Effect of Al(III) and curcumin on silk fibroin conformation and aggregation morphology

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Misfolding or β-sheet nano-fibrillation of specific proteins is considered to be an underlying pathogenic mechanism of neurodegenerative diseases. It was found previously that Al(III) can affect the β-sheet nano-fibrillation and deposit neurodegenerative disease related proteins, and that curcurmin can interact with metal ions and those proteins. In this work, silk fibroin (SF) was used as a model fibrillation protein for the investigation of the influence of Al(III) and curcumin on SF conformation transition. The effects of Al(III) and curcumin on SF were investigated using circular dichroism, thioflavin T fluorescence, 1-anilino-8-naphthalene sulfonate fluorescence, turbidity assays, atomic force microscope and Fourier transform infrared. This research demonstrated that Al(III) can bind with specific amino acid residues of SF, and then accelerate the formation of intermediates and also the formation of nanofibrils. The concentration of Al(III) is an important factor that influences the folding speed of SF. Furthermore, curcumin cannot only restrain the conformation transition of silk fibroin, but can also reverse the conformation transition of SF and Al(III)-induced SF from insoluble β-sheet to soluble random coil. Curcumin may prevent the neurodegenerative related proteins from nano-fibrillating and aggregating.

1. Introduction

Neurodegenerative diseases including Alzheimer’s disease (AD), prion disease and Parkinson’s disease have threatened human health for years.1-4 One of the pathogens involves the abnormal aggregation of neurodegenerative related proteins such as amyloid β (Aβ) peptide.5-7 The nano-fibrillation and aggregation of Aβ40 and Aβ42, which are derived from the proteolytic cleavage of an amyloid precursor protein in senile plaques and neuritic plaques are the main characteristics of AD pathology.8-10 It is found that the typical hydrophobic sequence, VGGAVVAGV, in the Aβ peptide is similar to the peptide segment, GAGVGAGYG, which is abundant in the silk fibroin (SF) of the silkworm, Bombyx mori,11,12 and the mechanism of nano-fibrillation is also similar between the two proteins.13 Accordingly, we used SF, an easily available protein, as a model fibrillation protein to simulate the aggregation process of Aβ. SF has two types of conformers: silk I (random coil and helix-like forms) and silk II (β-sheet and β-sheet-like forms).14-15 The morphology of silk I solid is amorphous while that of silk II solid is crystalline in β-sheet fibrils. It has been reported that the process of protein self-assembly into nanofibrils was stabilized by cross-β interactions.16 Previously, we demonstrated that Ca2+, Cu2+, K+ and Zn2+ ions could affect the secondary structures of the regenerated silk fibroin and accelerate the conformation transition from silk I to silk II at certain concentrations.17-22

Al(III), enriched in senile plaques, is related to the fibrillation, aggregation and toxicity of Aβ.23,24 However, the detailed mechanism of Aβ nano-fibril formation is still unclear. In fact, research on the spinning mechanism of silkworms shows that the process of forming SF fibrils has a similar dynamic behavior to that of denaturizing Aβ.25 On the basis of the resemblance between Aβ and SF, SF was used as a model protein to study its interaction with Al(III).

A previous report revealed that chelators such as desferrioxamine and clioquinol have anti-AD ability.26 In addition, it was found that a gel form of SF was caused by crosslinking via metal ions present in the solution. Furthermore, the solution could be converted from a gel to sol state by EDTA.27 Therefore, in this research we tried to find a physiological, non-toxic substance capable of chelating the metal ions efficiently, and stabilizing the fibroin in solution at a state of random-coil conformation, or even reversing the preformed β-sheet conformers into random-coil ones.

Fig. 1 Molecular structure of curcumin.
Curcumin, a planar, biphenoilic yellow pigment (Fig. 1) found in turmeric and commonly used in India and other Asian countries, is reported to have some therapeutic effects on AD. Its ability to cross the blood–brain barrier and its non-toxicity make curcumin more appealing than many other molecules. In addition, Yang et al. suggested that curcumin could bind with amyloid, and then inhibit Aβ aggregation. Garcia-Alloza et al. reported that curcumin could disrupt the existing plaques, and partially restore the distorted neurites in an Alzheimer’s disease model mouse. Baum and Ng suggested that curcumin could interact with copper and iron, reducing amyloid aggregation or oxidative neurotoxicity. Although the research on the effects of curcumin on Aβ is underway, there are few reports about the interaction of curcumin with Al(III).

