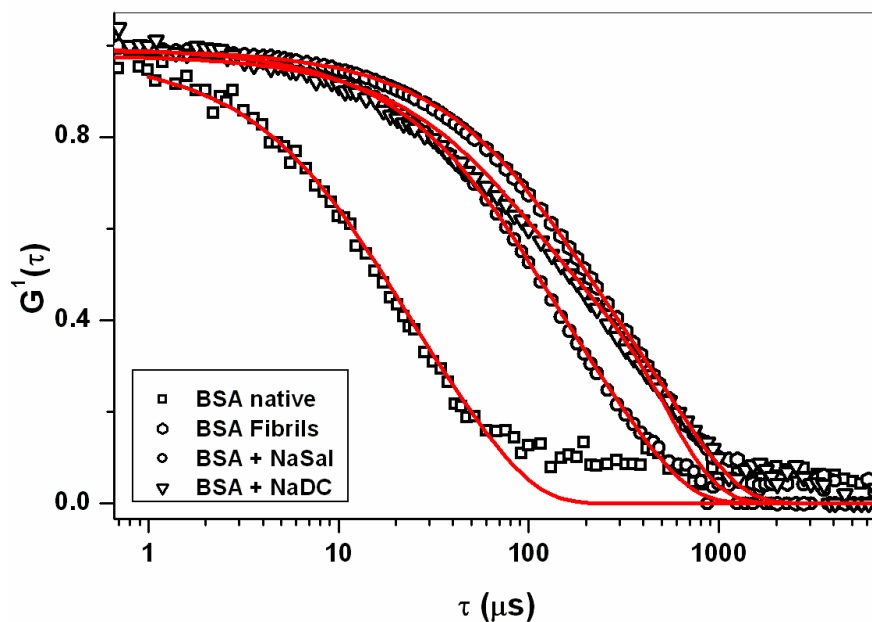


Fig.5. Variation of the electric field correlation function with lag time, measured from suspensions of BSA protein incubated with and without additives. The concentration of the additives is 50 mM and the measurements were done after 30 minutes.

Table1. Values of mean relaxation time and polydispersity index at 30 and 230 minutes of incubation.

Sample	Mean Relaxation Time (micro seconds)	Polydispersity Index
After 30 minutes of incubation		
Pure BSA	22 ± 1.1	0.38 ± 0.02
BSA fibrils	236 ± 12	0.53 ± 0.03
BSA + NaSal	135 ± 7	0.37 ± 0.02
BSA + NaDC	167 ± 8	0.84 ± 0.04
After 230 minutes of incubation		
BSA fibrils	349 ± 17	0.64 ± 0.03
BSA + NaSal	157 ± 8	0.50 ± 0.03
BSA + NaDC	31 ± 2	0.46 ± 0.02

From Fig.5 and Table 1, it can be clearly seen that the relaxation time of BSA increases drastically on fibrillation. The mean relaxation time of pure BSA is 22 microseconds which corresponds to an equivalent sphere hydrodynamic diameter of 8 nm, consistent with globular monomeric protein. Upon fibrillation, the correlation function shifts to longer time scales and the relaxation time reaches to 236 microseconds indicating the aggregation of proteins. However, upon addition of NaSal and NaDC, the relaxation time decreases as compared to that of fibrils. In the presence of NaDC, after 30 minutes of incubation, an increase in the polydispersity (PDI) is observed and is attributed to the presence of both monomers and aggregates as revealed by the intensity weighted size distribution plot (Fig. S5), obtained from inverse Laplace transformation of the data using CONTIN program. It may be noted that though in the presence of additives also there is an increase in the aggregate size with the incubation time, the average aggregate size is less in the presence of additives as compared to pure BSA fibrils, at both incubation times. This indicates that in the presence of additives this fibrillation process is slowed down and exists as oligomers and aggregates with a broad distribution of sizes. The slowing down of the aggregation process in the presence of additives (10 mM) is evident from the variation in the effective diameter of the BSA fibrils as a function of incubation time (Fig S6). The correlation function measured after 230 minutes of incubation and the corresponding fits are shown in Fig.8. The fitted parameters are included in Table 1.

