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Differential Effects of Ionic and Non-ionic Surfactants on Lysozyme Fibrillation†

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Abstract

Fibril formation is a common property of many proteins, though all are not associated with the diseases. Proteins' surface charges and the added co-solvents play vital role in determining fibrillation pathway and kinetics. In order to understand these phenomena, the effects of anionic, cationic and non-ionic surfactants on lysozyme fibrillation were studied. Lysozyme forms fibrils in 2 M and 4 M urea solutions by following nucleation-dependent and -independent pathways, respectively, at neutral pH. Under these conditions, the effects of sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), and triton X-100 (Tx) were investigated on lysozyme structure and fibrillation. The results indicate that there are differential effects of ionic and non-ionic surfactants. In the presence of SDS and CTAB, at above their critical micelle concentrations (CMC), lysozyme could not form fibrils. However, the non-ionic Tx do not inhibit fibril formation at all the concentrations. The time for complete fibril formation is increased by Tx. All of the surfactants are found to increase the initial nucleation phase; the extent of increase is less at near-CMC of the ionic surfactants and at above the CMC of Tx. The rates of fibril elongation show varying effects in the presence of different surfactants. The results suggest that the nucleation phase of lysozyme fibrillation is primarily controlled by charge interactions and micellation of the surfactants, but multiple factors might influence the fibril elongation. Further, the surfactants do not alter the fibrillation pathway from nucleation-dependent to -independent or *vice versa* in the studied conditions.

1. Introduction

Formation of fibril structures by proteins has various implications in biomedical and biotechnology research. *In vitro* studies on protein fibrillation suggest that the tendency of proteins to form fibrils is not limited to the disease-associated proteins and it is a common property of many proteins, perhaps the all under appropriate conditions.[1-4] Though these proteins do not show sequence homology or structural similarity, they can still form amyloid fibrils with similar morphology.[3] When the structure of native protein is altered with solution conditions to populate partially unfolded structures, the protein can form oligomeric structures which can further rearrange themselves into the amyloid structure.[4] These fibrils are formed by cross beta-assembly with beta-strands perpendicular and beta-sheets parallel to the fibril axis.[5-7] However, the exact molecular mechanism of conversion of soluble globular proteins into the cross-beta fibrillar form is yet to be clearly understood.

Lysozymes are glycosidases involving in the lyses of bacterial cell walls. Two of its natural mutants Ile56Thr and Asp67His are known to cause hereditary non-neuropathic systemic amyloidosis in human.[8] Studies show that these variants and the native form of the protein have similar structures. Also, the morphology of the fibrils formed by these variants is similar to the fibrils formed by the native protein at different conditions.[9] However, the mutants have lesser activity at physiological conditions and reduced stability against temperature.[10] Therefore, structural and fibril studies of human lysozyme and its mutants are considered as a good model to probe the fibrillation mechanism. The human lysozyme has nearly 60% homology with the hen egg white lysozyme (HEWL). Hence, HEWL is also considered as a good model to study the fibrillation mechanism of proteins *in vitro*. Hen egg white lysozyme is a globular protein with 129 amino acids, mainly containing alpha-helical

structure (Fig. 1). Various fibril forming conditions have been identified for lysozyme. Most of these studies were carried out in highly acidic conditions,[9,11-16] and the other solution conditions such as neutral pH and high temperature[17-19] and alkaline pH,[20-22] in the absence or in the presence of added denaturants have also been used to form the fibrils of lysozyme. These studies suggest that the partial denaturation of the protein by solvent environment[4] or mutation[10] is a prerequisite for fibril formation. They also emphasize the role of protein's surface charge neutralization on aggregation and fibril formation at different pHs.[12,15,22] The studies carried out at lower pH show that the rate and extent of fibril formation depends on the pH and temperature.[11] However, slight changes in pH, ionic strength and post-translational modifications such as glycation could shift the fibrillation pathway into spherical aggregates.[11,15,23] Studies on inhibition of fibrils by small molecules formed at different pH conditions also help to understand the interactions and mechanism of fibrillation. For instance, polyphenols, and indole derivatives are found to inhibit HEWL fibril formation majorly by aromatic and hydrophobic interactions at the pHs 12.7 and 2.5, respectively.[24,25]

Lysozyme-surfactant complexes are widely studied to understand the charge and hydrophobic interactions between the protein and the surfactants.[26-32] HEWL has a net charge of +8 at neutral pH due to 18 cationic and 10 anionic sites and its pI is ~11.[30] Neutralization of this net positive charge with oppositely charged surfactant such as sodium dodecyl sulfate (SDS) has been studied at various conditions to understand the role of charge on the solubility of lysozyme and its stability. Lysozyme shows turbidity in lower concentrations of SDS and it is completely soluble in higher concentrations of SDS.[26] This behavior varies with pH and added compounds.[33] From the binding isotherms[27] and neutron scattering experiment,[30] it has been proposed that initial charge neutralization followed by hydrophobic association are the factors behind the solubility changes. These intriguing characters of