In our previous work, Al(III)-curcumin–SF samples in the solid state were studied. Because, the drying process involved a concentration change which also influenced the SF conforma
tion transition and the formation of aggregations, in this work, Al(III)–SF samples were studied in the solution state. We investigated the chelating sites of Al(III) in SF, and the effects of Al(III) on the conformational transition and fibrillation of SF. Furthermore, using the research on the SF–Al(III)–curcumin mixture solution we deduced the effect of curcumin on SF and the Al(III)–SF system. This work would help to reveal a way to prevent and cure AD.

2. Materials and methods

2.1 Materials

The cocoons of the silkworm, Bombyx mori, were purchased from the Yuxing Textile Factory in Tongxiang, Zhejiang province, China. Curcumin was purchased from Alfa Aesar (Ward Hill, MA). 8-Anilino-1-naphthalene sulfonate (ANS), thioflavin T (ThT) and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma-Aldrich, USA. Lithium bromide (LiBr), silver nitrate (AgNO₃), sodium carbonate (Na₂CO₃), hydrochloric acid (HCl) and aluminum chloride (AlCl₃) were purchased from Sinopharm, China.

2.2 Preparation of SF solution

The raw silk fibers from Bombyx mori were degummed twice by boiling water with 0.5 wt% Na₂CO₃ for 40 min each time to remove sericin, and then the degummed silk fibers were thoroughly rinsed with ultrapure deionized water (resistivity ~18.2 MΩ cm) and dried overnight at 60 °C in a drying oven. Degummed silk fibers (6 g) were dissolved in 100 mL, of a solution of 9.3 M LiBr. After the silk fibers were completely dissolved, the solution was filtered and dialyzed against ultrapure deionized water with a 14 000 g mol⁻¹ dialysis tube to remove LiBr which was probed by 0.1 M AgNO₃ aqueous solution. The final concentration of the SF solution was about 1.5 wt%.

2.3 Preparation of Al(III)–SF solutions and films

SF solution (10 mg mL⁻¹) was mixed with different concentrations (0, 5, 10 mM) of Al(III) solution to obtain 6 mL of the mixture. The resulting solutions were cast on 30 × 30 cm polyester dishes, and then dried for three days in the fume hood. The films obtained contained 60 mg of SF in each one. The drying process was devised to mimic the spinning process of the silkworm, to get from an aqueous solution to insoluble fibers.

2.4 Preparation of samples of mixtures of SF, Al(III) and curcumin

To study the interaction of Al(III) with SF, SF solutions were mixed with various volumes of 2 mM AlCl₃ aqueous solution and then diluted with an aqueous solution Tris–HCl (20 mM, pH = 7.4) to 26 ml to give an SF concentration of 0.5 mg mL⁻¹, and Al(III) concentrations of 0, 10, 15, 20, 40, 50, 60, 80, 100 μM. Curcumin–SF solutions were prepared with 15 μM curcumin and 0.5 mg mL⁻¹ SF, and an Al(III)–curcumin–SF solution was prepared with 15 μM AlCl₃, 15 μM curcumin and 0.5 mg mL⁻¹ SF. To study the morphology of SF aggregation, 0.5 mg mL⁻¹ SF was incubated for 10 days to produce precipitates, and then diluted using ultrapure water and then mixed ultrasonically.

2.5 Circular dichroism

A circular dichroism (CD) spectrum of SF was recorded using a Jasco J715 spectropolarimeter with a quartz cell of 1 mm path length. All CD spectra were recorded by five scans at a speed of 100 nm min⁻¹ with a response time of 0.5 s. The range of the scan was from 190 to 250 nm with a nitrogen flow 15 mL min⁻¹.

2.6 Fluorescence spectroscopy

A fluorescence spectrum (FLS) was recorded using an Edinburgh Instruments FLS920 fluorescence spectrophotometer with a quartz cell with a 1.0 cm path length. For fluorescence measurement, the interaction of 30 μM ANS with SF was monitored by fluorescence emission at 500 nm (λex = 360 nm), and that of 20 μM ThT with SF by emission at 485 nm (λex = 450 nm).

