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Cationic gemini surfactant (16-4-16) interact electrostatically with anionic plant lectin and facilitates amyloid fibril formation at neutral pH

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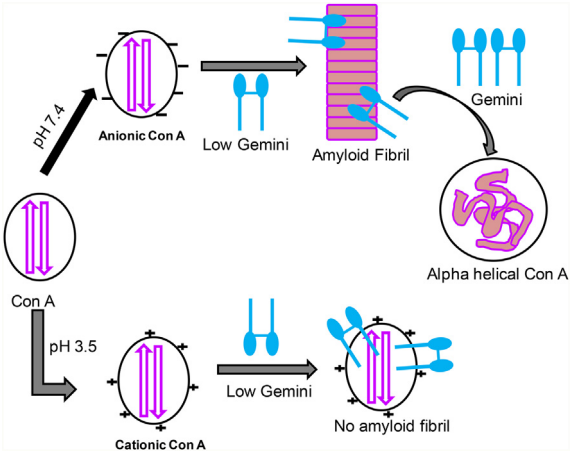
HIGHLIGHTS

- Low concentrations of gemini surfactant induces amyloid fibril in Con A protein.
- Higher concentrations of Gemini surfactant induces alpha helix in beta sheet Con A protein.
- Gemini surfactant interacts electrostaticaly and hydrophobicaly with anionic Con A protein.

GRAPHICAL ABSTRACT



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ABSTRACT

Amyloid are fibrous clumps or aggregates of protein which usually deposited in various organs and tissues which direct their degenration. In neurodenrative disease, these proteincious aggregates leads degeneration of neuronal tissues/organs. In order to develop drug condidate which can dissolve the amyloid fibrils and turned protein functional, it is urgent need to elucidate the mechanism of amyloid fibril formation under different conditions. In this study, we have taken a step to find the mechanism of amyloid fibril formation in concanavalin A (Con A) protein via cationic gemini surfactant (16-4-16) at two different pHs (7.4 and 3.5). We used several biophysical techniques such as Rayleigh light scattering, turbidity, ThT dye binding, intrinsic fluorescence, extrinsic fluorescence, far-UV CD and transmission electron microscopy to characterize the amyloid fibril formation of Con A by cationic gemini surfactant. The results suggest that the Con A form amyloid-like aggregates in the presence of very low gemini concentrations (2.5–125 μM) at pH 7.4 while in the presence of higher concentrations (125–1000 μM),

Abbreviations: ThT, Thioflovin-T; Con A, Concanavalin A; AFM, Atomic force microscopy.

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Con A remained soluble. The Con A was not forming any aggregates or amyloid in the presence of same gemini concentrations at pH 3.5. The possible cause of gemini surfactant-induced amyloid fibril formation of Con A is electrostatic as well as hydrophobic interaction at pH 7.4 and strong electrostatic repulsion at pH 3.5. The far-UV CD spectra of Con A transformed into a cross β-sheet structure when incubated with low gemini surfactants while at higher concentrations the β-sheet structures of Con A transformed into α-helix.

1. Introduction

Amyloid fibrils have a direct connection with almost 30 types of human diseases, and the large number of cases are incurable at some stages. The known amyloid fibril linked diseases are Amyloid Lateral Sclerosis, Huntington's diseases, Perkinson, Diabetes and Alzheimer [1,2]. Amyloid fibrils are fiber like aggregate, unbranched, nano- to micrometer in length and encompass cross-β like structure [3]. For the treatment of amyloid diseases, it is crucial to understand the molecular mechanism of amyloid fibril formation. The amyloid fibril formation mechanism is broadly classified in two ways, i.e. nucleation-polymerization and isodesmic polymerization [4]. Nucleation dependent polymerization begins with the fibril nuclei formation, which is the rate limiting step. However, in isodesmic method, protein directly transformed into mature fibrils without going through fibril nuclei formation [5]. Recently, functional amyloid is also discovered in bacteria, fungi, and human [6]. The functional amyloid fibrils provide extra stability due to tight packing of many fibrils. The inter- and intramolecular interactions are involved in the tight packing of the fibrils. The amyloid fibril formation can be promoted by external factors, i.e. interaction with particular ligand and interaction with the environment [7]. In order to understand the trigger and mechanisms of fibril formation, it is indispensable to study the amyloid fibril formation under *in vitro* conditions. Various *in-vitro* factors, particularly temperature, pH, pressure, salts, interaction with lipid and surfactants are used to induce amyloid fibril in both human and plant proteins [8–10]. In the current work, we have tried to understand the mechanisms of gemini surfactant-induced amyloid fibril formation in Con A lectin.

Usaally, surfactants are used as mimic membrane model in protein-membrane interaction study. These mimic model membrane are very commonly used in pharmaceutical industry like, drug delivery, cosmetics and other biotechnological studies [11]. It is reported that both (cationic and anionic) surfactant intracted with lysozyme at different sites [12,13]. It is also found that these surfactant play significant role in induction of protein fibril along with simulteniously destabilizing proteins and or transforming unstructured into structured conformation [14,15]. Several categories of surfactants i.e. cationic, anionic and hydrophobic are known to induce the amyloid fibril in proteins [16]. The micromolar concentrations of sodium dodecyl sulfate accelerate amyloid fibril formation in recombinant α-synuclein proteins [17]. Cationic surfactants such as cetyltrimethylammonium bromide (CTAB) and dodecyl trimethylammonium bromide (DTAB) are reported to facilitate amyloid fibril formation in many proteins, namely hen egg white lysozyme and beta-amyloid [18,19]. Single chain cationic surfactants (CTAB and DTAB) are extensively used to check the aggregation propensity in various proteins. Only a few reports are available about gemini surfactant-induced amyloid fibril formation in proteins [20]. Gemini surfactant is a dimeric surfactant, consist of two positively charged heads, two sixteen carbon tails and one four carbon spacer groups shown in Fig. 1A. Gemini surfactants are better than available conventional cationic surfactants because of low CMC, unusual viscosity, low Krafft temperature, specific aggregation and high affinity towards oppositely charged surfactants. gemini surfactant effectively interacted with the bovine serum albumin proteins compared to conventional single-chain cationic surfactant (CTAB and DTAB) [21].

