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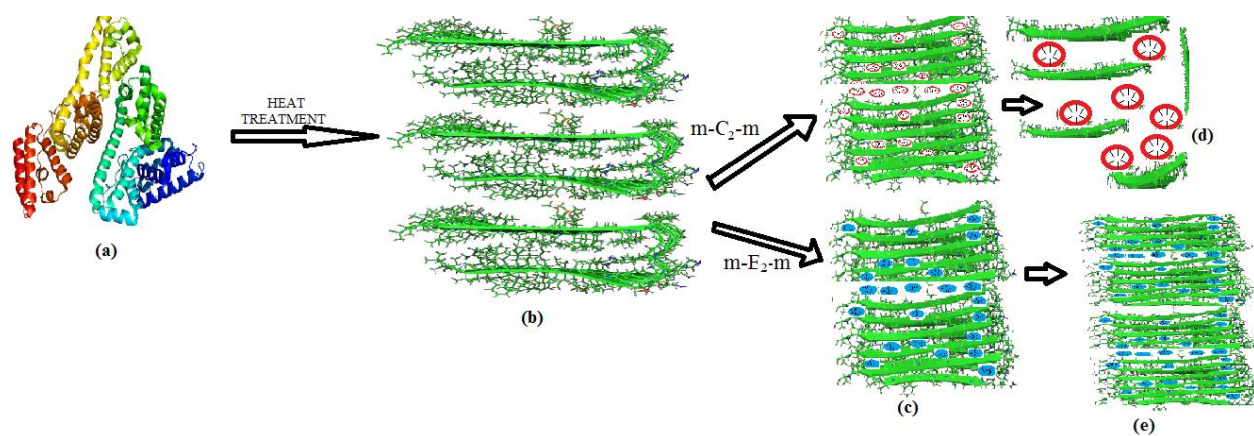


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Modulation of Bovine Serum Albumin fibrillation by Gemini surfactants

**MODULATION OF BOVINE SERUM ALBUMIN FIBRILLATION BY ESTER
BONDED AND CONVENTIONAL GEMINI SURFACTANTS**

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Protein fibrillation has been associated with various neurological disorders. Knowledge about molecular mechanisms of protein aggregation modulators is potentially helpful for therapeutic purposes. In-order to find out the key strategies for promotion and inhibition of protein fibrillation and to create better functional designs, we have studied the effect of gemini surfactants on the bovine serum albumin fibrillation. The ThT fluorescence emission spectrum indicates disintegration of BSA fibrils in presence of m-C2-m gemini surfactants (conventional). However, enhancement in BSA fibril formation in presence of m-E2-m diester bonded gemini surfactants. Although, with increase in tail length of m-E2-m gemini surfactant, slight disintegration of fibrils also occurs. Circular dichroism data suggest decrease in the β -content of the BSA fibrils in presence of m-C2-m surfactant, while as increase in β -content of fibrils in presence of m-E2-m surfactants. FTIR, TEM, and confocal microscopic results also show that m-C2-m disintegrates and m-E2-m prompts the fibrillation. The electrostatic/hydrogen bonding/hydrophobic balance play important roles in determining the BSA fibrillation and disintegration. Micelles of m-C2-m surfactants disrupt the hydrogen bonding in between the β -strands, hence result in fragmented fibrils. Presence of diester group in m-E2-m surfactant forms hydrogen bonds in between the β -strands resulting in enhancement of fibril formation.

1 Introduction

Self aggregation of proteins and peptides leads to different morphological oligomers under various biophysical circumstances. This aggregation results in fibril formation.¹⁻⁴ Protein self-assembly have been closely associated with various neurodegenerative diseases such as Prion, Parkinson's, Huntington's and Alzheimer's.^{5,6} Appearance of amyloid β -peptide ($A\beta$) in the patients is feature of Alzheimer's disease. A strong correlation between the appearance of fibrillar plaques and disease progression has been shown, and also suggested that early intermediate aggregates are more toxic.^{7,8} Presently, no effectual treatment can avoid neurodegenerative diseases in humans, and existing drugs can only slightly assuage the symptoms.⁹

Amyloid fibrils are thermodynamically stable,¹⁰ and hence the conversion of these fibrils into small fragments does not occur of its own. Therefore, in the recent past several approaches have been explored to break, destabilize and/or disrupt amyloid fibrils.¹¹ Ultrasonification,¹² high temperature (above 100 °C) and pH change results in fibril destruction.^{13,14} Electrostatic repulsive force needed to hold neighboring proto-fibrils together have been blocked by addition of strong ionic liquids, leading to destabilization of amyloid fibrils.¹⁵ Several studies have also been devoted to the effect of lipids on $A\beta$ aggregation pathway,^{16,17} as in vivo lipids are known to be associated with amyloid fibrils.^{18,19} On the same lines, surfactants like SDS (sodium dodecylsulphate) and LiDS (lithium dodecylsulphate) have been chosen as model systems for studying amyloidogenic co-aggregate intermediate forms, as these surfactants favor β -rich structures.²⁰ Ruhs et al.²¹ studied the interaction of sulphonic acid polyethylene glycol (surfactant) with amyloid fibrils. The synergistic interaction between the fibrils and polyethylene glycol chains reduces the entropy of amyloid fibril structure and converts it into amorphous globular ending structure. Sabate et al.²² have reported that alkylammonium surfactants below their cmc (critical micelle concentration) favor fibril formation and above the cmc can delay $A\beta$ amyloid fibril formation, however, no idea about disintegration of fibrils. Han et al.⁹ proposes that gemini surfactant, $C_{12}C_6C_{12}$. Br₂- hexamethylene-1,6-bis(dodecyldimethylammonium bromide), has stronger interaction with amyloid fibrils based on the thermodynamical profile. Cao et al.²³ studied the effect of surfactant concentration on the dynamics of $A\beta$ fibrillogenesis. All these studies suggest that surfactants represent one of the important categories of molecules which