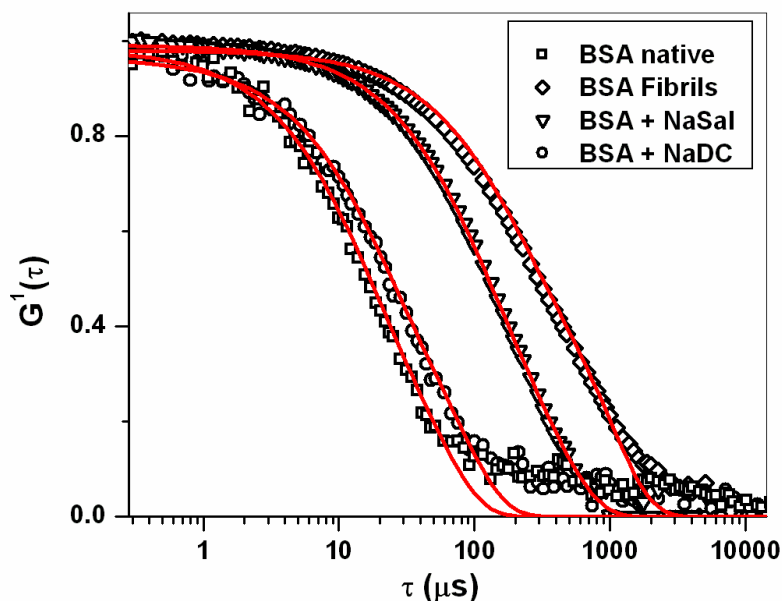


Fig.6. Variation of the electric field correlation function with lag time, measured from suspensions of BSA protein incubated with and without additives. The concentration of the additives is 50 mM and the measurements were done after 230 minutes.

After 230 minutes of incubation, it shows that both NaDC and NaSal suppresses the fibrillation process and NaDC effectively prevents the fibrillation of BSA molecules. These results are in agreement with our fluorescence and CD results. DLS studies were also carried out to understand the effect of NaSal and NaDC, if added after fibril formation. No significant change in the size distribution is observed when same concentration of additives were added after fibrillation. This is surprising, as it was noted from fluorescence and CD studies, it does show a change in protein secondary structure, even when added after fibrillation. This shows that the additives are effective in preventing fibrillation process if added to the protein in the native state. However, when added after fibril formation the additives do not revert the aggregated protein to

monomeric state, though the additives are able to induce changes in the secondary structure of the protein.

Having noticed a change in the protein aggregation behavior, when NaSal and NaDC were added before and after fibrillation, we explored the impact of these additives using SANS which has been extensively used for studying the microstructure of proteins. First, we monitored the protein structural changes using SANS, by adding NaSal and NaDC before incubation. The SANS data for 1 wt % pure BSA, BSA fibrils and that of BSA incubated in the presence of NaDC and NaSal are shown in Fig. 7. The solid lines are fit to the SANS data using an ellipsoid model and the fitted parameters included in table 2. The data of pure BSA has been fitted to an oblate ellipsoidal model and the fitting generated a value of semi major axes ($b=c$) = 37.5 Å and that of semi minor axis (a) = 12.5 Å, which are in good agreement with that of the reported values. Interestingly, upon formation of BSA fibrils, the scattering intensity at low Q increases drastically, indicating the formation of larger structures. The data is fitted using prolate ellipsoidal shape having values of semi major (a) and semi minor ($b=c$) axes as 152.7 and 34.9 Å respectively. This is in accordance to our DLS data, wherein also we obtained large increase in the average size of BSA on fibril formation

Analysis of the SANS data obtained from BSA incubated in the presence of 0.015 M NaDC shows that NaDC effectively prevents the fibrillation, and it does not alter the structure of BSA as well (remains oblate), even after 240 minutes of incubation. On the other hand, in the presence of 0.035 M NaSal the structure of the protein is different from that of pure BSA or BSA fibrils. NaSal does not prevent fibril formation completely, though it retards the formation of fibrils (Table 2). In the presence of NaSal, the proteins appear to form aggregates which are prolate in shape, but the length of the aggregates are smaller than that is observed in pure BSA

fibrils. This clearly suggests that both NaSal and NaDC, if added prior to incubation, are capable of retarding aggregation of BSA, the effect being more pronounced with NaDC. Thus, bile salts could act as potential adjuvants for suppression of protein fibril formation.