The aim of this work to probed the mode of gemini surfactant interaction with plant lectin i.e. Con A at neutral as well as acidic pH. Con A is carbohydrate binding proteins made up of four subunits. Con A is rich in β-sheet structures and possessing one carbohydrate and two metal (Ca^{2+} and Mn^{2+}) binding sites on each monomer of Con A [22]. Con A has a very important biological function i.e. agglutinates erythrocytes and kill the cancerous cells [23].

Con A and other lectin form aggregates at different unphysiological conditions like pH and temperature [24,25].

Understanding the mechanism of Con A aggregation with response to gemini surfactants is very important because of Con A has a similar resemblance to a human serum amyloid protein which is involved in amyloidosis diseases. This will be the first report about gemini surfactant induced amyloid fibril formation in Con A proteins. We used various types of spectroscopic and microscopic techniques to complete this study.

2. Materials and methods

2.1. Materials

Concanavalin A lectin (Con A), Tris–HCl, Thioflavin-T (ThT), and 8-anilino-1-naphthalene-sulfonate (ANS) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Other chemicals were used of analytical grade. Milli-Q water was used throughout the study.

2.2. Protein concentration measurements

Con A stock was prepared in 20 mM Tris-HCl buffer, pH 7.4. The stock concentrations of Con A was calculated via Perkin Elmer (Lambda 25) spectrophotometer, with molar extinction coefficients, $E^{1\%}_{1/4}$ =11.4 at 280 nm.

2.3. pH measurements

pH of every solution was prepared by the use of Mettler Toledo pH meter (seven easy S 20-K) with the least count of 0.01 pH units, using an expert "Pro3 in 1" type electrode. The buffers were filtered through syringe filters (Millipore Milex-HV).

2.4. Rayleigh light scattering (RLS) measurements

The RLS study was executed on Hitachi F-4500 Fluorescence spectrofluorometer. The role of gemini surfactant into Con A aggregation was measured by the Rayleigh light scattering (RLS) methods. All the samples with and without gemini surfactant were excited at 350 nm and emission were taken at the same wavelength. The excitation and emission slit width were kept constant 5 nm for all samples. The 0.2 mg ml^{-1} of Con A was incubated with and without different concentrations of gemini surfactant (0–1000 μM) at

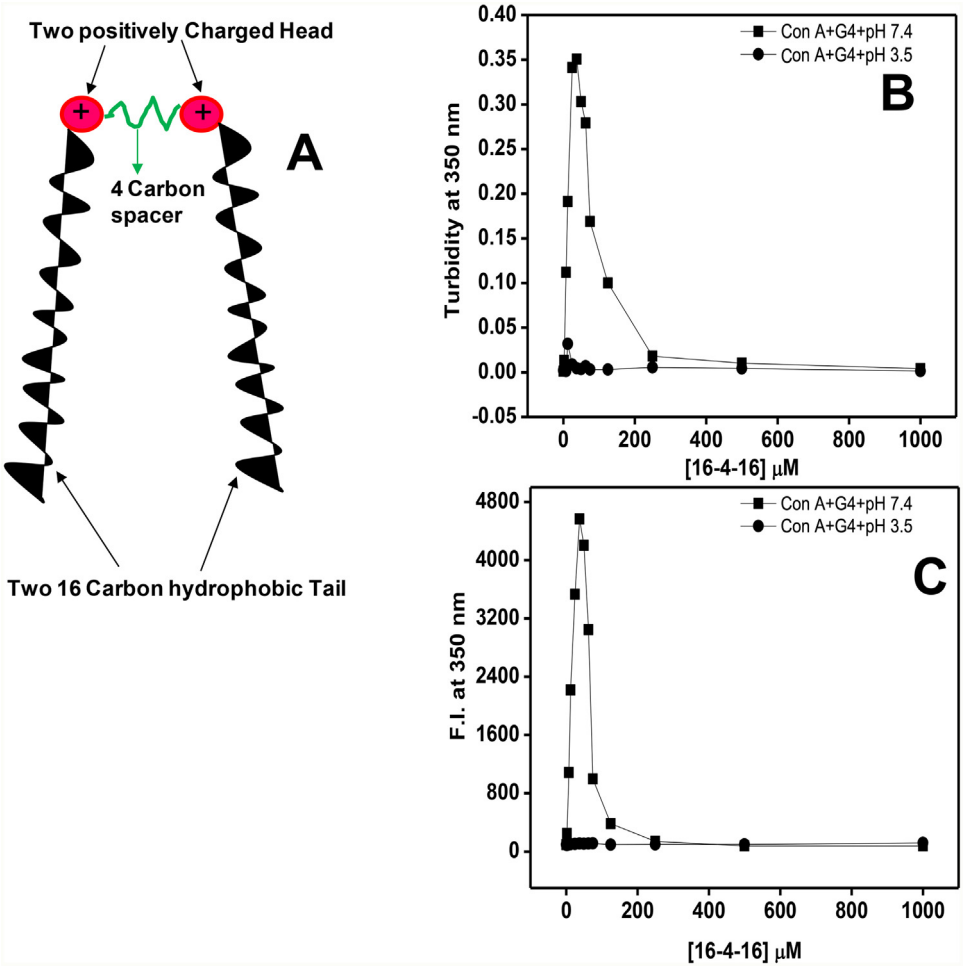

Fig. 1. Panel 1A; Schematic structure of cationic gemini surfactant. Panel 1B; showed the turbidity profile of Con A protein versus gemini surfactant concentration 90–1000 μM) at pH 7.4 (-■-) and 3.5 (-●-). Panel 1C; display fluorescence intensity at 350 nm versus different gemini concentrations (0–1000 μM) at pH 7. 4 (-■-) and 3.5 (-●-). The Con A concentrations was fixed (0.2 mg ml^{-1}) in both the measurements and the strength of both the buffers are 20 mM.

neutral pH 7.4. Another case, same concentrations of Con A and same concentrations of gemini surfactant were also incubated at pH 3.5. All the samples were incubated 12 h before the measurements.