2.7 Turbidity assay

The relative turbidity of a turbid SF solution was recorded by measuring UV-Vis scattering at λ = 400 nm. The UV-Vis scattering measurement was obtained using a PerkinElmer Lambda 35 UV/Vis spectrometer.

2.8 Atomic force microscope images

A portion (5 μL) of Al(III)–curcumin–SF mixture sample was freshly collected and swiftly diluted to a 5 μg mL⁻¹ solution with water. Diluted solution (10 μL) was mounted onto freshly cleaved mica for 5 min, gently rinsed with water, and dried under vacuum overnight. Images were acquired under air atmosphere using a silicon probe in tapping mode packaged in a Bruker MultiMode 8 system.
2.9 Attenuated total reflection-Fourier transform infrared spectroscopy

Attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) measurements were performed using a Thermo Nicolet Nexus 470 FTIR spectrometer equipped with an ZnSe crystal as window material and Omni sampling accessories.

3. Results

3.1 Conformation transition of SF induced by Al(III) in a solution state

To study the effects of Al(III) on SF conformation transition, the conformation of SF in solution was detected by CD, fluorescence and UV-Vis scattering.

The secondary structure of SF has special elliptical bands between 178 and 250 nm in CD. Random coil conformation has a negative strong band at 195 nm and the β-sheet structure has negative broad band at 217 nm and a positive strong band at 197 nm.[14,15]

ThT is a fluorescent dye that can selectively associate with the β-sheet or the aggregated forms of protein, resulting in a fluorescence enhancement,[16] therefore it is generally used to probe hydrophobic β-sheet conformation.

In addition, ANS usually also interacts with the hydrophobic blocks of partially folded proteins and oligomers because of its hydrophobicity, resulting in the enhancement and blue shift of ANS fluorescence.[17,18] Therefore, ANS was used to probe the intermediates of folded proteins and oligomers.

The turbidity of the Al(III)-SF solution was monitored by UV-Vis scattering at 400 nm. The increase of UV-Vis scattering intensity implies the aggregation of SF.

Another important factor, which impacts on the conformation transition of SF is pH.[17] To explain the effect of Al(III) on SF clearly, it should be pH effects should be excluded. The pH of the low concentration of Al(III) solution was kept to between 7.33 and 7.27 by using Tris–HCl buffer.

Fig. 2A shows the conformation transition of SF detected by CD at different incubation times with Al(III). It was found that the amount of β-sheet conformers of SF was increased during incubation, and the amount is higher in Al(III)-SF solution than in SF solution, based on the ellipticity at 217 nm. Fig. 2B shows the ThT fluorescence intensity in SF in the absence and presence of Al(III). After the Al(III)-SF solution was incubated for a given time, ThT was added to the solution. The fluorescence curve fitted a sigmoidal model, showing a low plateau phase at the beginning, called the lag phase, followed by a rapid increase, and finally reached a high plateau phase after 26 h of incubation in both the absence and presence of Al(III). The results indicate that Al(III) accelerated the formation of β-sheet conformer. Fig. 2C shows the impact on ANS fluorescence of the interaction of SF with different Al(III) concentrations after the solution was incubated for 10 h. It is found that the intensity of the ANS fluorescence increased as the Al(III) concentration increased, indicating that more intermediates of β-sheet SF were formed at higher Al(III) concentrations. Fig. 2D shows the ThT fluorescence of Al(III)-SF solutions after incubation for 10 h. Fig. 2E shows the turbidity of SF solution at different Al(III) concentrations. The trend of Fig. 2E is similar to that of Fig. 2D, indicating that the formation of β-sheet conformers and their precipitates depended on the Al(III) concentration.

All these results demonstrate that Al(III) can promote the formation of β-sheet conformers, leading to the fibrillation and aggregation of SF.