modulate the amyloid fibrils. In spite of various studies, much remain to be elucidated regarding the structure of surfactant molecules, especially of gemini surfactants, on the inhibition and formation of amyloid fibrils.

Gemini surfactants possess two hydrophilic head groups covalently connected by a spacer. They have lower cmc and stronger ability to self-aggregate as compared to conventional surfactants. The motivations in picking the cationic gemini surfactants for modulating protein aggregation are (a) their structural analogy to complex cationic lipids, hence, represent the molecular aspect of membranes in amyloid formation²⁴ (b) they can serve as drug delivery carriers.²⁵ Recently, our group have revealed the gene transfection efficiency of biocompatible diester bonded gemini surfactants.²⁶ These surfactants also show strong ability in stabilizing the aggregated form of bile salts and organic hydrotopes.^{27, 28} In the present study, we have chosen two sets of gemini surfactants (a) m-E2-m (E2 represents diester spacer) and (b) m-C2-m (C2 represents conventional spacer with two methylene groups), where m=12, 14 and 16, depicts the number of carbon atoms in surfactant tail. Observably, the chosen surfactants have different structure as shown in Scheme 1. The main objective is to reveal the mode of binding of these surfactants with BSA fibrils. Secondly the effects of alkyl tail length and the nature of spacer in surfactants on the BSA fibril modulation. To get a deeper perceptive of the interaction, a series of techniques have been used to obtain broader and more information. These studies shed light on the hydrophilic/hydrophobic effects which play important role in bringing about reflective perturbations in secondary structure of BSA fibrils. Also, it is quite interesting to analyze the effect of diester groups in the spacer of m-E2-m gemini surfactants on the BSA fibrils.

2 Experimental section

2.1 Materials.

Bovine Serum Albumin (BSA) and Thioflavin T (ThT) were purchased from Sigma Chemical Co. (St. Louis, USA) and used as received.

Synthesis of gemini surfactants. Following the proper procedure as ascribed in the literature^{29–32} for the synthesis of m-C2-m 2Br- and m-E2-m 2Cl⁻ Gemini surfactants. Further characterizations of surfactants were done by ¹H NMR and FT-IR, and the data agrees well

with the reported values. Purity of surfactants was also confirmed by non appearance of minima in their surface tension profiles.³³

Preparation of BSA fibrils. Solution of BSA protein in phosphate buffer at pH 7.4 was prepared and the concentration was measured (molar extinction coefficient $\epsilon_{280}=6.5 \text{ M cm}^{-1}$).³⁴ 150 μM of BSA solution was incubated for 6 hr at 65°C in 60 % (v/v) ethanol and then followed by incubation for 2 days at room temperature,³⁴ hence, leads to the formation of BSA fibrils.^{35,36} Further dilution of BSA fibrils was done with phosphate buffer to achieve a final concentration of 30 μM . Gemini Surfactants were added at a concentration of more than 20 times of their respective cmc values to assure that they exist as micelles, taking into fact that cmc values may get slightly altered in buffer solutions.³⁴ Mixtures were shaken thoroughly and kept at room temperature for 2 days.

2.2 Measurements.

ThT fluorescence assay. ThioflavinT (ThT–benzothiazole dye) exhibits increase in fluorescence emission intensity at 490nm upon binding with amyloid fibrils. However, the ThT does not bind to non amyloid structures as well as fragmented fibrils.³⁷ Thioflavin T fluorescence of the fibril-surfactant solutions were measured using Shimadzu spectrofluorimeter-5000 (Japan). Aliquots of BSA fibrillar solutions with and without surfactants were diluted using phosphate buffer to achieve final protein and ThT concentrations of 1 μM and 5 μM , respectively. Samples were excited at a wavelength of 450 nm with a scanning range of 470-580 nm.

Circular dichroism measurements. Far UV-CD spectra were recorded on Applied Photo-physics (U.K.) (model CIRASCAN) spectrophotometer, equipped with a Peltier temperature controller. Samples were taken in rectangular quartz cuvette of path length 10 mm, and the concentration of fibrils in each sample was 5 μM . Smoothing of the spectra were done by the Savitzky–Golay method with 25 convolution widths. The numerical estimations of secondary structure content of the fibrillar protein with and without surfactants were obtained by using the DichroWeb online CD analysis software server,³⁸ employing the CONTINLL analysis program^{39,40} along with ‘Set#4’,^{41,42} reference set in DichroWeb.