2.5. Turbidity measurements

The Perkin-Elmer double beam UV–vis spectrophotometer (Lambda 25) was used to measure the turbidity of all the samples. The turbidity of samples, which were incubated with Con A (0.2 mg ml^{-1}) and different concentrations of gemini surfactants (0–1000 μM) at pH 7.4 as measured by taking optical density (OD) at 350 nm. In control samples, 0.2 mg ml^{-1} of Con A and similar concentrations of gemini surfactants at pH 3.5 were incubated for 12 h and then OD at 350 nm was measured.

2.6. Dynamic light scattering (DLS) measurements

DLS is used to measure the size of protein and protein-aggregates. DynaPro-TC-04 (Wyatt Corporation) instruments were used which were equipped with temperature controller micro-sampler. The Con A and gemini surfactant were spun at 7000 RPM for 5 min separately, and then the supernatant was taken and filtered through 0.22 μm pore size micro-filter. After centrifugation, Con A was incubated with and without gemini surfactants at pH 7.4. The Con A concentration was fixed 1.0 mg ml^{-1} in all the samples and different concentrations of gemini was used. Every samples were scanned 50 times, and average scans were taken. Dynamic 610.0.10 software analyzed the DLS data. The mean hydrodynamic radii (*Rh*) and polydispersity (Pd) were evaluated from an autocorrelation analysis of scattered light intensity based on the translational diffusion coefficient from the Stokes–Einstein equation

$$Rh = \frac{kT}{6\pi\eta D_{W}^{25^{\circ}C}}$$

Where *Rh* is the hydrodynamic radius, k is the Boltzmann's constant, T is the absolute temperature, Z is the viscosity of water and $D_{w}^{25^{\circ}C}$ is the translational diffusion coefficient.

2.7. Fluorescence spectroscopy

The intrinsic and ANS fluorescence study was carried out on Shimadzu spectrofluorometer attached with paltier. The intrinsic and ANS fluorescence measurements were done at room temperature in 1 cm path length cuvette. In intrinsic fluorescence, the Con A samples with and without gemini surfactant at pH 7.4 were excited at 280 nm, and the emission spectra were taken in the range of 300–400 nm.

The ANS binding was seen to the samples which contain Con A with and without gemini surfactant at pH 7.4. The ANS binding samples were excited at 380 nm and emission was recorded in the

range of 400–600 nm. Before ANS fluorescence measurements, The samples which contain Con A and gemini surfactant, and incubated with 50 fold more ANS for 30 min in the dark at pH 7.4. The Con A concentration was fixed 0.2 mg ml^{-1} in both intrinsic as well as ANS fluorescence measurements. In both the cases excitation and emission slit width were fixed 5 nm.

2.8. Thioflavin (ThT) dye binding assay

The ThT binding assay was done on Jasco FP-750 spectrofluorometer which is attached with water circulator. ThT was dissolved in double Milli Q water and filter it through 0.45 μ Millipore filter. ThT concentration was measured by taking OD at 440 nm with the use of extinction coefficient 36000 M^{-1} cm^{-1}. The ThT (5 μM) was added to the samples which contain Con A and gemini surfactant at pH 7.4 and further incubated half an hour in dark before measurements. The ThT incubated samples were excited at 440 nm and emission were taken in the range of 460–600 nm. The Con A and gemini surfactant were kept same which were used in RLS and turbidity measurements.

2.9. Far-UV CD measurements

Far-UV CD study was done on Applied Photophysics, Chirascan-Plus, UK spectropolarimeter. The Con A (0.2 mg ml^{-1}) was mixed with a different concentrations of gemini surfactant at pH 7.4 in 20 mM Tris–HCl buffer and incubated for 12 h prior measurements. The far-UV CD spectra of all samples were collected in the wavelength range of 200–250 nm in a cuvette of 0.1 cm path length. Every spectrum was collected on average of three scans and smoothened by Svestky Gloy methods.

2.10. Transmission electron microscopy (TEM) measurements

TEM experiments were carried out on a JEOL transmission electron microscope operating at an accelerating voltage of 200 kV. The Con A (0.2 mg ml^{-1}) was incubated with 50 and 75 μM gemini surfactant at pH 7.4 for 12 h. The 50 μl was taken from the aggregated samples and placed in TEM grid for proper adhesion. The grid was left for air drying. The dried grid was further negatively stained with 2% uranyl acetate for 10 min and then washed with distilled water. The uranyl acetate stained grid was also dried in a desiccator to eliminate moisture from the grid. The other sample which contains Con A protein and 50 μM gemini were incubated only 30 min and then run the TEM expriments. The TEM images were taken at high resolutions.

3. Results

3.1. Turbidity

Turbidity at 350 nm is used to identify the protein aggregates in the solutions. It is reported that the optical density at 350 nm was found to be high due to protein aggregation [26]. From Fig. 1B, it was found that the optical density (OD) changed concerning gemini surfactant at pH 7.4 and 3.5. The optical density profile of Con A found high in the range of 2.5–125 μM of gemini surfactant at pH 7.4 while at higher concentrations (125–1000 μM) the OD was found to be negligible and almost negligible at the same pH. The OD of Con A was also measured in the presence of same gemini concentrations at pH 3.5. It was observed that OD did not change at any concentration of gemini. The increase in OD at pH 7.4 suggested that the Con A formed aggregates in the presence of low concentration of gemini surfactants (2.5–125 μM). The possible reason of gemini-induced aggregation is electrostatic interaction. The positively charged head of gemini surfactant interacted with negatively charged amino acids of Con A protein at pH 7.4, and induced aggregation. On the other hand, at pH 3.5 Con A possesses the net positive charge at pH 3.5 and gemini also contains a positive charge on the head, resulting electrostatic repulsion did not induce any aggregation.

Table 1
Hydrodynamic radii of Con A in the presence and absence of Gemini surfactant at different sets of conditions.