lysozyme further led to the studies on the effect of SDS on lysozyme fibril formation.[12,20-22] The studies were nevertheless carried out either at acidic or at alkaline pH conditions. Since charge interactions are imperative in protein-surfactant interaction,[34,35] thus influencing its fibril forming propensity, the investigation at near neutral pH would provide better view on these aspects. To the best of our knowledge, only two solution conditions have been identified for lysozyme fibril formation at neutral pH.[17,18] These studies show that the partially unfolded states of lysozyme obtained at near neutral pH with the most commonly used denaturants, urea and guanidinium hydrochloride (Gdn) can form amyloid fibrils at the temperatures between 45 and 55 °C. However, Gdn, an ionic denaturant might interfere with the charge interactions occurring between the protein and the surfactants. Therefore, using the conditions similar to Wang *et al*.[18] would be more appropriate to follow the effect of surfactants on the protein fibrillation. Further, the earlier studies are limited to smaller range of surfactant concentrations and mostly specific to anionic surfactants.[12,20-22] Considering these factors, our present work attempts to re-optimize the fibril forming conditions for HEWL at neutral pH and to follow the effects of anionic, cationic, and non-ionic surfactants at wider concentration range on the fibril formation. The results suggest that ionic surfactants at the concentrations above critical micellar concentration (CMC) inhibit the fibril formation of lysozyme, whereas non-ionic surfactant does not inhibit the fibril formation at any concentration. Also, the surfactants affect the nucleation and elongation phases distinctively. It emphasizes the crucial role of charge interactions between protein and surfactant on protein fibrillation.

2. Experimental Methods

2.1. Materials

Hen egg white lysozyme, urea, thioflavin-T (ThT), cetyltrimethylammonium bromide (CTAB), and *p*-toluidinyl-6-naphthalenesulfonate (TNS) were purchased from Sigma-Aldrich. Phosphate salts, sodium dodecyl sulfate (SDS), triton X-100 (Tx), and safranin were obtained from SRL, India.

2.2. Sample Preparation

Varying concentrations of surfactants ranging from 0.1 mM to 25 mM of SDS, 0.01 mM to 20 mM of CTAB, and 0.001 mM to 10 mM of Tx were prepared in 10 mM phosphate buffer at pH 7. Lysozyme was added directly into the aliquots from stock solutions to obtain the final protein concentration of 2.5 mg/mL or 15 μM for fibril or unfolding studies, respectively. Fibrils were formed by keeping the protein samples at 60 °C for 10-12 h. To follow the kinetics of fibril formation, the samples were heated inside the spectrophotometer using an air-cooled Peltier in the presence of 40 μM of ThT. The earlier studies show that the micelles of ThT efficiently binds to protein fibrils,[36] therefore the concentration of ThT was kept well above its CMC of 4 μM.

The exposure of hydrophobic residues of HEWL was followed using an external fluorophoric probe, TNS.[37,38] Samples with varying concentrations of SDS, CTAB, and Tx were prepared in the presence and the absence of urea with 40 μM of TNS in 10 mM phosphate buffer at pH 7. Fluorescence emission of TNS was measured between 380 and 500 nm after exciting the samples at 320 nm. Then, HEWL was added into the samples in such a

way that the changes in volume of the samples were ≤1 % only. TNS emission spectra were measured again after equilibrating the samples for an hour.

2.3. Spectroscopic and Light Scattering Measurements

ThT emission spectra were recorded between 460 and 600 nm, after exciting the samples at 440 nm using excitation and emission slit widths of 4 nm. For kinetic measurements, the samples were excited at 440 nm and the change in emission intensity at 485 nm was followed. Fibril kinetic data were analyzed using an empirical equation,[39]

$$Y = (y_i + m_i x) + \{(y_f + m_f x) / (1+e^{(x-x_o/t)})\} \quad (1)$$

Where, Y is the observed fluorescence, $(y_i + m_i x)$ and $(y_f + m_f x)$ are the initial and final baselines, t is time constant and x_o is the time to reach half of the maximum fluorescence. Lag (nucleation) time is equal to x_o-2t. The protein's fluorescence changes were measured by exciting the samples at 280 nm and the spectra were recorded from 300 to 400 nm. All the spectra were obtained using a Horiba Jobin Yvon – fluoromax-3 spectrofluorometer. Ellipticity changes at far-UV region were measured in a Jasco-810 spectropolarimeter. Due to the interference of Tx in the measurements, probably because of its weak absorption at 280 nm,[36] the ellipticity changes could not be followed for lysozyme in Tx. Thermal denaturation studies were carried out by following the ellipticity changes at 220 nm for the temperature interval of every one degree from 20 to 90 °C.

2.4. Microscopic Imaging

10 µL of the protein samples were placed on 200 mesh carbon coated copper grids obtained from Ted Pella and incubated for 20 min at room temperature. The samples were stained for

30 sec with 2% uranyl acetate and visualized under Tecnai transmission electron microscope (TEM) at an accelerating voltage of 200 kV. For atomic force microscopic (AFM) images, the samples were placed on a freshly cleaved mica surface for a few minutes, washed with deionized water, and dried. The samples were then imaged in SPA400 (Seiko) using tapping mode with the scan rate of 1-2 Hz and the resonance frequency of 110-150 Hz.

3. Results

3.1. Lysozyme Fibril Formation

HEWL in varying concentrations of urea at neutral pH was heated at 60 °C to identify an appropriate condition for fibril formation. The protein in 2 M and 4 M urea turned turbid after heating for 10-12 h, but no turbidity was observed in the absence of urea. All the samples were assayed with ThT fluorescence. The samples with urea showed increase in ThT emission intensity which is the characteristic of fibril formation whereas without urea the intensity was slightly decreased. The samples were imaged under TEM for further characterization. TEM images clearly showed that in the presence of urea lysozyme forms fibrils, but in the absence of urea the protein forms small aggregates which are not the characteristic of amyloid fibrils. All the results are presented in Fig. 2.