Fourier transform infrared spectroscopy (FTIR). A Shimadzu-8300 FTIR spectrophotometer was used to record the spectra. 20 μL aliquot of samples was loaded between two flat CaF_2 windows with a path length of 20 μm .

Transmission electron microscopy. BSA fibrillar suspensions with and without surfactants were diluted 10 fold and applied to carbon coated TEM grids. Excess of fluid was removed after 2 min and the grids were then negatively stained with 2% (w/v) uranyl acetate and scanned in JEM-2100F (JEOL, JAPAN) transmission electron microscope operating at an accelerating voltage of 200 kV. Length and width of BSA fibrils were then quantified by using Image J software.

Confocal microscopy. Specimens for fluorescence imaging was prepared by incubating 10 μL of sample solutions with 5 μL of 1 mM ThT to achieve the required staining. Images were recorded in a Nikon Real Time Laser Scanning Confocal Model A1R Microscope equipped with Spectral imaging Model Ti-E.

3 Results

ThT has been widely used to study fibril formation in a number of proteins aggregates including α -synuclein, $A\beta$ amyloids, lysozyme, and $\beta 2\text{m}$. ThT molecules bind to the “channels” formed in β -sheets due to the interactions between the backbone C=O from one strand and N-H from the other strand that form the fibrils.⁴³ The extent of ThT binding to amyloid samples depends on the accessibility of these binding grooves. Once β sheet starts forming, ThT binding is initiated, and it increases in proportion to the content of β -sheet in the system. The aggregation of $A\beta$ fibrils in the absence of surfactant shows sigmoid ThT fluorescence trace, Fig. 1a. Gemini surfactants m-C2-m show significant decrease in the ThT fluorescence intensity. The order of decrease in the intensity of ThT is: 12-C2-12 < 14-C2-14 < 16-C2-16, although the difference in lowering of fluorescence intensity between these gemini surfactants is very less (Fig. 1b). The reduction observed in the ThT intensity implies that there is a decrease in fibrillar species present in solution. Within this set of surfactants, 16-C2-16 represents the best one in disintegration of fibrillar species. However, the gemini surfactants m-E2-m show interesting results, in which 12-E2-12 and 14-E2-14 increase the binding grooves for ThT binding on amyloid fibrils, which result in increase in the ThT fluorescence intensity as compared to that of fibrils without surfactants (Fig. 1b).

Furthermore, with increase in the hydrophobic tail length of m-E2-m surfactants, as in case of 16-E2-16, there is 11% reduction in the ThT fluorescence intensity with respect to the fibrils without surfactant.

We have also applied circular dichroism spectroscopy for exploring the secondary structural changes of BSA fibrils in presence of surfactants. Proteins dominant with α -content and $\alpha+\beta$ content proteins exhibit two negative peaks, one at 208 nm and other at 222 nm, while β -content proteins show a single negative peak between 215 nm and 222 nm. From Fig. 2, it is quite evident that BSA fibrils and BSA fibrils in presence of m-E2-m surfactants show only a single negative broad peak in between 212nm to 220 nm, indicating the dominance of β -sheets. However, in presence of m-C2-m, the BSA fibrils show two negative peaks (208nm and 223nm), confirming the presence of $\alpha+\beta$ content. Table 1 summarizes the results of secondary structural changes in BSA fibrils in presence of different surfactants. The CD data depicts that BSA fibrils are rich in β -sheets. In case of BSA fibrils in presence of m-C2-m surfactants, there is decrease in the β -sheet and increase in α -helix. In presence of m-E2-m surfactants, there is increase in the β -sheet content of the BSA fibrils. These results suggest the disintegration of BSA fibrils in presence of m-C2-m surfactants while as m-E2-m assists fibril formation, which are in total agreement with fluorescence results.

FT-IR spectroscopy has been extensively used to analyze amyloid formation⁴⁴, as it enables secondary structural fluctuations from the initial monomer to the amyloid formation. A prominent peak at approximately 1654cm^{-1} is characteristic feature of albumin proteins. This band can be attributed to the amide I modes of these predominantly α -helical proteins.⁴⁵ Fig. 3 shows FTIR spectra of BSA fibrils and BSA fibril–surfactant complexes. BSA fibrils and complexes of BSA fibrils +m-E2-m surfactants does not show band in vicinity of 1650cm^{-1} . However, there is appearance of band near 1650cm^{-1} in BSA fibrils + m-C2-m surfactant systems. These results are in agreement with CD results, which clearly indicate that there is increase in α -helical content in presence of m-C2-m surfactants.