S. No.	Conditions	*Rh (nm)*	Polydispersity
1.	Con A at pH7.4	4.2	16.2
2.	ConA + 50.0 μM Gemini + pH 7.4	6.3, 30.7	30.21, 40.54
3.	ConA + 75.0 μM Gemini + pH 7.4	7.6, 32.5	32.09, 45.45
4.	ConA + 1000 μM Gemini + pH7.4	4.8	10.32

3.2. Rayleigh light scattering (RLS) measurements

Rayleigh light scattering is another probe to detect the soluble and insoluble aggregates in solutions. A light scattering of gemini surfactant in the presence of Con A was examined at two different pH (7.4 and 3.5). From Fig. 1C, it was observed that the light scattering of Con A at 350 nm is very low in the absence of gemini surfactants at both pHs. At pH 7.4, the light scattering was significantly higher in the presence of very low concentrations (2.5–125 μM) of gemini surfactants. However the light scattering significantly reduced at higher concentrations of the surfactant. The scattering results at pH 7.4 suggested that the possible cause of aggregation in is electrostatic interaction. When Con A was incubated at pH 3.5, the light scattering was insignificant in the absence and presence of 0–1000 μM of gemini surfactant concentration. The scattering results at pH 3.5 demonstrated that gemini was unable to induce aggregation in Con A due to the electrostatic repulsion between positively charged ConA and positively charged surfactant molecules.

3.3. DLS study

DLS is highly used techniques for detecting the changes in hydrodynamic radii of proteins and polymers. It is also used to characterize the size of aggregates in solutions. We performed DLS study to discover the hydrodynamic radius of Con A protein in the presence of gemini surfactant. From Fig. 2A, it was observed that the hydrodynamic radii of Con A at pH 7.4 is around 4.2 nm and light scattering is low. On the other hand, the hydrodynamic radii increased when protein was incubated with 50 and 75 μM of the gemini surfactant at pH 7.4 as shown in Fig. 2B and C. In the presence of higher concentrations (1000 μM) of gemini surfactant, the hydrodynamic radii was found to be around 4.8 nm which is very close to hydrodynamic radii of native Con A protein as shown in Fig. 2D. The hydrodynamic radii and polydispersity values at every concentration of gemini surfactant are shown in Table 1. The DLS data pattern suggests that the Con A become aggregated in the presence of very low concentrations of gemini surfactants and but no aggregation was observed at higher concentration of the surfactant. The hydrodynamic radii at higher concentrations are suggesting some conformational changes in Con A protein.

3.4. Intrinsic fluorophores

Two amino acids in proteins give an idea about protein folding and misfolding, i.e., Tryptophan (Trp) and tyrosine (Tyr). These residues act as an intrinsic fluorophore in proteins. Consequently, Trp residue is most common fluorophore used to study the protein aggregation, unfolding and dynamics. In this work, we have seen

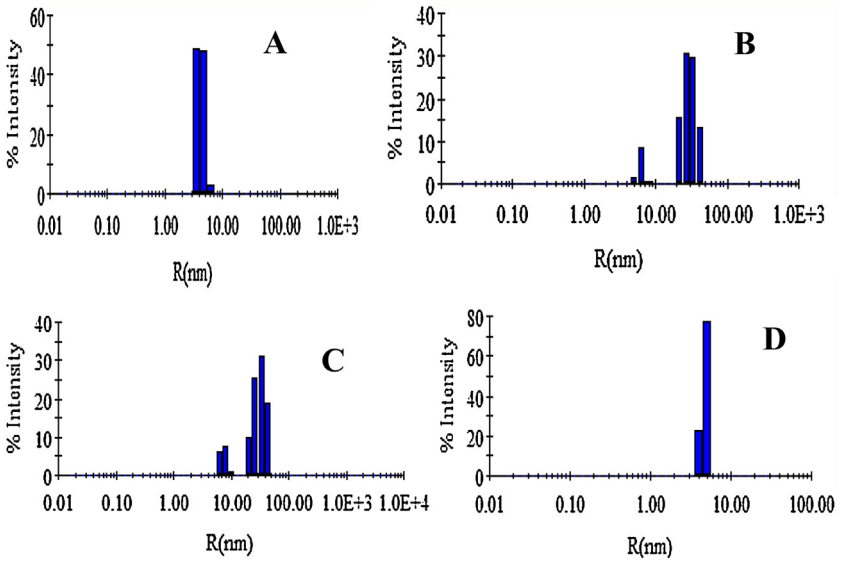

Fig. 2. DLS measured hydrodynamic radii of Con A and its aggregates. The hydrodynamic radii of Con A at pH 7.4 is shown in panel A, and in the presence of 50.0 μM (B), 75.0 μM (C) and 1000 μM (D). The Con A concentrations were taken 1.0 mg ml^{-1} in all the conditions.

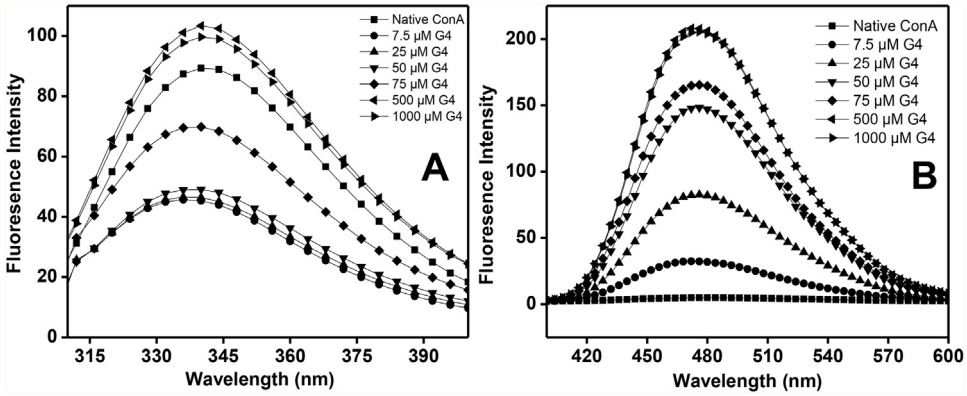

Fig. 3. Identification of tertiary structural alteration in Con A protein, intrinsic as well as ANS fluorescence measurements were done. The intrinsic fluorescence (panel A) and ANS fluorescence (panel B) spectra of Con A (0.2 mg ml^{-1}) with and without different concentrations of gemini surfactant was plotted in panel A. The intrinsic and ANS fluorescence spectra of Con A without gemini (-■-) and with 7.5 μM (-●-), 25 (-▲-), 50 μM (-▼-), 75 μM (-♦-), 500 μM (-◄-) and 1000 μM (- &z.rtrif; -) of Gemini surfactant was plotted in both the panels.

