STRUCTURAL ORIGIN OF SILK NANOPARTICLES AND THEIR STABILIZATION

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A naturally occurring biopolymer known as silk fibroin (SF) has several distinctive qualities that make it an ideal vehicle for the delivery of drugs and a variety of therapeutic agents. It has been demonstrated that drugs and biomolecules can be successfully delivered via SF matrices. In this study homogenous SF nanoparticles (SFNP) obtained with a size ranging between 171-207 nm. SFNPs were stabilized with GA (glutaraldehyde) which is known as a cross-linking agent was used to stabilize the SFNPs. The stabilized SFNPs were consistent for 4 days. Circular dichroism (CD) and Fourier-transform infrared spectroscopy (FTIR) studies revealed random-coil to beta structure transition with beta structure reaching up to 59 %. Observed birefringence of Congo red staining in a polarized microscope indicates beta amyloid formation. Thus, SFNPs composed of amyloid nanoparticles.

Keywords: silk fibroin, silk nanoparticles, cross linker, amyloids.
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1. INTRODUCTION

Silk fibroin is a natural biomaterial with important biological and mechanical properties. SF retains a superb blend of mechanical and biological characteristics that are difficult to find together in other natural or synthetic materials, and which are mainly determined by its secondary structure. It is one of the toughest biomaterials found in nature, with toughness higher than Kevlar (a para-aramid synthetic fiber used to make bulletproof vests), showing high tensile strength, stiffness and remarkable stability, especially when processed at high temperatures (above 250°C), without altering its stability and structure [1]. As compared to other silks from the silkworm family, B. mori SF has the highest tensile strength and modulus.

There are numerous and inexpensive sources of this natural polymer because silkworms are widely cultivated for the textile industry. The raw cocoon filaments are made up of fibroin (75%) and sericin proteins (25%) where two parallel fibroin fibers are bound together by a coating of sericin proteins [2-4]. For medical applications, the sericin component must be properly removed so the fibroin fibers can be further processed into various materials [5].

Nowadays, the use of nanoparticles to deliver small molecules is rapidly growing in many fields. The unique properties of nanoparticles could overcome the limitations of using small molecules as therapeutic agents in biomedical applications. The fabrication of silk nanospheres is a more challenging area of research than the fabrication of silk microspheres because of the high molecular weight and protein nature of silk [6]. Many different techniques are used to produce nanoparticles from regenerated SF by taking advantage of the hydrophilic and hydrophobic chain interactions that govern silk's ability to self-assemble. In this study we used dehydration method to produce silk nanoparticles which then treated with glutaraldehyde (GA) as the cross-linking agent to stabilize SF nanoparticles. Crosslinking rigidifies and deactivates many biological functions, so in this way, glutaraldehyde solutions are used as biocides and as fixative [7]. Among the many protein crosslinking substances on the market, GA has unquestionably found the broadest use in a variety of industries, including biomedical and pharmaceutical sciences, histochemistry, microscopy, cytochemistry, the leather tanning industry, enzyme technology, and chemical sterilization. A clear, colourless to pale straw-coloured, fragrant oily liquid, GA is a linear, 5-carbon dialdehyde that is soluble in all quantities in water, alcohol, and organic solvents. It is mostly offered as acidic aqueous solutions with a pH range of 3.0-4.0, with concentrations ranging from less than 2% to 70% (w/v).

The results provided here reveals, that GA can stabilize the silk nanoparticles for several days without any aggregation which prolongs the life of silk nanoparticles by reducing the cost of production. For medical purposes it could increase the storage time and target particles for drug delivery. Using acetone as dehydrating agent we fabricated silk nanoparticles of 171-207 nm size. SFNPs treated with glutaraldehyde (GA) are able to preserve their stability up to 4 days in a row. Results from CD and FTIR concluded a transformation from random coil to beta structure to 59%. Amyloid formation was verified with Congo red staining on a birefringence in a polarized microscope.

2. EXPERIMENTAL SECTION

2.1 Sample preparation

A commonly used method was applied to extract SF from the silk cocoons made by B. mori silkworms [8]. To remove sericin, silk cocoons were cut into small pieces and cooked for 30 minutes in 0.02 M Na_2CO_3 (Fisher Science). The resulting SF was dissolved in 10.5 M LiBr solution (Sigma-Aldrich) at

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60ºC for 4 hours and dialyzed against deionized water at 4ºC for 43 hours. After dialysis, the final concentration of SF was between 23 and 27 mg/mL. UV measurements of the SF concentration were made using the formula ε^{275nm}= 1.064 cm-1(mg/mL)$^{-1}$ [9] and MW = 390 kDa.