Transmission electron microscopy (TEM) was used to verify the effect of the surfactants on the fibrillar morphology, during the formation and disintegration process. The BSA fibrils and fibrils treated with surfactant were subjected to negative TEM analysis. Fig. 4 represents the morphology of BSA fibrils. The length of the fibrils was measured and found that more

than 65% of populations have an average length of 1000 ± 350 nm with average diameter of 22 ± 4 nm. BSA fibrils treated with m-C2-m type of surfactants show disappearance of the fibrils. More specifically, in 14-C2-14 and 16-C2-16 there is almost complete absence of fibrils, however, the fibrillar density is less in case of 12-C2-12 (Fig 4 b). Various research groups have used negative-stain TEM to estimate the length or width of the $A\beta$ fibrils, which was found to be in quite good agreement with cryo-EM technique.⁴⁶⁻⁴⁸ Considering that the average length and width of 12-C2-12 treated BSA fibril resulted in shorter but similar thickness as that of surfactant free BSA fibrils, indicates these are fragmented type of fibrils. All the surfactants from m-E2-m category show the presence of fibrils when treated with BSA fibrillar solution. 12-E2-12 treated BSA fibrillar solution shows dense fibrillar formation as represented in Fig. 4c. With increase in the tail length, the density of fibrils decreases as in case of 14-E2-14 and 16-E2-16 (Fig. 4d and 4e). The TEM results are well supported by the confocal microscopy images. The presence of BSA fibrils and its morphology in absence and presence of gemini surfactants was also studied via confocal microscopy. A green fluorescence was observed when the ThT-stained fibrils were excited at 450nm using an appropriate filter. CM images initially show ribbon-like fibrils in case of BSA, whereas in presence of m-C2-m show almost complete disappearance of fibrils (Fig. 5). The BSA fibrils treated with 12-E2-12 surfactant show enhancement in the fibrillar density, and elongated fibrils from morphological point of view. In case of 14-E2-14 surfactant, the fibrillar density decreases and is scattered ribbon-like in comparison to that of 12-E2-12, although more than that of BSA fibrils without surfactants. In 16-E2-16 treated BSA fibrils, the density of fibrils is less. These results are in total agreement with that of ThT fluorescence and CD.

4 Discussions

Herein, we demonstrate that m-C2-m gemini surfactants inhibit the BSA fibril formation. Among m-C2-m category, 16-C2-16 represents the most effective in disintegrating the fibril formation. In each case the concentration of surfactant is above than that of cmc, and exist as prolate ellipsoidal in the chosen concentration range.^{49, 50} From TEM analysis it is evident that the BSA fibrils in presence of 12-C2-12 surfactants get shorter in length, hence results in fragmented β -fibrils. Also CD results suggest that there is decrease in β -sheet content in presence of m-C2-m surfactants. In order to explain this phenomenon, we have to focus on

the mechanism of fibril formation by increasing temperature treatment. One of the major theory on protein fibrillation states that the protein only partially unfolds^{51,52} and then forms inter-aligned β -sheets between the partially unfolded patches of protein.^{53,54} This conformational fluctuation initiates the protein fibril formation. Another theory states that protein fibrillation at high temperature involves not only denaturation but also hydrolysis of the protein into peptide fragments, which then assemble into the fibrils.⁵⁵⁻⁵⁷ The secondary structure of the α -helix is formed by the hydrogen bond between the NH and CO groups of the same strand whereas the β -strands form hydrogen bonds with other β -strands. These β -strands stabilize each other through intermolecular hydrogen bonding. At pH 7.4, BSA fibrils carry net negative charge; hence the micelles of dicationic Gemini surfactants m-C2-m get adsorbed on the fibrils via electrostatic interaction. The positively charged head groups of the gemini surfactants intercalate in between the NH terminus and CO terminus of the other strand, hence inhibits their involvement in hydrogen bonding. Thus, m-C2-m micelles pull BSA fibril sheets apart, results in breaking long, thick fibrils into short, fragmented pieces. These results also support that fibrillation of protein on heat treatment occurs via hydrolysis of the protein into peptide fragments, which then aggregate into fibril formation. With increase in the tail length of gemini surfactants their self-aggregation ability increases which, inturn, increases their ability to interact with the hydrophobic tails of the peptide molecules. These findings reveal that both electrostatic as well as hydrophobic forces are a prerequisite for disaggregation of fibril formation but electrostatic interaction plays a primary role. BSA fibrils in presence of m-E2-m surfactants show interesting results. Gemini surfactants 12-E2-12 and 14-E2-14 tend to prompt the BSA fibril formation, 16-E2-16 results in slight disintegration of fibrils. The diester dicationic gemini surfactants (m-E2-m) micelles bind on the BSA fibrils due to electrostatic interactions. However, the two CO groups in the spacer part of gemini surfactant get involved in the hydrogen bonding between the β -strands, hence holding them intact. Therefore, m-E2-m surfactants aids in the BSA fibril formation. With increasing hydrophobicity (by increase in the surfactant tail length, as in case of 16-E2-16), the fibrils get slightly destabilized. The basis for the slight disintegration of fibrillar assembly by 16-E2-16 compared to the others in the m-E2-m series could be ascribed to two factors – lower cmc value³⁰ which is due to its longer hydrophobic tail and larger ellipsoidal dimensions. A comparison of the ellipsoidal dimensions of 16-E2-16 (Semi-major axis $a=50.5 \text{ \AA}$, semi-minor axis, $b=c=18.2 \text{ \AA}$) micelles compared to 12-E2-12 micelles ($a=32.6 \text{ \AA}$, $b=c=13.4 \text{ \AA}$)⁴⁹ indicates that the long hydrocarbon chain would allow better spatial

interaction with the hydrophobic portion of the BSA fibrils thus making it more effective in causing a breakdown of the fibrillar assembly of BSA. Therefore, in m-E2-m gemini surfactants, hydrogen bonding plays a crucial role in prompting the fibril formation, although this efficacy gets faded with increase in hydrophobicity (Scheme 2).