the binding of gemini surfactant with Con A protein at physiological pH as shown in Fig. 3A. Trp residues of Con A at pH 7.4 was excited at 295 nm and emission was found maximum at 339 nm. When Con A was incubated with low concentrations of gemini surfactant, the wavelength maximum was shifted towards lower wavelength, and fluorescence intensity was dropped down. Moreover, in the presence of higher concentrations of gemini the wavelength maximum was not shifted, but fluorescence intensity was found high compared to native Con A. The change in wavelength maximum and fluorescence intensity suggested that the Con A Trp residues are internalized inside the core of Con A due to aggregation while at higher concentration the Con A Trp residues are exposed towards the polar environment.

We further checked the exposure of hydrophobic residues by using of an extrinsic fluorophore, i.e., 8-anilino-1-naphthalenesulfonic acid (ANS). ANS is an azo dye highly used to determine the exposure of hydrophobic residues in proteins. As shown in Fig. 3B, the ANS was not showing any binding with Con A at pH 7.4. However, in the presence of gemini surfactant a massive increase in ANS fluorescence intensity was seen. The ANS binding results showed that Con A underwent aggregation. The results obtained from both intrinsic and extrinsic fluorescence measurements, demonstrated that gemini surfactant is destabilizing Con A protein structure resulting in aggregate formation.

3.5. Thioflavin-T (ThT) binding

ThT is a very useful probe to identify amyloid fibril under *in-vitro* and *in-vivo* conditions. ThT binds with amyloid fibril and produces a high fluorescence intensity at 485 nm [27]. In Fig. 4A, the ThT fluorescence spectra of Con A alone and in the presence of different concentrations of gemini surfactant was plotted. ThT was showing insignificant binding with native Con A. In the presence of 2.5–125 μM gemini surfactants, the ThT fluorescence intensity was found to be very high. In the presence of higher concentrations (125–1000 μM) of the surfactant, fluorescence intensity was very low indicating poor ThT binding. Fig. 4B shows, ThT fluorescence intensity at 485 nm plotted against gemini concentrations. It is evident from the figure, the ThT fluorescence intensity continuously increased from 2.5 μM to 125 μM of gemini surfactant and the maximum ThT fluorescence intensity was noticed at 37.5 μM. The fluorescence intensity dropped above 125 μM concentrations of gemini surfactants. The ThT result also suggested that the gemini surfactants induced amyloid-like aggregates in Con A protein.

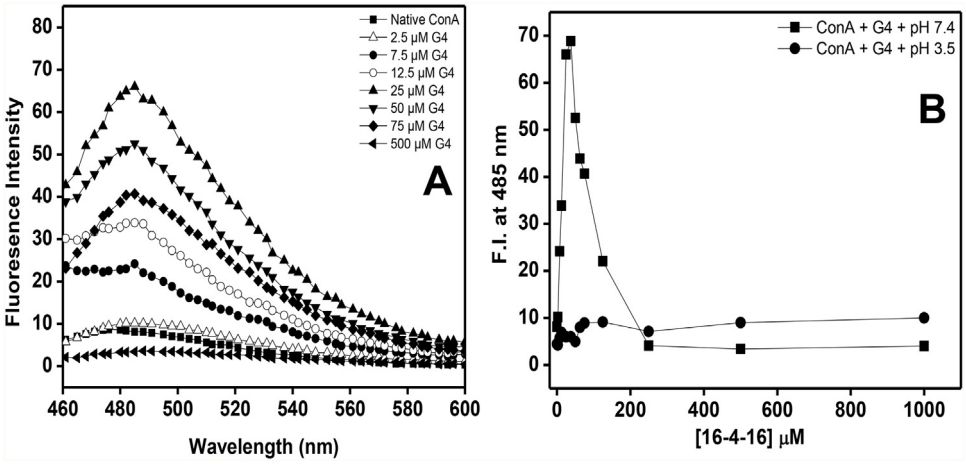

Fig. 4. ThT dye binding was carried out to characterize the morphology of gemini-induced aggregates. The ThT fluorescence spectra of Con A (0.2 mg ml^{-1}) in the presence and absence of gemini are shown in panel A. The ThT fluorescence spectra of Con A alone (-■-) and with gemini surfactant 2.5 μM (-Δ-), 7.5 μM (-●-), 12.5 μM (-◯-), 25 μM (-▲-), 50 μM (-▼-), 75 μM (-♦-) and 500 μM (-◄-) were plotted in panel A at pH 7.4. The fluorescence intensity of ThT at 485 nm was plotted against gemini surfactant concentration (0–1000 μM) at two different pHs 7.4 (-■-) and 3.5 (-●-).

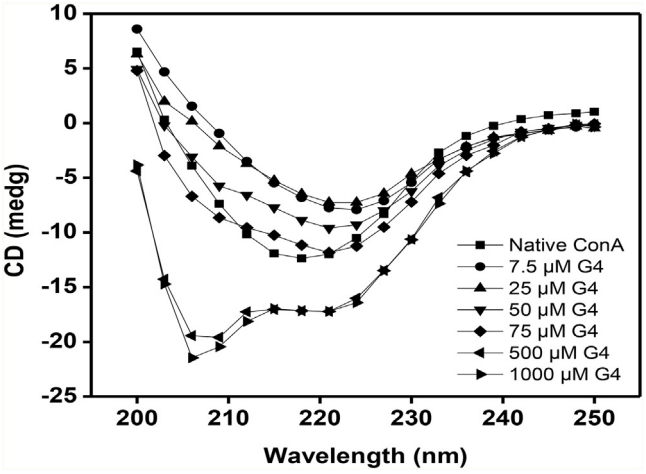

Fig. 5. Secondary structure transformation was measured in the presence and absence of gemini surfactant at pH 7.4. The far-UV CD spectra of Con A (. 0.2 mg ml^{-1}) without (-■), and with 7.5 μM (-●-), 25 μM (-▲-), 50 μM (-▼-), 75 μM (-♦-), 500 μM (-◄-) and 1000 μM (-●-) gemini surfactant at pH 7.4.