3.2 Effect of curcumin and Al(III) on the secondary structure of SF

In order to investigate the effects of curcumin, ThT fluorescence in SF, Al(III)-SF, curcumin-SF and Al(III)-curcumin-SF solutions were measured. Fig. 3A shows that the ThT fluorescence intensity is in the order of: Al(III)-SF > SF > curcumin-SF. Also, the ThT fluorescence intensity of the Al(III)-SF solution was higher than that of Al(III)-curcumin-SF (where the Al(III) to curcumin ratio was 1 : 1) mixture solution incubated for 35 h. The results indicate that Al(III) could accelerate the conformation transition and fibrillation of SF, and that curcumin could inhibit Al(III)-induced SF conformation transition.

Fig. 3B shows the CD spectra of Al(III)-SF solutions which were pre-incubated for 35 hours, and then curcumin was added for an additional 1, 3, or 6 h incubation. It is found that the negative peak at 217 nm became weak, which indicates that the conformers of the β-sheet decreased gradually, namely that the curcumin could remold the preformed β-sheet into a random coil. Fig. 3C shows ThT fluorescence in Al(III)-SF solutions which were pre-incubated for 35 hours, and then curcumin was added for an additional 5, 10, 15, or 20 h incubation. ThT fluorescence intensity decreased during incubation, indicating that curcumin could disaggregate the preformed SF fibrils, which is the same as the results from the CD measurement.

3.3 Morphologies of aggregates detected by AFM

Fig. 4 shows the atomic force microscopy (AFM) images of SF samples. Fig. 4A shows the nanofibrils with a height of about 2.5 nm where SF precipitates were formed by evaporating water from the SF solution. This process is similar to that of silkworm spinning together with the water removed from the gland and thus, the protein concentration is increased. Fig. 4B–E are images for SF (B), Al(III)-SF (C), curcumin-SF (D) and Al(III)-curcumin-SF (E) samples which were incubated for 35 h, where the heights of particles are about 4.5 nm (B), 14.0 nm (C), 3.0 nm (D), 6.5 nm (E). Fig. 4F–1–4F-4 are images of Al(III)-SF samples pre-incubated for 35 h and then treated by curcumin for another 5 h (F-1), 10 h (F-2), 15 (F-3), and 20 h (F-4) incubation, where the heights of particles are about 6.5, 6.5, 3.5, 4.5 nm, respectively.

The sizes of Al(III)-SF particles (Fig. 4C) are larger than those of SF (Fig. 4B), which demonstrates that Al(III) promoted the SF aggregation. Comparison of Fig. 4B and C with Fig. 4D and E, shows that curcumin inhibited the SF and Al(III)-induced SF aggregation. Fig. 4F-1–F-3 show the process of nanofibrils disappearing gradually to form oligomers when curcumin was added, and 20 hours later (4F-4), the oligomers become larger.
and the heights become higher, which demonstrated that the oligomers formed the amorphous aggregates.

The morphology of SF precipitates shown in Fig. 4A is different from that shown in other images in Fig. 4, indicating that nanofibril formation may require a higher concentration of protein than particle formation does. The environmental interference from the presence of Al(III) and curcumin in the protein influences the formation of nanofibrils.

3.4 Interaction of Al(III) with SF – an FTIR study

For insight into the interaction between Al(III) and SF on a molecular level, FTIR spectroscopy was used. Fig. 5A shows that in SF films containing 5 mM Al(III) a new broad peak appeared near 680 cm⁻¹, which was not present with the pure SF film. When the concentration of Al(III) was increased to 10 mM, two new sharp peaks at 694 cm⁻¹ and 673 cm⁻¹ clearly existed. It was reported that the stretching vibrations of Al–N bonds...
appear at 621, 672 cm\(^{-1}\), and that those of Al–O bonds appear at 500–750 cm\(^{-1}\). Therefore, it was speculated that the peaks at 680 or 694 and 673 cm\(^{-1}\) might result from the stretching vibration of octahedral Al–O and Al–N bonds, respectively.