3.2. Effect of Surfactants on Lysozyme Fibrillation

Having identified the conditions for fibril formation at neutral pH, the effects of surfactants, SDS (anionic), CTAB (cationic), and Tx (non-ionic) on the fibrillation were analyzed. Surfactants exist as monomers at lower concentrations and form micelles at higher

concentrations (above CMC). The concentration at which a surfactant form micelles is generally affected by the buffer conditions and added co-solvents, particularly the CMC of surfactants increases with the addition of urea.[41,42] Therefore, the CMC of the selected surfactants in the presence of 2 M and 4 M urea were calculated using fluorescence probes such as safranin and TNS for the buffer conditions used in our experiments. Fluorescence intensity of these probes increases steeply when the surfactants form micelles. This property was used to measure the CMC of the surfactants in the presence and the absence of lysozyme (Fig. S1 in ESI†). The results showed that all the three surfactants form micelles at higher concentrations in the presence of urea (Table S1 & Fig. S2 in ESI†). Further, in the lower concentrations of SDS, lysozyme formed precipitate which dissolved into the solution at higher concentrations of SDS as found in the earlier studies.[26] It was then examined whether this transition occurs in the presence of urea and in the case of other surfactants, CTAB and Tx, as well. The protein solutions prepared in CTAB and Tx did not show any turbidity. Moreover, the protein showed complete dissolution in lower concentration of SDS in the presence of 2 M and 4 M urea (Fig. S3-A in ESI†).

In order to evaluate the effect of surfactants on lysozyme fibrillation, different concentrations of the surfactants ranging from their monomeric to micelle concentrations were added to the protein solution in the presence of 2 M or 4 M urea and heated at 60 ^{o}C for 10-12 h. ThT fluorescence intensity was measured before and after incubating the samples at higher temperature and the increase in ThT fluorescence was considered as the formation of fibrils. ThT is known to show higher fluorescence in the presence of SDS micelles, but not in CTAB and Tx.[43] To avoid the surfactant's effect, the fluorescence intensities were normalized with the fluorescence of respective samples measured before heating. In the presence of 2 M urea, ThT showed an increase in fluorescence intensity for the lysozyme

samples containing lower concentrations of SDS and CTAB. However, in the presence of higher concentrations, that is above the CMC of SDS and CTAB in 2 M urea, fluorescence intensity was similar to the samples measured at room temperature (Figs. 3A and 3B). Lysozyme in the presence of all the concentrations of Tx showed increase in ThT fluorescence upon incubation at higher temperature (Fig. 3C). There were considerable changes in the visual appearance of the samples as well. In the presence of lower concentrations of SDS and CTAB, the samples showed turbidity, but in the presence of micellar concentration of these surfactants the samples were clear and transparent. In Tx, irrespective of the surfactant concentration, lysozyme showed turbid insoluble component (Fig. S3-C in ESI†). The insoluble components which showed turbid appearance might be due to fibril formation of the protein under these conditions.

For further characterization, the samples were imaged using TEM and AFM techniques. The TEM images showed that the samples contained long and twisted fibrils, though the dimensions of the fibrils were slightly varying with the surfactants used (Figs. 3D-F). The similar morphologies were obtained from AFM also (Fig. S4 in ESI†). The same experiments were repeated for lysozyme in the presence of 4 M urea. The results were found to be similar with 2 M urea samples. ThT fluorescence of lysozyme samples in the lower concentrations of SDS and CTAB and in all the concentrations of Tx showed increase in intensity, but at above the micellar concentrations of SDS and CTAB, ThT did not show any increase in the fluorescence intensity (Figs. 4A-C). Moreover, the samples with higher fluorescence intensity were turbid (Fig. S3-D in ESI†). The TEM and AFM images of these samples also showed long and twisted fibrils with slightly different morphologies (Figs. 4D-F and Fig. S4 in ESI†). The experiments carried out in the absence of urea did not show any significant change in the sample appearance, (Fig S3-B in ESI†) and ThT fluorescence (Fig

S5A-C in ESI†). TEM images of the representative samples at different conditions which did not show any characteristic of fibrils are presented in Fig. S6 (in ESI†).

3.3. Kinetics of Fibril Formation

The fibril formation was followed using the change in ThT fluorescence. Lysozyme in the absence of urea did not show any significant change in ThT fluorescence over time (Fig S5-D in ESI†) whereas in the presence of 2 M and 4 M urea showed different kinetic profiles (Fig. 5). The kinetic traces were analyzed using eqn (1)[39] and the resultant parameters are presented in Table 1(and Fig. 6). In 2 M urea, lysozyme showed an initial nucleation or lag phase followed by a fibrillation or elongation phase. In the absence of any surfactant molecule, lysozyme showed ~2 hours of initial lag phase in 2 M urea. The initial lag phase was extended for lysozyme in the presence of all the three surfactants at the fibril forming conditions, except in SDS at near CMC concentration. In the case of SDS and CTAB, even within the concentrations below CMC two distinct regions were observed. The lag phase was longer at the lower concentrations compared to the lag time at the concentrations near CMC. In Tx, the lag phase of lysozyme was extended at the concentrations below CMC compared to the lag time at the micellar concentrations. However, the rate of fibril elongation did not change significantly in the presence of any of the surfactants, except slower rates were observed in higher concentrations of SDS (~2 mM). Above the CMC of SDS and CTAB, no increase in the fluorescence of ThT was observed which is consistent with the results discussed in the previous section.