3.6. Secondary structure conversion during amyloid fibril formation

Far-UV CD is one of the best spectroscopic technique to characterize the change in secondary structure of proteins during amyloid fibril formation [28]. It is widely reported that the amyloid fibril has β or cross β structure. It is also found that during amyloid formation, the α-helical proteins convert into β structure and β-sheet proteins retains their β conformation [29,30]. Secondary structural changes of Con A in the absence and presence of gemini surfactant were determined using far-UV CD. As shown in Fig. 5, in the absence of gemini surfactant, the CD spectra of Con A was showing single minima around 218 nm which indicated that the Con A protein is rich in a β-sheet structure. The CD spectra of Con A in the presence of different concentrations (7.5, 25, 50 and 75 μM) of gemini surfactant showed single minima between 222 nm to 225 nm. The shift in wavelength is suggesting that the β-sheet structure of Con A is not true β, this could be a cross β-sheet structure of amyloid. This kind of CD spectrum was also found when hyperthermophilic proteins were incubated with SDS at pH below two units of their respective pI [26]. The change in secondary structure of Con A in the presence of low concentrations of gemini surfactant indicated that the Con A protein formed amyloid-like aggregates and have cross β-sheet structure. Upon addition of higher concentrations of gemini surfactants (500–1000 μM), the β-sheet conformation of Con A transformed into α-helical structure. It was noted that the single minima at 218 nm converted into two minima at 208 and 222 nm. This kind of far-UV CD spectrum is commonly observed for proteins that are rich in α-helix, for example, human serum albumin [31]. It is speculated that β to α transition were occurring due to intra-chain hydrogen bonding. Overall CD results suggested that Con A form cross β-sheet structure in the presence of low gemini concentrations while α-helix conformation was acquired by the protein at higher surfactant concentrations.

3.7. Verification of the amyloid morphology by transmission electron microscopy (TEM)

Transmission electron microscopy was used to determine the shape and size of gemini-induced aggregates of Con A lectin. TEM is an excellent tool to differentiate between amyloid-like structure and amorphous aggregates. Various types of amyloid morphology have been observed in proteins for example rod-shape, beadlike, wormlike, ribbon shape, and straight fiber [32,33]. The TEM image of Con A aggregates in the presence of different concentrations (50 μM and 75 μM) of gemini, overnight incubated is shown in Fig. 6A, B. From Fig. 6A, B, it was noticed that Con A form long straight fibril in the presence of the gemini surfactants at pH 7.4 while sample containing 50 μM gemini and Con A incubated only for 30 min are showing big oligomers and immature fibrils shown in Fig. 6C. The oligomers and immature fibrils are marked in Fig. 6C. Thus, the results obtained from ThT fluorescence, far-UV CD and TEM confirmed that the gemini surfactant is inducing amyloid-like aggregates in Con A protein when samples were incubated over night at pH 7.4.The Con A incubated with same concentrations of gemini for very less time form large oligomer and immature firbils at same pH.

4. Discussion

In this study, we have explored the gemini surfactant-induced amyloid fibril formation in plant lectin protein (Con A) by using various biophysical techniques at pH 7.4 and 3.5. It is reported that the Con A become aggregated and form amyloid fibril at pH 8.9 and