In addition, the infrared absorption frequency of amide groups in protein is quite sensitive to the secondary structure. The infrared spectral region within 1700–1600 cm\(^{-1}\), namely amide I, shows high intensity and little interference from other group vibrations, compared with amide II (1600–1500 cm\(^{-1}\)) and amide III (1330–1220 cm\(^{-1}\)), thus amide I is often used to analyze the conformational change of proteins. In the amide I band, the adsorption peaks are generally assigned as: 1653 ± 4 cm\(^{-1}\) to the helix, 1645 ± 4 cm\(^{-1}\) to a random coil, 1625 ± 5 cm\(^{-1}\) and 1675 ± 5 cm\(^{-1}\) to a β-sheet, and 1663 ± 4 cm\(^{-1}\) to a β-turn. Fig. 5B shows the spectra of SF incubated with Al(III) at various concentrations. It is found that the adsorption band at 1625 cm\(^{-1}\) corresponding to the antiparallel β-sheet increased, and the band at 1647 cm\(^{-1}\) corresponding to the random coil decreased as the concentration of Al(III) increased. The results reveal that Al(III) can interact with SF and result in the transition from a random coil to a β-sheet, which is demonstrated by the results from CD in Fig. 2.

4. Discussion

In combination with the results from CD, ANS-FLS, ThT-FLS, UV-Vis scattering, AFM, as well as ATR-FTIR, this research provides an insight into the role of Al(III) and curcumin in affecting the conformation transition of SF.

4.1 Interaction between Al(III) and SF

The FTIR results show that Al(III) can bind to oxygen atoms (absorption peaks at 680 and 694 cm\(^{-1}\)) and nitrogen atoms (absorption peak at 673 cm\(^{-1}\)) (Fig. 5A). There are relatively high contents of serine (Ser, 10.8%), tyrosine (Tyr, 4.9%) and aspartate (Asp, 1.5%) in SF. With –OH groups in the side chains of Ser and Tyr, and –COO in Asp, these amino acid residues are the correct sites for the binding of Al(III) and other metallic ions such as Ca\(^{2+}\) or Mg\(^{2+}\). In addition, lysine, arginine and histidine (His) have nitrogen atoms at the side chain. Therefore,
it is supposed that the bonded nitrogen atoms might come from these amino acids.

4.2 Effect of Al(III) on SF conformation transition and fibrillation

This work shows that Al(III) can promote SF conformation transition. The process of SF aggregation undergoes “nucleation-dependent polymerization”, and Al(III) ions accelerate the process (Fig. 2). SF gradually changed its conformation from random coil to β-sheet as shown by CD measurements (Fig. 2A) as well as by ThT fluorescence (Fig. 2B) where ThT was selectively associated with the β-sheet conformation of protein, and then partially formed the β-sheet folded proteins or folded oligomers, namely the “nucleus” or intermediates which were verified by ANS fluorescence where ANS interacted with hydrophobic blocks of β-sheet proteins because of its hydrophobicity (Fig. 2C). Al(III) ions accelerate the formation of β-sheet SF, which was demonstrated by the ThT fluorescence measurements (Fig. 2D). The resulting aggregates were detected by UV-Vis scattering (Fig. 2E) as well as using AFM images (Fig. 4). During the process of fibrillation and aggregation of SF, Al(III) promoted and accelerated the protein folding. It has been reported that the heavy chain (H-chain) silk fibroins contain Asp and glutamic acid (Glu) at the N-terminus and C-terminus,11,46 and have –COO at the side chains, which causes the terminus to be highly hydrophilic. In addition, there are some hydrophilic spacers, GTGSSGFGPYVA (N/H), GGYSGYEYAWSSESDFGT, located between the long hydrophobic blocks in the central protein region.11 H-chain fibroins have a isoelectric point (pI) of

![Fig. 4](image_url) AFM images of SF precipitates (A); SF incubated for 35 hours (B); SF incubated for 35 hours in the presence of Al(III) (C); SF incubated for 35 hours in the presence of curcumin (D); SF incubated for 35 hours in the presence of Al(III)–curcumin (E); SF and Al(III) co-incubated for 35 hours and curcumin was added for an additional 5 (F-1), 10 (F-2), 15 (F-3), or 20 hours (F-4), respectively.