In 4 M urea, lysozyme fibrillation did not show any lag phase (Figs. 5D-F). ThT fluorescence was exponentially increasing from the onset of the kinetics, suggesting the presence of only the fibrillation phase. In the lower concentrations of SDS and in the higher

concentrations of CTAB and Tx, lysozyme fibrillation was slowed down. In other concentrations of the surfactants, fibrillation rate was not significantly affected (Table 1 and Fig. 6C). Similar to the results observed in 2 M urea conditions, the micellar concentrations of SDS and CTAB did not increase the ThT fluorescence, reiterating the absence of fibril formation at these concentrations. The amplitude of ThT fluorescence was similar in the case of CTAB and Tx samples, but varying with SDS concentration. This could be due to the varying interaction of SDS with ThT, which alter the fluorescence emission of ThT.[43] However, the amplitude change between the samples with 2 M and 4 M urea might be due to the differences in morphology of the fibrils formed under these conditions.

Further, to characterize the protein in lag phase and elongation phase, TEM images were obtained during the fibrillation process, i.e., after 80 minutes of initialization of the kinetics (Fig. S7 in ESI†). The images showed that the protein formed aggregates at the initial stages of kinetics in 2 M urea which could be the nucleation for further fibrillation. However, in 4 M urea condition, formation of thin fibrils at the early stage suggesting the nucleation-independent pathway.

3.4. Surfactant-induced Denaturation

To investigate the conformational changes of lysozyme in the presence of surfactants, change in the intrinsic fluorescence of the protein was measured. HEWL has six tryptophan residues, among which four of them are in the α-domain and the other two are in the loop region connecting the α- and β-domains (Fig. 1). Upon excitation at 280 nm, in the presence of surfactants, lysozyme invariably showed the emission maximum at 350 nm. The fluorescence emission intensity changes are presented in Fig. 7. Due to the insolubility of lysozyme in

SDS at lower concentrations in the absence of urea, fluorescence changes could not be measured for those samples.

When lysozyme was titrated against SDS in the presence and the absence of 2 M and 4 M urea, it showed a two-state cooperative transition (Fig. 7A). Up to 0.4 mM of SDS, the fluorescence intensity was not altered. Further increase in SDS concentration increased the intensity cooperatively until ~5 mM. Increasing the surfactant concentration above 5 mM (in 4 M urea) or 6 mM (in 2 M urea) or 6.5 mM (without urea) did not alter the fluorescence emission. However, in the case of CTAB the pre-transition region was also affected by the presence of urea (Fig. 7B). Lysozyme fluorescence emission was not changed up to 0.1 mM of CTAB which increases after the addition of CTAB up to 0.7 mM. Further addition of CTAB did not change the fluorescence in the absence of urea. The fluorescence intensity of the protein started to increase from 0.2 mM and 0.4 mM of CTAB concentrations in 2 M and 4 M urea, respectively. There were no fluorescence changes observed above 0.95 mM and 1.4 mM of CTAB in 2 M and 4 M urea, respectively. Also, the protein showed a two-state cooperative transition against the titration of Tx (Fig. 7C). Up to 0.2 mM Tx, the protein fluorescence was not altered. Further addition of Tx up to 2 mM, increased the fluorescence intensity. Increasing the surfactant concentration above 2 mM did not affect the fluorescence. The presence of urea did not affect these transitions caused by Tx.

Further, to investigate the interaction between HEWL and the surfactants, TNS was used as an external fluorphoric probe. The fluorescence quantum yield of TNS increases when it interacts with hydrophobic surfaces compared to polar surroundings.[42] This property of TNS was used to probe the exposure of hydrophobic residues of proteins upon their interaction with denaturants.[37,38] In general, the fluorescence emission of TNS varies with

varying concentrations of surfactants.[42] Therefore, the emission intensity of TNS was obtained in the presence and the absence of the protein at different concentrations of the surfactants (Fig. S8 in ESI†). In order to understand the change in TNS fluorescence due to the protein-surfactant interactions, the change in fluorescence intensity of TNS after the addition of HEWL into surfactant solutions was calculated (Fig 8). Since the protein was insoluble at lower concentrations of SDS in the absence of urea, the data could be obtained for the SDS concentrations above 1 mM only. TNS fluorescence was increased when HEWL was added into 1 mM of SDS. However, the intensity came down upon increasing SDS concentration and no change in TNS intensity was observed above 6.5 mM of SDS. In the presence of 2 M and 4 M urea, TNS fluorescence increases at the concentrations of SDS above 0.2 mM and 0.3 mM, respectively. In both the cases, addition of SDS above 0.6 mM reduced the florescence intensity until 6 mM and no difference in fluorescence was observed at the concentrations of SDS above 6 mM.

TNS fluorescence at the lower concentrations of CTAB was decreased upon addition of HEWL in the absence of urea which increased as the concentration of CTAB was increased above 0.1 mM. At the concentrations above 0.8 mM of CTAB, TNS fluorescence was decreased and above 3 mM of CTAB fluorescence changes of TNS was little. In the presence of 2 M and 4 M urea, TNS fluorescence was not altered up to 0.2 mM and 0.4 mM of CTAB concentrations, respectively, by the addition of HEWL. Above these concentrations, TNS fluorescence increased with increasing CTAB concentration which again decreased at above 1 mM and 2 mM of CTAB upon addition of the protein in the presence of 2 M and 4 M urea, respectively. At the concentrations above 4 mM of CTAB, no change in TNS fluorescence was observed in both the cases. In case of Tx, the difference in

TNS fluorescence before and after the addition of protein was insignificant both in the presence and the absence of urea.

To analyze the changes induced by the surfactants on the secondary structure of lysozyme, change in ellipticity of the protein was measured at far-UV region in varying concentrations of the surfactants (Fig. S9 in ESI†). The changes in ellipticity at 220 nm are shown in Fig. 9. SDS in the presence and in the absence of urea increased the ellipticity of the protein up to 1.5 mM; however, the protein lost the increased ellipticity upon further addition of SDS. It was also observed that in 4 M urea, the extent of increase in the secondary structure of lysozyme is less compared to the samples in 2 M urea and in the absence of urea. In contrary to SDS, CTAB showed no significant change in the secondary structure of the protein in the absence and the presence of 2 M urea. In the presence of 4 M urea, up to 3 mM of CTAB the ellipticity of lysozyme was slightly increased. Further addition of CTAB destabilized these secondary structures, thus decreased the ellipticity.