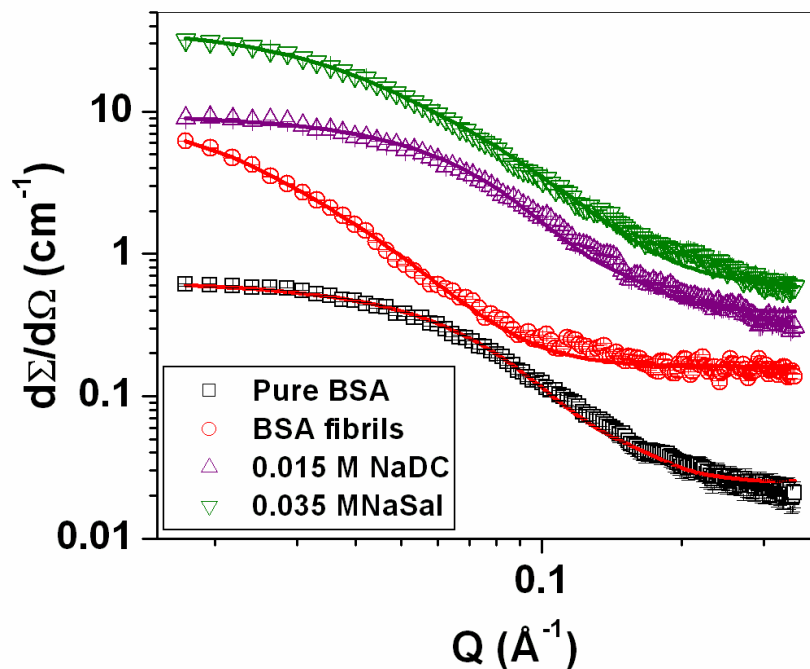


Fig.7. SANS data for 1 wt % BSA fibrils as a function of [NaDC]. The concentrations of additives are mentioned in the inset. The measurements were carried out after 240 minutes of incubation.

Table 2. Fitted parameters of SANS analysis of BSA and BSA fibrils with varying amounts of NaSal and NaDC.

System	a (Å)	b=c (Å)	Shape	Polydispersity
Pure BSA	12.5 ± 0.4	37.5 ± 0.8	Oblate	
BSA fibrils	153.0 ± 5.0	35.0 ± 1.0	Prolate	0.4 ± 0.1
0.015 M NaDC	12.5 ± 0.4	38.0 ± 0.9	Oblate	
0.035 M NaSal	86.0 ± 3.0	16.0 ± 0.5	prolate	0.4 ± 0.1

Next, we looked upon the effect of NaSal and NaDC, on the structure of preformed fibrils using SANS . The ability of additives in defibrillating preformed fibrils will be advantageous to use these additives as drugs for curing protein fibril related diseases. The concentration of NaSal has been varied upto 2.0 M, as it was observed from fluorescence studies that at high additive concentration there exists significant change in the secondary structure of protein fibrils.

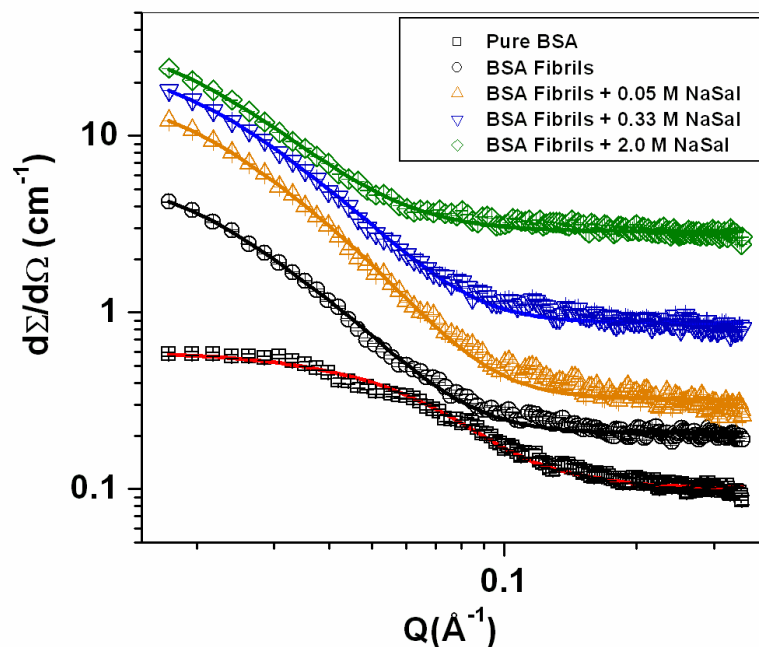


Fig.8. SANS data for 1 wt % BSA fibrils as a function of [NaSal]. The concentrations of additives are mentioned in the inset. The plots have been shifted vertically for clear presentation. BSA fibrils x 2, BSA fibrils + 0.05 M NaSal x 6, BSA fibrils + 0.33 M NaSal x 10, BSA fibrils + 2.0 M NaSal x 20.