5 Conclusion.

We have investigated the effect of gemini surfactants m-C2-m and m-E2-m on the BSA fibrils by using a broad range of biophysical techniques. All the results suggest that m-C2-m gemini surfactants destabilize the BSA fibrils, and m-E2-m surfactants prompt the fibrillation. Micelles of dicationic m-C2-m gemini surfactants get adsorbed on BSA fibrils via electrostatic and hydrophobic interactions. These micelles inhibit the hydrogen bonding inbetween β -strands, hence disintegrate them into small fragments. However, the presence of diester group in the spacer of gemini m-E2-m involves in hydrogen bonding between the β -strands and keeps them intact, hence aids in fibril formation. Increase in tail length, however, as in 16-E2-16, also tries to destabilize the β sheet framework.

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FIGURE CAPTIONS.

Scheme 1: Structure of surfactants (a,b&c) m-C2-m (d,e &f) m-E2-m.²⁸⁻³¹

Scheme 2: Mechanism of the gemini surfactants disintegration/formation of the BSA fibril.

(a)BSA protein (PDB: 1E7I) (b) BSA fibrils (c) Binding of the gemini surfactants onto the fibril surface. (d) Breaking down of a long fibril into fragmented fibrils. (e) Prompt the BSA fibril formation by m-E2-m surfactants (values are means \pm SD of three experiments).

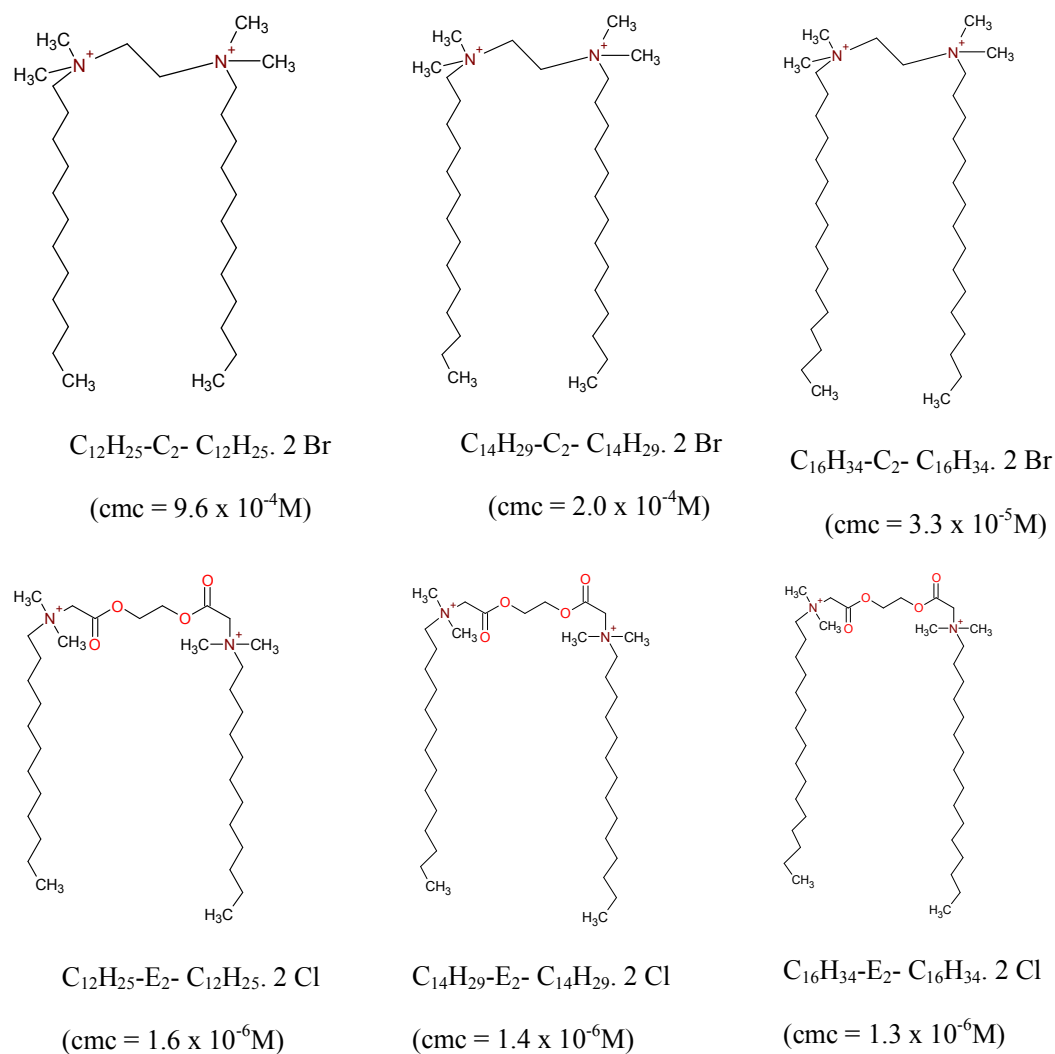
Fig. 1. (a) Representative ThT fluorescence spectra of BSA fibril with and without gemini surfactants (b) histogram of ThT fluorescence intensity change showing the effect of gemini surfactant on the BSA fibrils.