3.5. Thermal Denaturation

Though the addition of urea did not show any considerable change in the overall structure of the protein, it altered the fibril forming propensity of HEWL. In order to evaluate the stability changes implied by urea, change in ellipticity of the protein in the far-UV region at higher temperature was compared with the spectra obtained at room temperature. The results suggested that at room temperature lysozyme did not show considerable changes, but at higher temperature the protein displayed relatively higher loss of the secondary structure in the presence of urea (Fig. 10A). For further evaluation, thermal denaturation of lysozyme was followed using ellipticity changes at 220 nm in the presence and the absence of urea (Fig. 10B). As the concentration of urea was increased, the thermal denaturation midpoint (T_m)

decreased to 70 and 64 °C in 2 M and 4 M urea, respectively compared to the T_m value of 74 °C for lysozyme in the absence of urea at pH 7.

4. Discussion

4.1. Lysozyme Fibril Formation at pH 7

Though the fibril formation of lysozyme has been extensively characterized at non-neutral pH conditions, the studies at neutral pH are very limited.[17,18] Since our study is aimed to probe the effect of surfactants on the fibril formation and to determine the role of charge interactions, it would be advantageous to monitor the changes at neutral pH. The change in pH alters the surface charges on the protein and the micelle forming concentrations of surfactants. However, lysozyme does not form fibril at neutral pH at normal conditions. Therefore, a moderate non-ionic denaturant, urea was added at low concentrations to facilitate the fibrillation. Addition of 2 M - 4 M urea to lysozyme at higher temperature induced fibril formation which was evident from the ThT assay, TEM images, and AFM images (Fig. 2 and Fig. S4 in ESI†). These conditions were used for analyzing the effects of surfactants on the fibrillation. Earlier crystallographic studies[46] show that urea does not implicate conformational changes in lysozyme, except on the flexible side chains on the surface. The all atom root mean square differences (RMSD) of the 4 M urea bound lysozyme against the native lysozyme is 0.66 Å, which is small and can be considered as a minor conformational change. However, far-UV CD spectra of lysozyme in urea at room and higher temperature (Fig. 10A) evidently show that at higher temperature (60 °C) urea could induce more denaturation compared to the room temperature (25 °C). This effect has been observed

even at 50 °C for lysozyme-urea system.[18] Moreover, the thermal denaturation of lysozyme exhibits a linear decrease in T_m value as urea concentration is increased. This further evidences for the differential urea-induced conformational changes of lysozyme at higher temperature. This partially unfolded conformational state of lysozyme in urea might provide a favorable condition to the protein which could lead to the fibril formation. Further, from the fibrillation kinetic studies of lysozyme in 2 M and 4 M urea (Fig. 5 and Table 1), it is evident that the change in the initial conformational state could completely alter the fibrillation mechanism.[4] In 2 M urea, lysozyme shows an initial lag phase followed by a fibril elongation phase suggesting a nucleation-dependent fibrillation pathway. However, in 4 M urea where the protein loses slightly more secondary structural contents at higher temperature does not show any lag phase and follows a nucleation-independent fibril formation. The nucleation-independent pathway is observed in HEWL at pH 2 and high salt concentration conditions[15] and also in a few other proteins such as bovine serum albumin.[44] This pathway might occur through classical coagulation or downhill aggregation mechanism (for more details reader may refer (45)). Analyzing the effects of surfactants in both of these conditions could provide valuable information on the role of surfactants at different stages of fibril formation.

4.2. Lysozyme-Surfactants Interaction

Conformational changes followed with intrinsic fluorescence of lysozyme shows that SDS has no considerable effect up to 0.4 mM. As the concentration of SDS increases, the protein fluorescence displays a cooperative increase in the intensity (Fig. 7A) which might arise from the tertiary structural changes of the protein. Further insight on the structural changes with an external fluorophoric probe, TNS, (Fig. 8A) suggests that at the lower concentrations of SDS the hydrophobic residues of the protein is partially exposed which is occupied by TNS

molecules, thus enhancing its fluorescence. As the SDS concentration is further increased (above ~ 0.6mM), SDS molecules interact with the protein through hydrophobic interactions and replace the bound TNS. The TNS molecules released from the protein surface to the solvent reduces its fluorescence intensity. Above 6 (±0.5) mM of SDS, the protein's surface might be completely occupied with SDS micelles and further increase in the surfactant concentration does not provide any conformational change on the protein. In these concentrations, free micelles of SDS (not interacting with the protein) also could be found in the free solvent. TNS might bind to these free micelles, thus its fluorescence is not altered compared to its fluorescence in the absence of HEWL. Far-UV CD spectra (Fig. 9A) demonstrate that up to ~1.5 mM of SDS, the secondary structure content of the protein is increased. It could be due to the property of SDS to induce helicity of lysozyme at lower concentrations.[47] Increase in SDS concentrations above 1.5 mM destabilizes these additional secondary structures. Further increase in the concentration of SDS (>6.5 mM in 0 M urea; >6 mM in 2 M urea; >5 mM in 4 M urea) does not affect the secondary or tertiary structure of the protein. By comparing these results with the earlier studies[26-32] on lysozyme-SDS interactions, it could be inferred that lysozyme interacts with SDS through ionic interactions at the concentrations below 0.5 (±0.1) mM and it initiates protein-associated early micelle formation of SDS (critical aggregation concentration-CAC[12,48]). Above this concentration of SDS, hydrophobic interactions predominate between lysozyme and SDS molecules which affect the tertiary structure of the protein. These tertiary structural changes are accompanied with the change in helicity of the protein. Such protein-SDS complexes, including for lysozyme, are well characterized by various techniques.[30,37,49,50] Though the presence of urea decreased the concentration for the complete protein-micelles complex formation, it has insignificant effect on critical aggregation concentration. Moreover, the slopes of

denaturation curves are similar in the presence and the absence of urea (Fig. 7A) suggesting that urea at the studied concentrations has less interference on the SDS interactions with the protein.