The SANS data in Fig.8 and the fitted parameters (Table 3) indicates that on addition of even upto 0.33 M NaSal, not much difference is observed in the scattering profile, and the BSA fibrils appears to remain unaffected by the addition of NaSal. However, at 2 M NaSal, the scattering pattern could not be fitted with prolate ellipsoid model, but gave good fit with the Debye-

Bauche model for polyelectrolyte chains with a correlation length of 37.4 Å. This indicates that at high NaSal concentration the protein fibrils rearranges to random polymer chains, possibly from the increased electrostatic repulsion of adsorbed NaSal molecules. This is consistent with the fluorescence measurements, where it is observed that beyond 0.33 M NaSal concentration, the protein undergo structural changes.

Table 3. Fitted parameters of SANS analysis of BSA and BSA fibrils with varying amounts of NaSal.

System	Semi major axis a (Å)	Semi minor axis (b=c) (Å)	Geometry	Polydispersity
BSA Fibrils	153.0 ± 5.0	35.0 ± 1.0	Prolate	0.4 ± 0.1
BSA Fibrils +0.05 M NaSal	157.0 ± 5.0	36.6 ± 0.7	Prolate	0.4 ± 0.1
BSA Fibrils +0.33 M NaSal	151.0 ± 5.0	34.4 ± 0.7	Prolate	0.4 ± 0.1

We also studied the effect of NaDC on the structure of preformed fibrils, as it was observed that NaDC is much more effective in preventing fibril formation. SANS spectra of preformed fibrils in the presence of NaDC shows characteristics of both micelles and fibers (Fig.9).

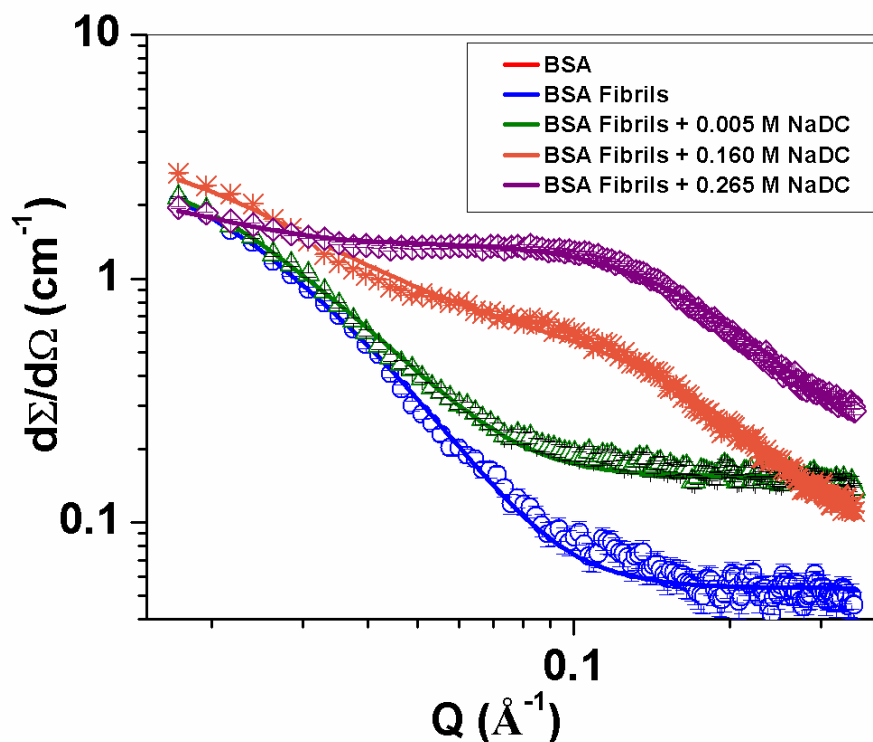
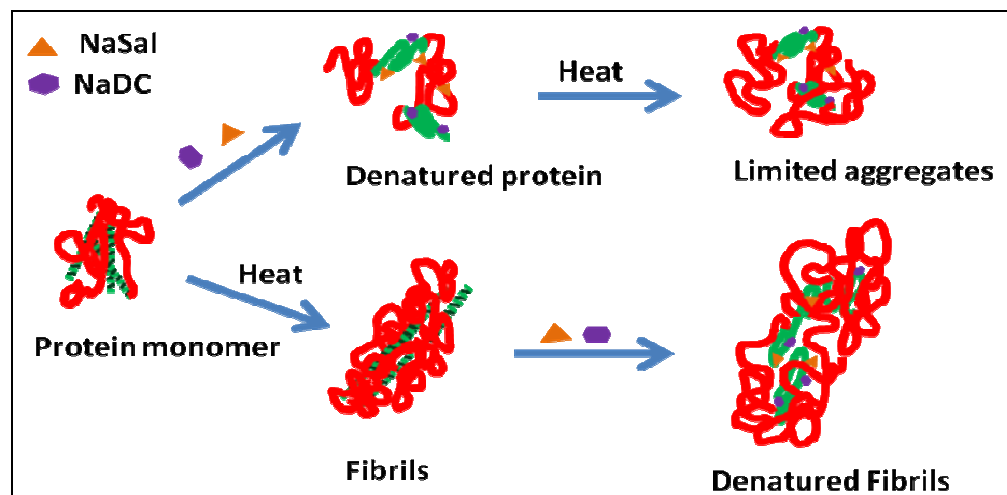