![Fig. 5](image_url) FTIR spectra of SF in the presence of Al(III) at various concentrations. (A) FTIR spectra of SF in the fingerprint region. (B) FTIR spectra of SF in the amide I region.
4.2, implying that at near neutral pH, the predominant negative charges in SF would prevent intra- and inter-molecular hydrophobic interaction by their strong static electric repulsion, which keeps the hydrophobic blocks from approaching and aggregating.\textsuperscript{26} Interestingly, of all those amino acids binding Al(m), acidic Asp predominates in the hydrophilic N-terminus of the H-chain fibrin, and Ser and Tyr are abundant in the hydrophobic central region. Therefore, in a neutral solution with a pH higher than the \textit{pl} of 4.2, the charges of the amino acids are negative, allowing Al(m) to easily bind to these amino residues, and effectively neutralizing the negative charges in SF, and then reducing the static electric repulsion among the hydrophobic blocks, resulting in the proteins approaching each other easily,\textsuperscript{47,48} and resulting in the intra- and inter-molecular hydrogen bonding between those hydrophobic blocks, and promoting the aggregation of SF. Furthermore, there are 17.2% amino acids such as Ser, Tyr, Asp and Glu,\textsuperscript{43} which are able to bind with Al(m) in SF. The 100 \textmu M Al(m) in the samples used in the fluorescence and turbidity analysis are half of the maximum concentration for binding those amino acids, which is far lower than the saturation concentration for binding, thus the more Al(m) added the more amino acids would be bound, with the acceleration of the SF aggregation. Therefore, Al(m) promoted SF oligomers and aggregation concentration-dependently, which were observed by ANS fluorescence (Fig. 2C), ThT fluorescence (Fig. 2D) and turbidity assay (Fig. 2E).

From the previous discussion, it is suggested that Al(m) can accelerate the nucleation and aggregation of SF. Al(m) in the brain could cause damage of the neural system, because the aggregative capacity of the Al(m)–protein could bring about the dramatic structural changes of the membranes seen in the early stages of AD,\textsuperscript{49,50} meanwhile, the nanofibrils and precipitates caused by Al(m) could induce the tangled structure of proteins and cause the disease to worsen. Therefore, the mechanism of the aggregation of neurodegenerative protein, induced by Al(m), might be similar to that of SF.

### 4.3 Influence of curcumin and Al(m) on SF

It is found that adding curcumin to the SF or Al(m)–SF solutions decreased the intensity of ThT fluorescence, as shown in Fig. 3A, indicating that the \(\beta\)-sheet conformers were decreased, meanwhile, the random coil conformers were increased, as demonstrated by the CD spectrum (Fig. 3B). Furthermore, the ThT fluorescence intensity was decreased (Fig. 3C) and the aggregate sizes were reduced (Fig. 4). Therefore, we suggest that curcumin could disaggregate the preformed \(\beta\)-sheet conformers or nanofibrils into the random coil conformers.

Curcumin, a planar biphenyl molecule, which is similar to ThT, can bind and insert into the plagues proteins.\textsuperscript{31,51} Curcumin may damage the hydrogen bonding between protein chains, and then inhibit the formation of \(\beta\)-sheet conformation, and also the formation of fibrils.\textsuperscript{24}

In addition, aromatic rings have been considered to play an important role during the process of protein fibrillation.\textsuperscript{52–54} Kamihira-Ishijima \textit{et al.}\textsuperscript{54} reported that a phenol ring in islet amyloid polypeptide,\textsuperscript{22–27} for example, can interact with the phenol rings in catechins. Therefore, phenol rings in curcumin were supposed to interact with those amino acids which have aromatic rings such as Tyr and tryptophan. Meanwhile, curcumin has the ability to chelate Al(m), thus inhibiting the process of Al(m)-induced conformation transition of SF from a random coil to \(\beta\)-sheet.\textsuperscript{55}

### 5. Conclusions

The results presented previously show that Al(m) could bind to amino acids (Ser, Tyr, Asp, His) in SF, reduce the static electric repulsion between the hydrophobic blocks in intra- and inter-molecular interactions and the energy barrier required for folding and aggregation of SF, and thus, accelerate nucleation and aggregation. This work also indicates that curcumin could prevent the aggregation of SF, and even disaggregate the preformed SF nanofibrils by changing the \(\beta\)-sheet conformations into random coil ones. Curcumin might be used as a potential drug to prevent and treat AD.

#### Conflict of interest

The authors declare that there are no conflicts of interest.

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