Fig. 2. CD spectrum of BSA fibrils without and with gemini surfactants.

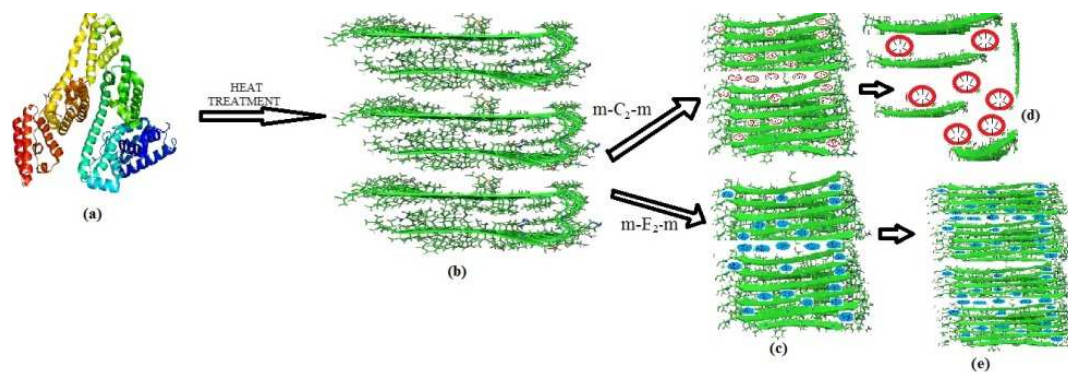
Fig. 3. FT-IR spectrum of BSA fibrils incubated without (*a*) and with 16-E2-16 (*b*), 14-E2-14 (*c*), 12-E2-12(*d*), 16-C2-16(*e*), 14-C2-14 (*f*), and 12-C2-12 (*g*).

Fig. 4. TEM images of BSA fibrils incubated without (*a*) and with 12-C2-12(*b*), 12-E2-12 (*c*), 14-E2-14 (*d*), 16-E2-16(*e*).

Fig. 5. Confocal microscopic images of BSA fibrils incubated without (*a*) and with 12-C2-12 (*b*), 14-C2-14 (*c*), 16-C2-16(*d*), 12-E2-12(*e*), 14-E2-14 (*f*), and 16-E2-16 (*g*). Scale bar of 500 nm.



Scheme 1.



Scheme 2.

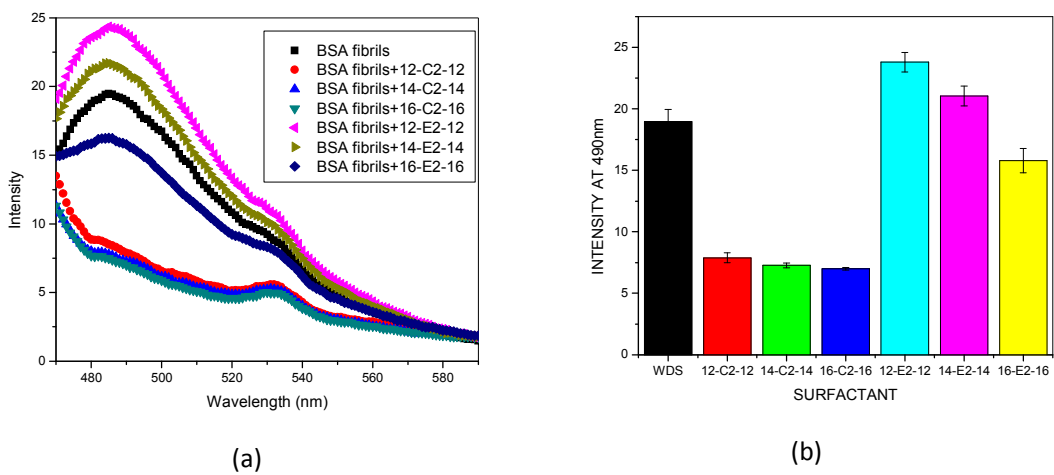


Fig. 1.

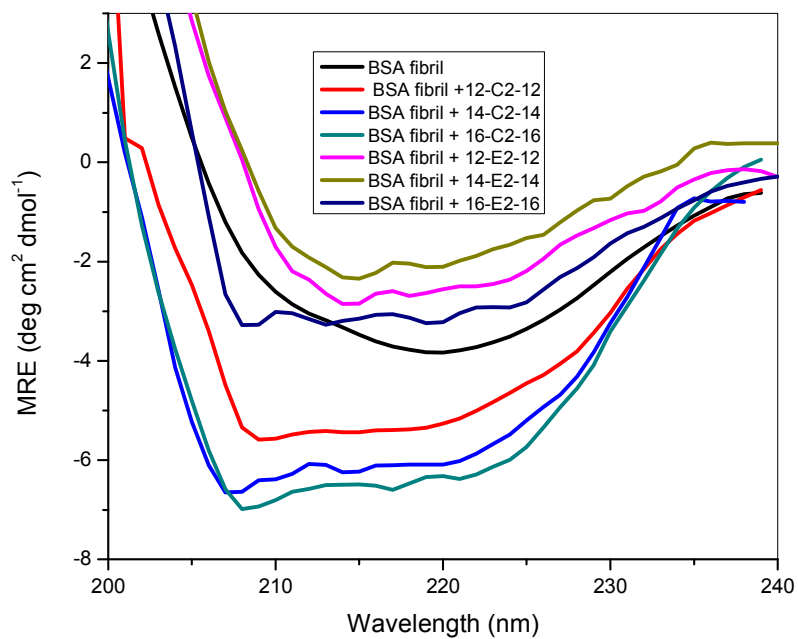


Fig. 2.