Protein-induced early aggregation is also observed in CTAB (Fig. 7B). In the case of CTAB, the slope of the denaturation curve is similar in all the urea concentrations, but the CAC is notably higher at higher urea concentrations. In 2 M and 4 M urea, the CAC values are 0.2 and 0.4 mM, respectively compared to 0.1 mM in the absence of urea. Urea also increases the required concentration of CTAB to form the complete micelles around the protein. It is evident from the saturation of lysozyme fluorescence which occurs at 1.0 and 1.5 mM of CTAB in 2 M and 4 M urea, respectively, whereas in the absence of urea it is observed at 0.8 mM of CTAB. These results are further verified with change in TNS fluorescence in CTAB upon addition of HEWL (Fig. 8B). In the absence of urea at above 0.1 mM of CTAB, TNS fluorescence increases with increasing CTAB concentration upon addition of HEWL suggesting the partial exposure of the hydrophobic residues of the protein. The CTAB concentration at which the TNS fluorescence starts increasing is shifted to higher surfactant concentrations in the presence of urea (0.2 and 0.4 mM in 2 and 4 M urea, respectively). This might be due to the simultaneous ionic and hydrophobic interactions of CTAB on the protein surface at lower surfactant concentrations which could be affected by urea. The studies[29,31] on the interaction of lysozyme with CTAB and dodecyltrimethylammonium bromide (DTAB, a cationic surfactant similar to CTAB) using calorimetric methods propose that initial interaction between HEWL and cationic surfactants at lower concentrations is due to the simultaneous electrostatic and hydrophobic interactions and at higher concentrations only hydrophobic interactions predominate. These interactions expose some of the hydrophobic sites of the protein which lead to conformational changes.

This could be correlated with the spectroscopic changes observed for HEWL in the presence of CTAB. In the absence of urea at above 0.8 mM of CTAB, TNS fluorescence is decreased. This might be due to the occupation of exposed hydrophobic sites of HEWL by CTAB through hydrophobic interactions by replacing the bound TNS. The similar effect is observed in the presence of 2 M and 4 M urea as well at the concentrations of CTAB above 1.0 and 2.0 mM, respectively. Further, CTAB does not show any significant secondary structural changes in the protein, except in the presence of 4 M urea. In 4 M urea, it is noted that CTAB slightly increases the secondary structure content of the protein which is destabilized on further increase in the surfactant concentration. These results suggest that lysozyme-CTAB interaction majorly affects the tertiary interactions only.

Tx, a non-ionic surfactant, also showed a cooperative transition which might correspond to the tertiary structural changes of the protein. Interestingly, urea has no notable effect on the CAC of Tx in lysozyme which is 0.2 mM in all the cases and it does not affect the concentration of complete micelle formation by Tx around the protein (at 2 mM) as well. In other words, the presence of urea has little or no effect on the protein – non-ionic surfactant interaction. Moreover, the change in TNS fluorescence intensity in Tx upon addition of HEWL is minimal both in the presence and the absence of urea. This suggests that the extent of protein denaturation is minimal in the presence of Tx. In the presence of all the studied surfactants, fluorescence emission maximum was observed at around 350 nm. The absence of any wavelength shift during the titrations against surfactants suggests that the extent of unfolding is less and the exposure of hydrophobic tryptophan residues to the solvent is minimal. This corroborates with the less or no changes observed in secondary structure content of the protein in the surfactants.

4.3. Effect of Surfactants on Fibril Formation

Change in fibrillation of lysozyme in the presence of surfactants can be broadly categorized into ionic and non-ionic surfactants effects. In the presence of ionic surfactants, SDS and CTAB, the fibrillation propensity (formation or inhibition) of lysozyme is similar, though the rates of fibril formation are different. In 2 M urea solution, lysozyme forms fibrils with nucleation phase of ~2.1 h and elongation rate of ~1.6 h^{-1}. Addition of SDS at lower concentrations where ionic interaction is predominated (<0.4 mM SDS), the nucleation period is marginally increased. However, at higher concentrations where the charge neutralization is complete and the tertiary structures are also slightly altered the nucleation time is reduced gradually. Addition of CTAB at lower concentrations where they do not show any tertiary structural changes also (<0.2 mM CTAB) extends the lag phase, but the change in fibrillation rate is minimal. In 2 mM SDS, the protein exceptionally had a shorter lag phase and slower fibrillation rate. At near the CMC of CTAB and above the CMC of Tx, the lag phase is reduced compared to the lower concentrations, but still longer than the time observed in the absence of surfactants. The fibrillation rate shows insignificant changes in these conditions. Overall, the initial binding of the surfactant molecules on the protein's surface residues increases the nucleation period. Binding of more surfactant molecules which alters the tertiary structures by hydrophobic interactions reduces this lag phase. By comparing the effect of different surfactants, it is clear that the surfactant having opposite charge (SDS) to the protein surface charge shows relatively less nucleation time. These results suggest that surface charge neutralization of the protein by SDS might reduce the initial lag phase, whereas similarly charged and uncharged surfactants mostly interacts with the hydrophobic surfaces extend the nucleation time.