Fig.9. SANS data for 1 wt % BSA fibrils as a function of [NaDC]. The concentrations of additives are mentioned in the inset. The experimental data for BSA fibrils without any additives is shown in blue color.

Table 4. Fitted parameters of SANS analysis of BSA and BSA fibrils with varying amounts of NaDC. A polydispersity of 0.4 is used for the fibril structures,

System	Fibrils Parameters		Micellar Parameters		
	Semi major axis a (Å)	Semi minor axis b=c (Å)	Semi major axis a (Å)	Semi minor axis b=c (Å)	Charge
BSA fibrils	153.0 ± 5.0	35.0 ± 1.0	-	-	
BSA fibrils+0.005 M NaDC	151.0 ± 5.0	34.6 ± 0.8			-
BSA fibrils+0.160 M NaDC	150.0 ± 5.0	34.5 ± 0.8	20.8 ± 0.6	8.0 ± 0.2	-
BSA fibrils+0.265 M NaDC	150.2 ± 4.8	34.0 ± 0.7	21.5 ± 0.5	8.0 ± 0.2	5.5 ± 0.2

At high NaDC concentration a characteristic correlation peak like pattern develops in the intermediate Q range of the SANS spectra with an increase in scattering at low Q region. This correlation peak is attributed to the presence of repulsive micellar aggregates which are formed, as the concentration of additive is well above the critical micelle concentration (cmc). Thus, the SANS data at high additive concentrations were fitted as a sum of contributions from micelles and fibrils. The fitted parameters are listed in Table 4. SANS analysis indicates that NaDC does not appear to effect the BSA fibrils, if added after fibrillation. Excess NaDC remain in solution as globular micelles without disrupting the fibril structure. Thus, DLS and SANS studies clearly indicate that NaSal and NaDC can retard or prevent the formation of fibrils in BSA, if added to the native form of protein. However, when added after fibril formation, not much change in the structure of the fibrils is observed at low additive concentrations. Based on the above observation, a scheme has been proposed for the mechanism of action of additives on BSA (Scheme 1)



Scheme 1. Schematic representation of the mechanisms of action of small molecule additives on BSA fibrillation.

The mechanism of action of various small molecule chemical chaperons in prevention or reversal of protein aggregation has been ascribed to both contraction of the unfolded state and denaturing effects.⁵⁷ The unfolded state contraction has been achieved by addition of protecting osmolytes which include certain aminoacids (glycine, proline etc), carbohydrates or methylamines. It is reported that the protecting osmolytes preferentially destabilizes the unfolded state of the protein. On the other hand, common denaturants like guanidinium hydrochloride or urea binds to the native state of the proteins, affecting the side chains rather than the backbone. Significant changes in the far UV CD spectra of BSA in the presence of NaSal and NaDC indicate that both additives influence the native state of the proteins. Several synthetic and natural small molecules have been explored as adjuvants for prevention or slowing down of amyloid formation. Small peptides consisting of natural aminoacids, amphiphilic block copolymers etc are also shown promising results as amyloid inhibitors. The present study reveals the hydrophobic association of small molecules such as hydrotropes (NaSal), bile salts (NaDC) etc with native BSA protein thereby limiting the aggregation process.