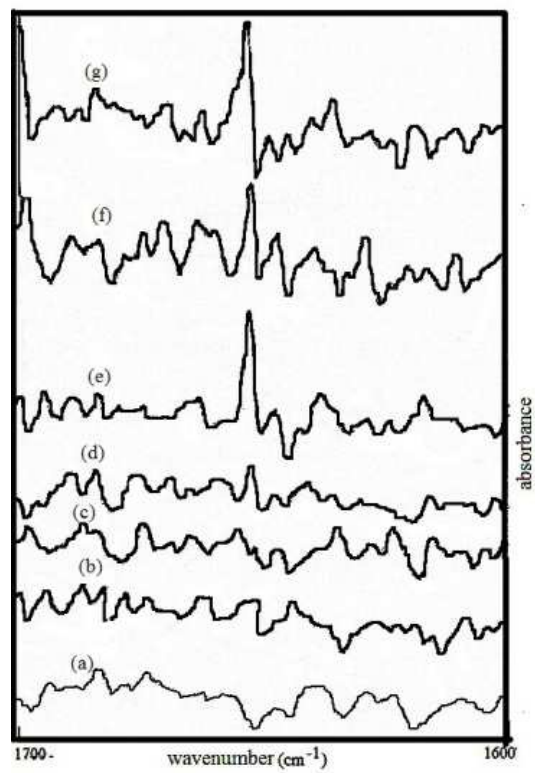


Fig. 3.