In the complete micelle forming concentrations, both SDS and CTAB inhibit the fibril formation. Addition of non-ionic surfactant does not inhibit the fibrillation of the protein, but it increases the lag phase. Surfactants are known to form micellar-like aggregates[49-51] around protein by interacting with the protein through their hydrophobic tails and exposing their charged head groups to the solvent, in the case of ionic surfactants. This charged surfaces of the protein-ionic surfactant complexes might reduce the interaction between the individual protein chains, thus inhibit the fibril formation. Nevertheless, the non-ionic surfactant cannot produce such a charged surfaces might lead to fibril forming aggregates. Earlier studies have shown that SDS would induce amyloid fibril formation of lysozyme at lower concentrations and above the CMC it inhibits fibrillation.[12,21] Also, SDS is found to evade the lag phase of lysozyme fibrillation at pH 2.0[12] and of apolipoprotein C-II at pH 7,[52] and facilitate the direct elongation phase. Further, increase in ionic strength of the solution is also shown to alter the mechanism of lysozyme fibrillation.[15] Though our results do not show complete abstain of nucleation phase, at near CMC, SDS shows shorter nucleation time. This might be due to the varying surface charge distribution of the protein at different pHs which could alter its interaction with SDS. SDS is also shown to induce fibrillation of other proteins as well at lower concentrations by converting α-helical structures of the proteins into β-sheets.[53-55] Recently, Khan *et al*.[56] has shown that 0.5 mM SDS induces aggregation of 25 different proteins, including lysozyme at the pH two units less than their pI. Also, molecular simulation studies on amyloid-β peptides suggest that the change in ionization states of charged amino acid residues due to change in solution pH could alter the electrostatic interactions within and between the peptides chains, thus influence fibrillation propensities of the peptides.[57,58]

Comparing these observations with our results reiterates the fact that surface charge neutralization by ionic surfactants could reduce the nucleation time, but the occupation of hydrophobic sites by the hydrophobic tail of similarly charged surfactant or non-ionic surfactant would show an inverse effect. Thus, it could be concluded that the nucleation phase of lysozyme fibrillation is mostly influenced by the charge interactions and micellation of the surfactant around the protein.

In 4 M urea conditions where no lag phase is observed, the initial binding of SDS to the protein slowed down the fibrillation rate, but accelerated by the further addition of SDS. Higher concentrations of CTAB and Tx are shown to reduce the rate of fibrillation. Though the exact reasons for these variations could not be deduced from the limitations of our experiments, the effects observed in these cases propose that the fibril elongation phase might be controlled by the additional factors[4,52] such as exposure of protein's hydrophobic residues, change in surfactant binding sites and their structural features. Moreover, the studies on the effect of amphiphiles and other small molecules on protein fibrillation[52,59] suggest that these molecules could have distinctive effects on different stages of protein fibrillation and aggregation. Therefore, it could be concluded that the different stages of lysozyme fibrillation, nucleation and elongation, are differently influenced by the surfactants. Comparing the kinetic profiles obtained in 2 M and 4 M urea conditions indicates that the surfactants do not switch the fibrillation mechanism from nucleation-dependent into nucleation-independent or the other way around, though the formation of protein-ionic micelle complexes inhibit fibril formation at all the conditions. Non-ionic micelles do not inhibit the fibrillation, but can affect the fibrillation process at each phase.

Interestingly, the fibrillation rate is five-fold slower in the presence of 4 M urea compared to the fibril elongation rate in 2 M urea. In the case of 2 M urea samples also where the shortest nucleation time is observed (in 0.2 mM of SDS) the elongation rate is reduced. This suggests that the initial nucleus formation before fibril elongation may accelerate the fibrillation rate. Nevertheless, considering the distinctive effects of surfactants on different phases of fibrillation and the urea-induced change in fibrillation mechanism, this assumption needs to be verified with further experiments on lysozyme fibril formation in similar conditions and with other fibril forming proteins as well.

5. Conclusions

Fibrillation of HEWL was achieved at neutral pH using mild denaturation with urea and temperature. Effect of ionic and non-ionic surfactants on each step of the fibril formation was investigated. All of the surfactants shows increase in the nucleation time of the protein, but the extent of increase is less at near the CMC of ionic surfactants and at above the CMC of non-ionic surfactant. This predicts that the lag phase is mainly controlled by the ionic interactions and the micellation property of the surfactants. The fibril elongation rate is not generally affected in 2 M urea conditions and has differential effects in 4 M urea. This indicates that the elongation phase is influenced by multiple factors. Nevertheless, ionic surfactants inhibit the fibril formation at above their CMC, but non-ionic surfactants could only extend the time for nucleation and the elongation.

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Figure Captions

Fig. 1 Cartoon diagram of hen egg white lysozyme (PDB id: 2HUB). Helices and sheets are shown in red and yellow, respectively. Tryptophan residues are shown as sticks in blue color.

Fig. 2 HEWL fibrils formed at pH 7. (A) ThT fluorescence spectra in lysozyme in the absence (black) and the presence of 2 M (cyan) and 4 M (red) urea measured before (dotted lines) and after (solid lines) heating the samples at 60 ^{o}C for 15 h. Inset is the photographic image of the final protein sample in (1) 0 M, (2) 2 M and (3) 4 M urea. (B-D) TEM images of lysozyme in the absence and the presence of urea after heating for 15 h.