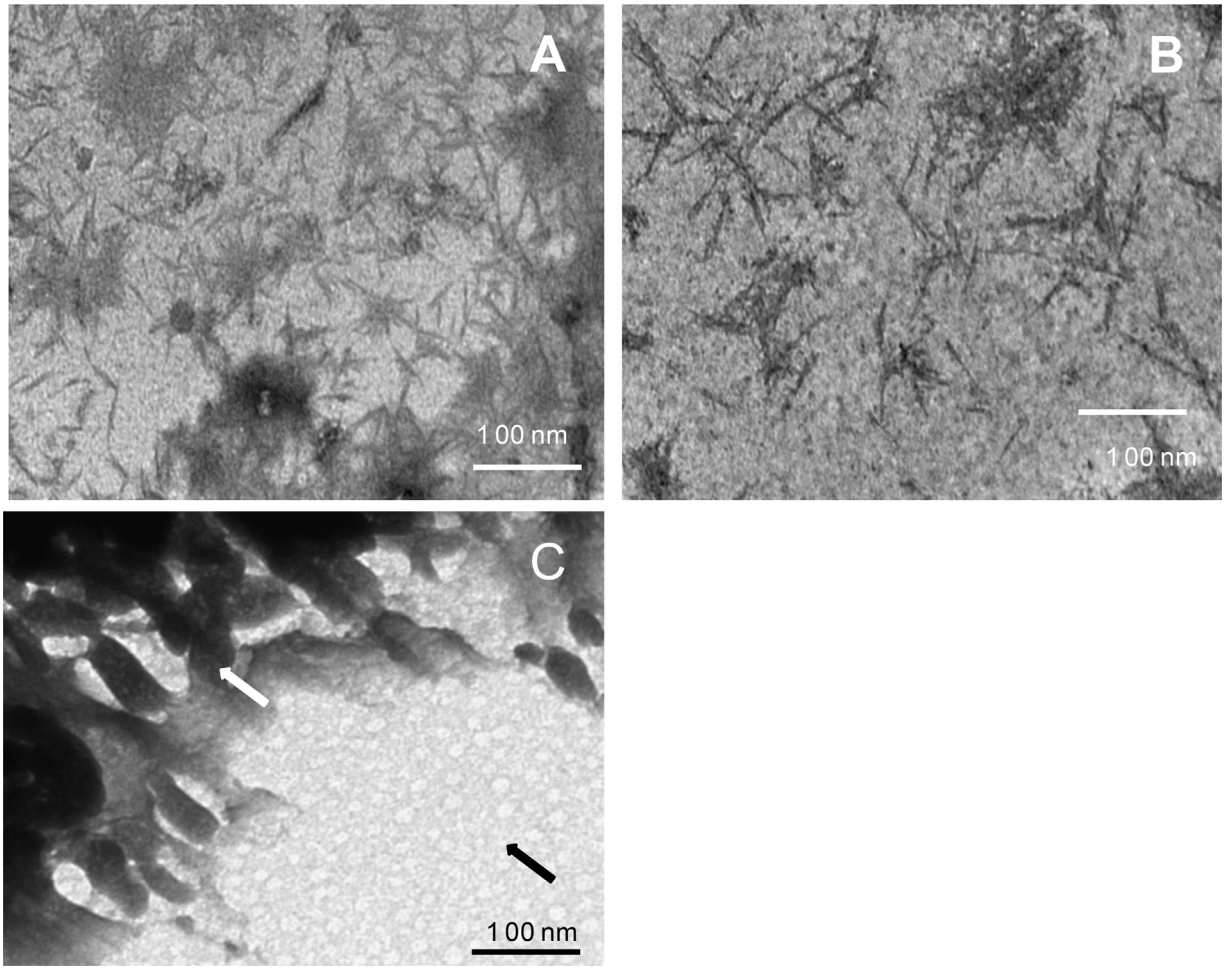

Fig. 6. Gemini-induced Con A aggregation was detected by TEM. The Con A (0.2 mg ml^{-1}) was incubated with 50 μM (panel A) and 75 μM (panel B) of gemini surfactant at pH 7.4 for overnight. Panel C, the Con A (0.2 mg ml^{-1}) was incubated with 50 μM of gemini for 30 min. The TEM images were captured after different incubation.

amorphous type aggregates at pH 5.1 at high temperature (40 °C) for longer time incubation [34]. It is important to see the amyloid fibril formation mechanism via surfactants (conventional and non-conventional) with Con A proteins because surfactant has a high tendency to affect the functional property of protein by alteration the conformation, polarity, and stability of proteins. The gemini surfactant is different from conventional surfactants in a sense that it contains two positively charged head, two hydrophobic tails and one spacer group. The spacer is connecting two monomers near the head of the surfactant [35]. The isoelectric point of Con A is reported around 5.43 [36]. Con A possesses the net negative charge at pH above two units of theire pI i.e., pH 7.4. The aspartic and glutamic acid residues are present in anionic form because these residue liberated its proton at pH 7.4. The online tools "PROTEIN CALCULATOR" was used to calculate the total charges on each subunit of Con A (−5) at both pHs. From the PROTEIN CALCULATOR, it was confirmed that the Con A is anionic in nature at pH 7.4 and cationic at pH 3.5. The low concentration of cationic surfactant (gemini) interacted with anionic residues (aspartic acid and glutamic acid) of Con A via electrostatic interaction and assisted in amyloid fibril formation. The positively charged head of gemini surfactant interacted with negatively charged amino acids of Con A and the hydrophobic tail of Gemini surfactant actively breaks all the Con A-solvent interaction and simultaneously increasing Con A-Con A-gemini and Con A-gemini-Con A interaction. On the other hand, at pH 3.5 Con A become positively charged due to protonation of arginine, lysine and histidine residues and amyloid fibril was not formed because both Con A and gemini surfactant have the same charge. Previously, we have discovered the same mechanism of aggregation with anionic surfactants (SDS), in that, we had reported that SDS was inducing aggregation in 25 types of different proteins when it was oppositely charged compared to SDS [36]. We also found that the negatively charged Con A transformed into amyloid fibrils when it binds with very low concentrations (0.0125–0.25 mM) of cationic surfactant (CTAB) at neutral pH [37]. It is now established form the current study that the electrostatic interaction is playing very important role in surfactant-induced amyloid fibril formation [38,39].

The role of gemini surfactant into amyloid fibril formation of Con A protein was characterized via the use of various biophysical techniques. The turbidity and light scattering results are suggested that low concentrations (2.5–125 μM) of gemini surfactant inducing aggregation and no aggregation was found at higher concentrations. The size of aggregates was increasing till 37.5 μM gemini concentration, and after that, the size of aggregates starts decreasing confirmed by turbidity and scattering measurements. A similar observation was also found when a partially unfolded state of stem bromelain was incubated with sub-micellar SDS concentration (50–1000 mM). At low concentration bigger size aggregates are formed, and no aggregates were formed beyond the micellar concentration. All these changes were confirmed by turbidity and Rayleigh scattering measurements [40]. DLS measured the hydrodynamic radii of aggregated species in the solution. DLS is very useful techniques to characterize the hydrodynamic radii of aggre-