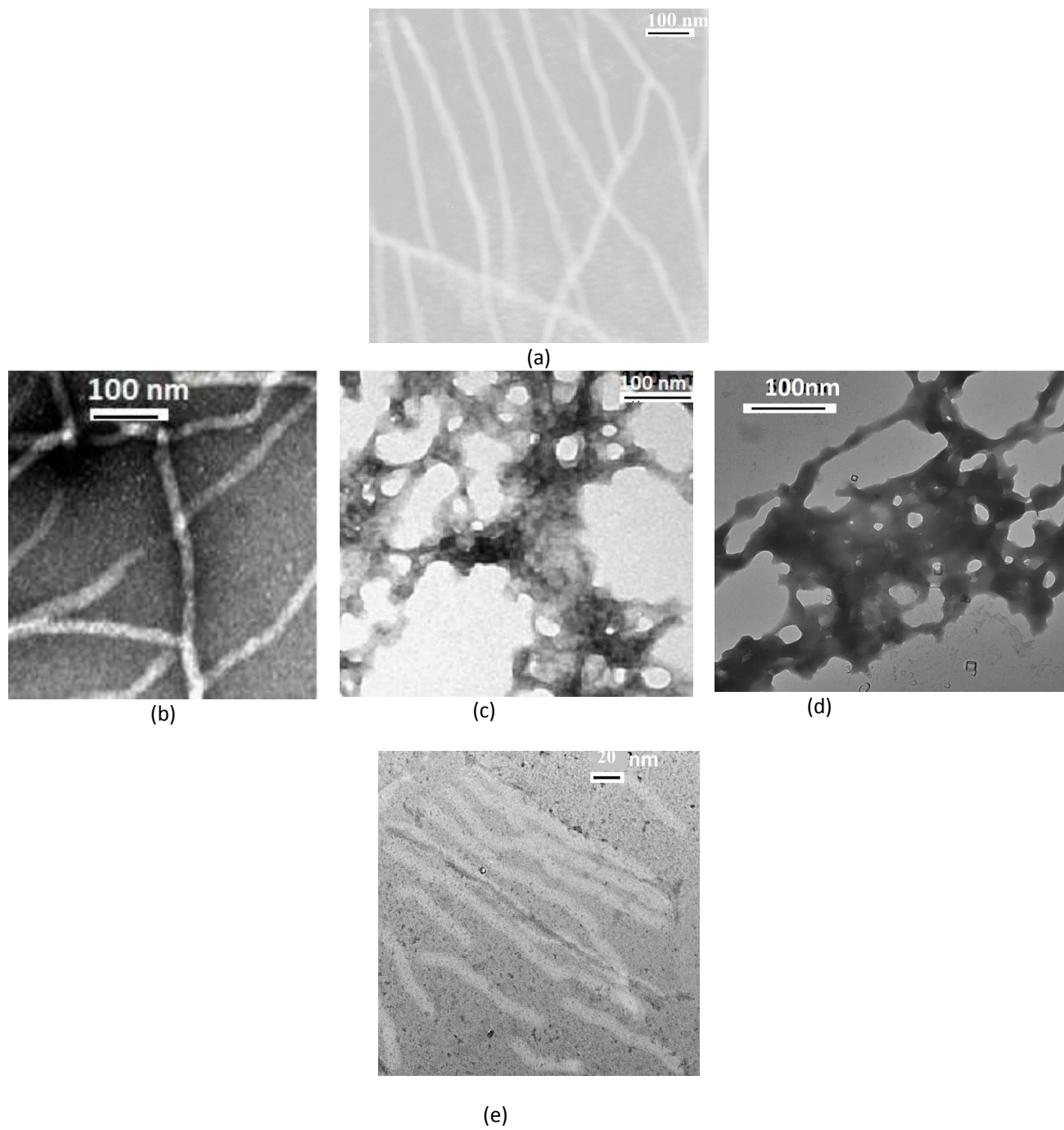


Fig. 4

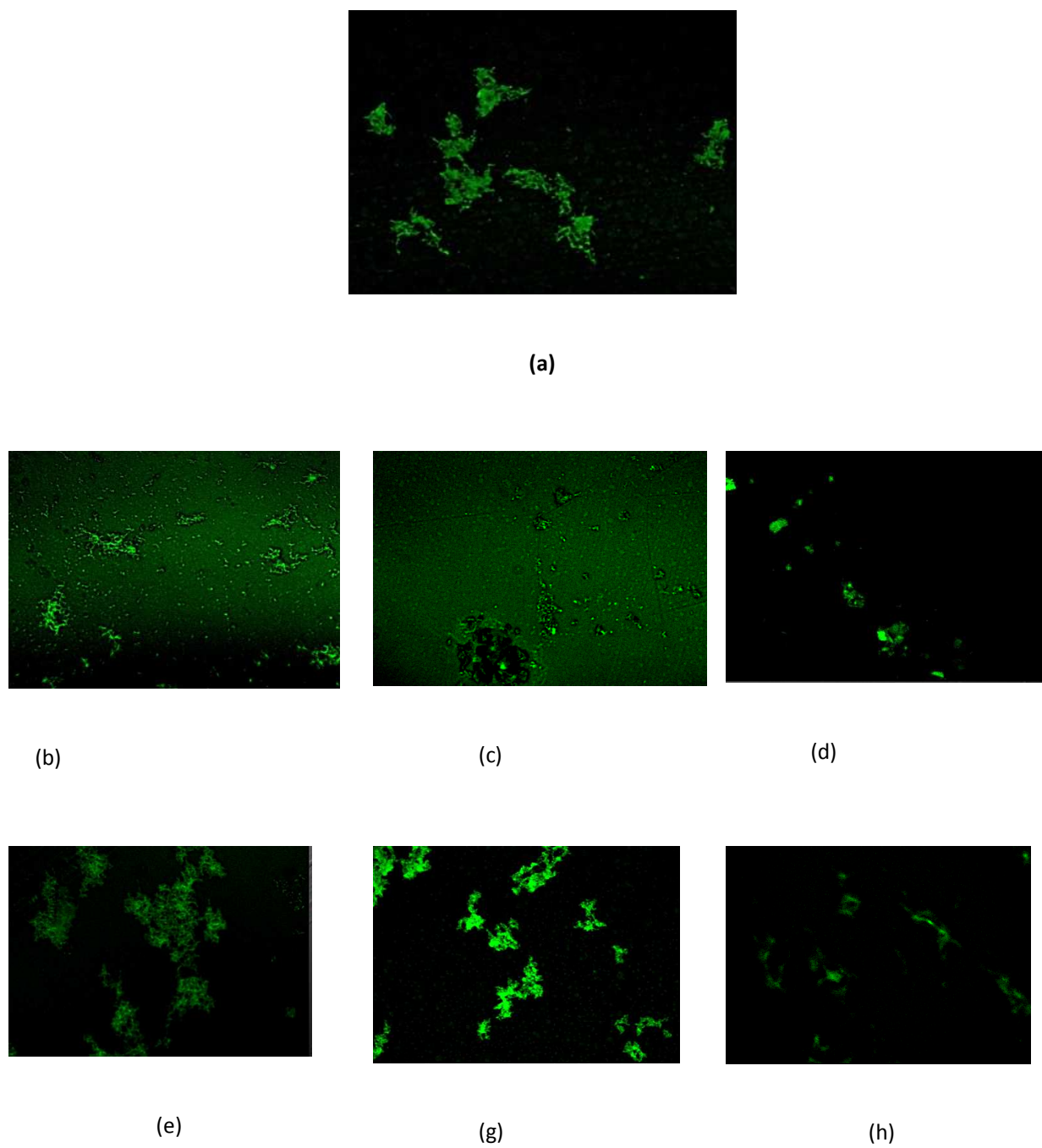
**Fig. 5.**

Table 1: Estimated secondary structure of BSA fibrils in different solutions.

System	α -helix(%)	β -sheet(%)	Random(%)
BSA Fibril	04.33	59.15	20.22
BSA Fibril + 12-C2-12	22.11	09.33	18.11
BSA Fibril + 14-C2-14	23.09	06.55	12.13
BSA Fibril + 16-C2-16	27.33	06.50	12.14
BSA Fibril + 12-E2-12	05.11	61.55	18.22
BSA Fibril + 14-E2-14	05.14	60.33	19.11
BSA Fibril + 16-E2-16	14.12	56.44	12.22