Fig. 3 Effect of surfactants on lysozyme fibrillation in 2 M urea. ThT fluorescence spectra were measured before (dotted lines) and after (solid lines) heating the samples at 60 ^{o}C. ThT fluorescence in the presence of (A) 0 (black), 0.1 (cyan), 1.0 (blue), 10 (red) and 20 mM (gray) of SDS, (B) 0 (black), 0.05 (cyan), 0.2 (blue), 5 (red) and 10 mM (gray) of CTAB, and (C) 0 (black), 0.01 (cyan), 0.2 (blue), 2 (red) and 5 mM (gray) of Tx. (D-F) TEM images of the fibrils formed in the presence of 0.2 mM SDS, 0.5 mM CTAB and 5 mM Tx, respectively.

Fig. 4 Effect of surfactants on lysozyme fibrillation in 4 M urea. ThT fluorescence spectra were measured before (dotted lines) and after (solid lines) heating the samples at 60 ^{o}C. ThT fluorescence in the presence of (A) 0 (black), 0.1 (cyan), 2.0 (blue), 10 (red) and 20 mM

(gray) of SDS, (B) 0 (black), 0.05 (cyan), 0.2 (blue), 5 (red) and 10 mM (gray) of CTAB, and (C) 0 (black), 0.01 (cyan), 0.1 (blue), 2 (red) and 5 mM (gray) of Tx. (D-F) TEM images of the fibrils formed in the presence of 0.2 mM SDS, 0.5 mM CTAB and 5 mM Tx, respectively.

Fig. 5 Kinetics of HEWL fibril formation followed by change in ThT fluorescence in (A-C) 2 M and (D-F) 4 M urea in varying concentrations of SDS, CTAB and Tx. The concentrations of surfactants are shown in each figure in the color corresponding to their kinetic curves. In the higher concentrations of SDS (A and D) and CTAB (B and E), lysozyme does not show any increase in fluorescence intensity.

Fig. 6 (A) Lag time and (B & C) elongation rate of HEWL fibril formation in varying concentrations of SDS (black), CTAB (green), and Tx (blue) in the presence of 2 M (A and B) and 4 M (C) urea followed using ThT fluorescence (Figure 5) and calculated using equation 1. The red squares represent the corresponding kinetic parameter in the absence of any surfactant. The numerical values of data are presented in Table 1. The horizontal lines represent the CMC of surfactants, SDS (black), CTAB (green) and Tx (blue) at the corresponding urea concentrations.

Fig. 7 Change in fluorescence emission of lysozyme at 350 nm with increasing concentrations of (A) SDS, (B) CTAB, and (C) Tx in the absence (gray circles) and the presence of 2 M (red triangles) and 4 M (cyan squares) urea.

Fig. 8 The difference in fluorescence intensity of TNS before and after the addition of the protein in varying concentrations of (A) SDS, (B) CTAB, and (C) Tx in the absence (gray circles) and the presence of 2 M (red triangles) and 4 M (cyan squares) urea. The actual fluorescence intensity changes of TNS are presented in Figure S8 in ESI†.

Fig. 9 Ellipticity changes of the protein at 220 nm measured in increasing concentrations of (A) SDS and (B) CTAB in the absence (gray circles) and the presence of 2 M (red triangles) and 4 M (cyan squares) urea.

Fig. 10 (A) Far-UV CD spectra of lysozyme in the absence (gray) and the presence of 2 M (red) and 4 M (cyan) urea measured at room temperature (solid lines) and at 60 ^{o}C (dotted lines). (B) Thermal denaturation of lysozyme followed by ellipticity change at 220 nm in the absence and the presence of 2 M and 4 M urea.

Table 1. Lag time and elongation rate of lysozyme fibrillation

surfactant	concentration (mM)	2 M urea		4 M urea‡
		lag time (min)	rate (min^{-1})	rate (min^{-1})
-	0	125.4	0.027	0.0053
SDS	0.1	188.2	0.019	0.0011
	0.2	136.2	0.037	0.0018
	2	67.4	0.005	0.0074
CTAB	0.01	398.5	0.025	0.0065
	0.05	386.2	0.029	0.0076
	0.2	278.9	0.034	0.0076
	0.7	315.4	0.020	0.0031
Tx	0.001	367.0	0.021	0.0059
	0.01	421.3	0.021	0.0050
	0.1	422.2	0.020	0.0049
	0.2	308.9	0.028	0.0025
	0.5	282.3	0.036	0.0029
	2	307.6	0.028	0.0022
	5	313.8	0.031	0.0023

‡ - no lag phase is observed

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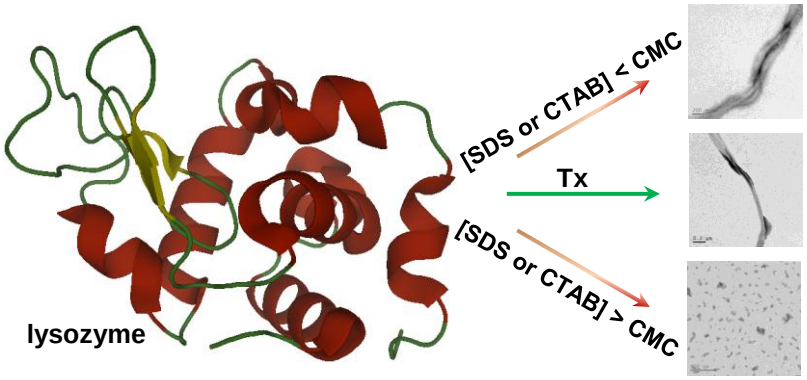

Micellar concentrations of ionic surfactants inhibit lysozyme fibrillation, but not the non-ionic surfactant