Conclusions

We have studied the effects of two bio-compatible additives on the fibre formation of BSA. The fibrillation of proteins is responsible for a number of diseases. Hence, the main objective of this investigation idea was to identify if fibrillation of proteins would be prevented or slowed down, by using some common biocompatible additives. The impact of additives if added before fibrillation and after fibrillation has been identified. The present results indicate that NaDC and NaSal show contrasting behavior if added before and after incubation. Significant reduction in the rate of fibrillation is observed, if the additives are added prior to the formation of fibrils. However, though the additives are able to adsorb on preformed fibrils, if added after fibrillation,

they have negligible effect on structure of fibrils. Fluorescence and CD studies reveal that both additives are capable of adsorbing on protein surface through hydrophobic or electrostatic binding. This process leads to significant alterations in the protein conformation, mostly the secondary structure. One important outcome of the present investigation is that though fluorescence and CD spectral studies provide information about the conformational changes in protein, this cannot be taken as a conclusive evidence for the tertiary structure of fibrils. Several groups have used such spectroscopic tools to indirectly assess the macroscopic structure of proteins. Monitoring the aggregate structure using DLS and SANS and comparing the results with other spectroscopic tools suggest that complementary tools are necessary to confirm the tertiary structure of proteins. The slowing down of fibril formation in BSA by the addition of NaSal and NaDC indicates its potential as adjuvants in storing proteins which are prone to fibril formation under normal conditions of temperature and pressure. The observation that additives added prior to fibril formation are more effective than added after the fibrillation process may be exploited in future studies with other bio-compatible molecules, which may be useful in designing formulations which can provide pathways for the treatment of amyloid related diseases.

Supporting Information

Figure S1: Fluorescence spectra of ThT intensity as a function of [NaDC]. The concentrations of NaDC are shown in the inset. Figure S2: Plots of variation of Th-T intensity as a function of [NaCl]. Fig.S3: CD spectra (at 25 °C) of BSA in the far-uv region showing secondary structural changes as a function of [NaSal]. The [BSA] = 0.1 wt. % and [NaSal] = 0.02 M. Fig.S4:

Thermal denaturation curves obtained by monitoring ellipticity at 222nm as a function of temperature. The concentration of BSA is 0.1 % w/w and that for NaSal and NaDC are 50 mM.

Figure S5: Plots of intensity weighted size distribution of BSA fibrils in the presence of SSal and NaDC

Figure S6. Variation in the effective diameter of BSA as function of incubation time with and without additives. The concentration of the additives used are indicated in the inset

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FIGURE CAPTIONS

Fig.1. (A) Fluorescence spectral changes of Thioflavin T (ThT) as a function of [NaSal]. The concentrations of NaSal are shown in the inset. (B) and (C) Plots of variation of Th-T intensity as a function of [NaSal] and [NaDC] respectively.

Fig.2 CD spectra (at 25 °C) of BSA in the far-uv region showing secondary structural changes as a function of [NaDC]. The concentrations of NaDC (in M) are shown in the inset. The [BSA] = 0.1 wt. % and [NaCl] = 0.1M.

Fig.3. Fluorescence spectral changes of ThT in the presence of 10 mM NaSal and NaDC. Inset shows the spectra of pure BSA for clarity. Spectra recorded after 240 minutes of incubation.

Fig.4. CD spectra (at 25 °C) of BSA in far-uv region showing secondary structural changes in the presence of NaSal and NaDC. The concentration of BSA is 0.15 % and that for NaSal and NaDC are mentioned on the inset. The spectra in presence of the additives and for BSA fibrils were taken after 240 minutes of incubation with 0.2 M NaCl and additives as mentioned in the inset.

Fig.5. Variation of the electric field correlation function with lag time, measured from suspensions of BSA protein incubated with and without additives. The concentration of the additives is 50 mM and the measurements were done after 30 minutes.

Fig.6. Variation of the electric field correlation function with lag time, measured from suspensions of BSA protein incubated with and without additives. The concentration of the additives is 50 mM and the measurements were done after 230 minutes.

Fig.7. SANS data for 1 wt % BSA fibrils as a function of [NaDC]. The concentrations of additives are mentioned in the inset. The measurements were carried out after 240 minutes of incubation.

Fig.8. SANS data for 1 wt % BSA fibrils as a function of [NaSal]. The concentrations of additives are mentioned in the inset. The plots have been shifted vertically for clear presentation. BSA fibrils x 2, BSA fibrils + 0.05 M NaSal x 6, BSA fibrils + 0.33 M NaSal x 10, BSA fibrils + 2.0 M NaSal x 20.

Fig.9. SANS data for 1 wt % BSA fibrils as a function of [NaDC]. The concentrations of additives are mentioned in the inset. The experimental data BSA fibrils without any additives is shown in blue color.