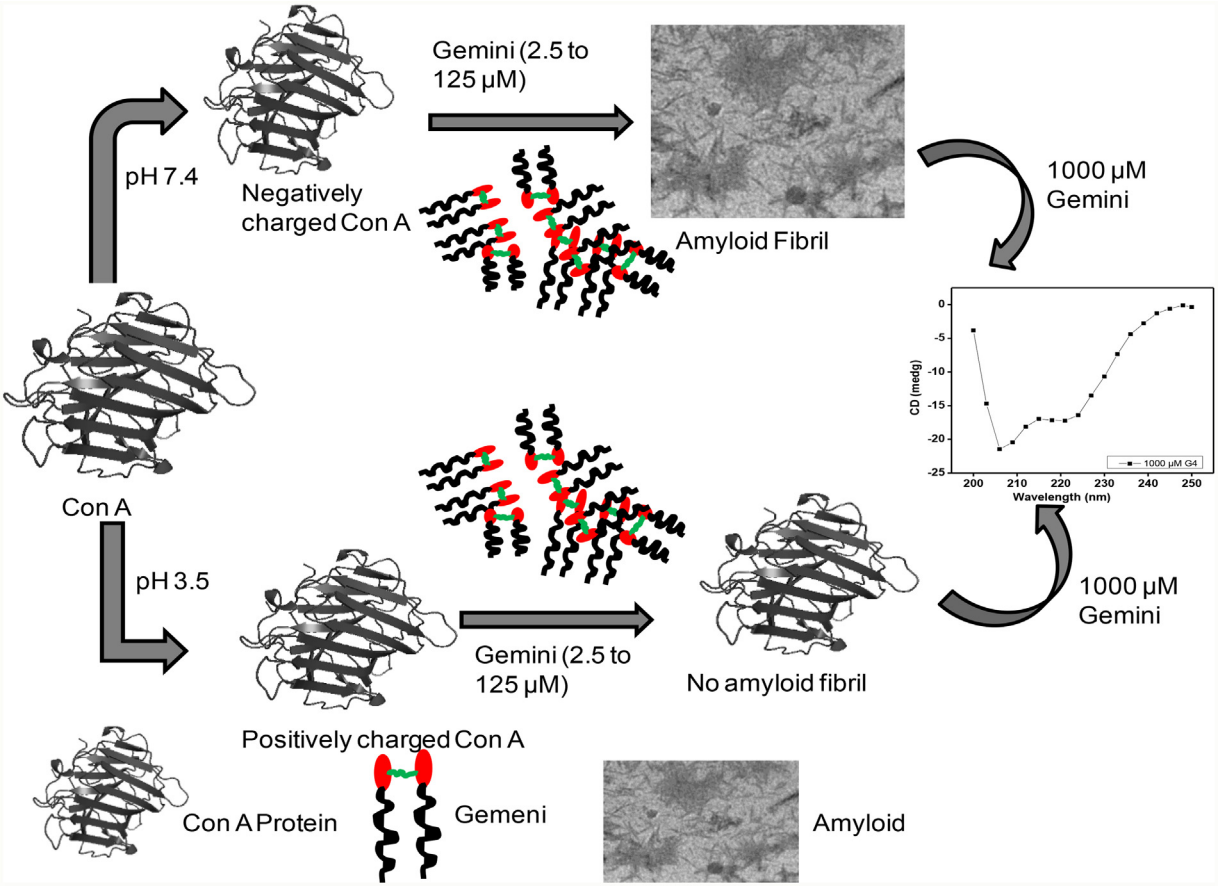

Fig. 7. Schematic presentation of gemini-induced amyloid fibrils formation in Con A protein at two different pHs.

gated and non-aggregated proteins and also giving a clear picture of oligomerization of proteins [41]. The Con A formed a bigger size aggregate when it was incubated with 50.0 and 75.0 μM gemini surfactant. The hydrodynamic radii were found 4.8 the presence of 1000.0 μM of gemini surfactant which is very close to the hydrodynamic radii of native Con A. The DLS results are also suggesting that Con A form bigger size aggregates at lower concentrations and no aggregation at higher concentrations. We also explored the position of tryptophan in gemini surfactant-induced amyloid fibril formation of Con A by the use of intrinsic and extrinsic fluorescence. It is reported that the Trp residues of protein either buried or exposed when proteins transform into amyloid fibrils and its wavelength maximum was shifted towards blue or red shifts [42]. In gemini surfactant-induced amyloid fibrils of Con A exhibited a blue shifte in emission maxima and showed high ANS binding. Further, we characterized the nature of the aggregates using ThT binding and TEM. It was found that the gemini surfactant is inducing amyloid-like aggregates because ThT is showing very strong binding with aggregated species and rod-shaped morphology was seen by TEM. The N-terminal fragment 1–29 of apomyoglobin from horse heart transformed into amyloid fibril at pH 2.0 and the fibrillation pathway is nucleation-dependent confirmed by ThT dye binding and TEM study [43]. Far-UV CD also characterized the secondary structural transformation. It is evident from various published works, and the amyloid fibril contains β-conformation [44,45]. The minima at 218 nm of Con A protein in the presence of gemini surfactant shifted to higher wavelength (222–225 nm). This kind of transformation indicated that the Con A formed cross β-sheet structure which is a characteristic of amyloid-like structure. This kind of secondary structural transition was also observated in hyperthermophilic proteins and mesophilic protein when it was incubated with low concentrations of anionic surfactant sodium dodecyl sulphate [26,36]. From all the biophysical methods, it is now confirmed that the gemini surfactant is inducing amyloid-like aggregates in Con A proteins at pH 7.4.

The overall results obtained from this study are summarized in Fig. 7. At pH 7.4, Con A was found negatively charged while it acquired positive charged at pH 3.5 (pH below two units of Con A pI). It is a well-known fact that the proteins acquire a net positive or negative charge when it was incubated at pH two units below and above the pI respectively [36]. In this aspect, Con A become positively charged at pH 7.4 due to the release of its proton from negatively charged amino acids. When lower concentrations of gemini surfactant (2.5–125 μM) were added, and then the positively charged head of gemini interacted electrostatically with negatively charged amino acids of Con A and concurrently neutralize all the interaction with solvents and increased the Con A-gemini interactions leading to the formation of amyloid fibrils. We also did the same experiments at pH 3.5 where Con A is positively charged due to protonation of positively charged amino acids. We added similar concentrations of gemini surfactant, but amyloid fibril formation did not occur because of the strong electrostatic repulsion between positively charged amino acids of Con A and gemini surfactant. The aggregates were solubilized in the presence of higher concentrations of gemini surfactant (125–1000 μM) inducing an α-helical structure in the β-sheet Con A protein.

5. Conclusions

We have demonstrated the role of gemini surfactant in the process of amyloid fibril formation of Con A at physiological and low pH. The results of this study are highlighted that the electro-

static interaction, as well as hydrophobic interactions, are playing a critical role in the gemini-induced amyloid fibril of Con A at physiological pH. The amyloid fibril was not formed when Con A was incubated at low pH due to electrostatic repulsion because both the molecules contain same charges. Gemini is having dual behavior in terms of reactivity. At low concentrations, gemini are inducing cross β-sheet structure in Con A protein while at higher concentrations inducing α-helical structure in β-sheet Con A protein at pH 7.4. The possible cause of α-helix induction in β-sheet protein is intrachain hydrogen bonding in Con A.

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