SF solution was added to acetone dropwise and at the end of the experiment the concentration of acetone was not below 70%.

GA was purchased from Sigma-Aldrich. GA was dissolved in water to obtain with 8% glutaraldehyde solution [9] which was then added into treated SF and kept on magnetic stirrer for 12 hours at a room temperature. Then SFNPs were extracted from the solution via centrifugation.

2.2 Circular dichroism spectroscopy

Circular dichroism spectra were measured on a Chirascan V100 (Applied Photophysics, UK) at room temperature under constant nitrogen flush over a wavelength range of 180-260 nm using step size of 0.5 nm and bandwidth of 1 nm. The quartz cuvette with path length of 0.2 mm was used for the measurements. Chirascan V100 is equipped with a solid state Large Area Avalanche Photodiode (LAAPD) detector, allowing good signal detection at 180 nm.

2.3 Fourier-transform infrared spectroscopy

Infrared absorption spectra were recorded with a Vertex 70 FTIR spectrometer (Bruker Optics, Ettlingen, Germany). FTIR is a useful tool to monitor SF structural changes in various conditions. Protein materials are characterized by the position and intensity of the amide I, amide II, and amide III bands. However, Amide I band was used to calculate the secondary structure of the protein. Amide I band of the proteins were fit to Gaussian components using Origin Lab 2016 Software.

2.4 Congo Red binding assay

Congo Red is one of the most widely used amyloid dyes. Because of its extensive β - sheet structure CR binds to amyloid proteins. When a dye binds to amyloid, its absorbance spectrum is changed [10].

The Congo Red (CR) concentration was calculated using the extinction coefficient ε_{490nm} = $3.3x10^4$ [11]. In deionized water, the dye was freshly produced, and samples were adjusted to achieve a final concentration of 20 μM. A Shimadzu UV-2700 spectrophotometer was used to record the mixture's absorption spectra from 400 to 700 nm.

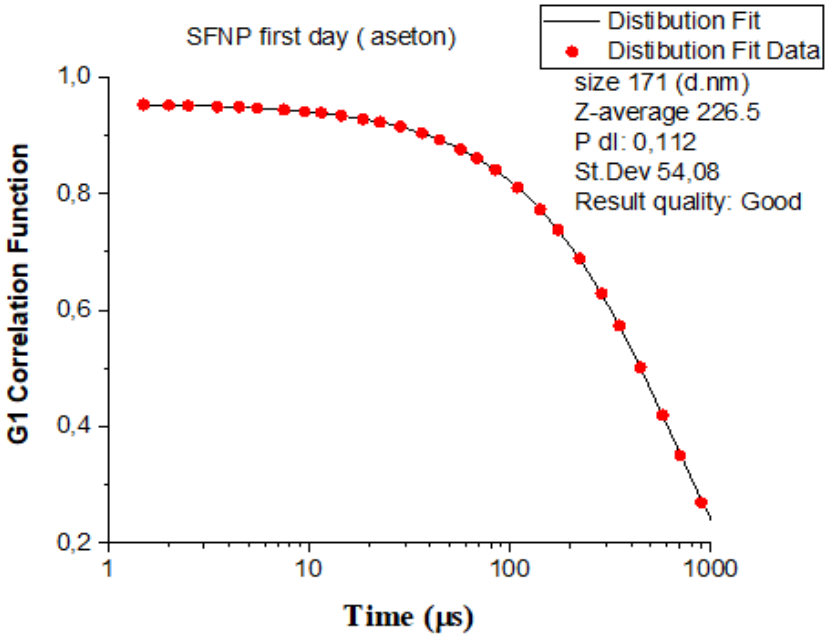
By using a microscope and crossed polarizers, CR staining experiments were carried out to observe the beta-amyloid aggregates of the SF solution. Congo red molecules are oriented on amyloid fibrils, which causes the dye to become birefringent. Amyloid stained by Congo red usually shows apple-green birefringence in cross-polarized light [12].

3. RESULTS AND DISCUSSION

3.1. Particle size and stability analysis

To find out the size of the obtained silk nanoparticles Dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) measurements operating at λ= 633 nm was used 4 days in a row. As shown in the figures below (Figure 1 and 2) the solution of fibroin nanoparticles prepared with acetone showed a single peak with a diameter of 171 nm on the first day following with 188 nm, 207 nm, 189 nm on other days respectively. Silk nanoparticles showed a good stability even after several days without aggregation.

As can be seen in the figure 2 obtained silk nanoparticles are basically homogenous. During 4 days we haven't seen any changes in size of the nanoparticles. On average the size of SFNPs are 189 ±15 nm.



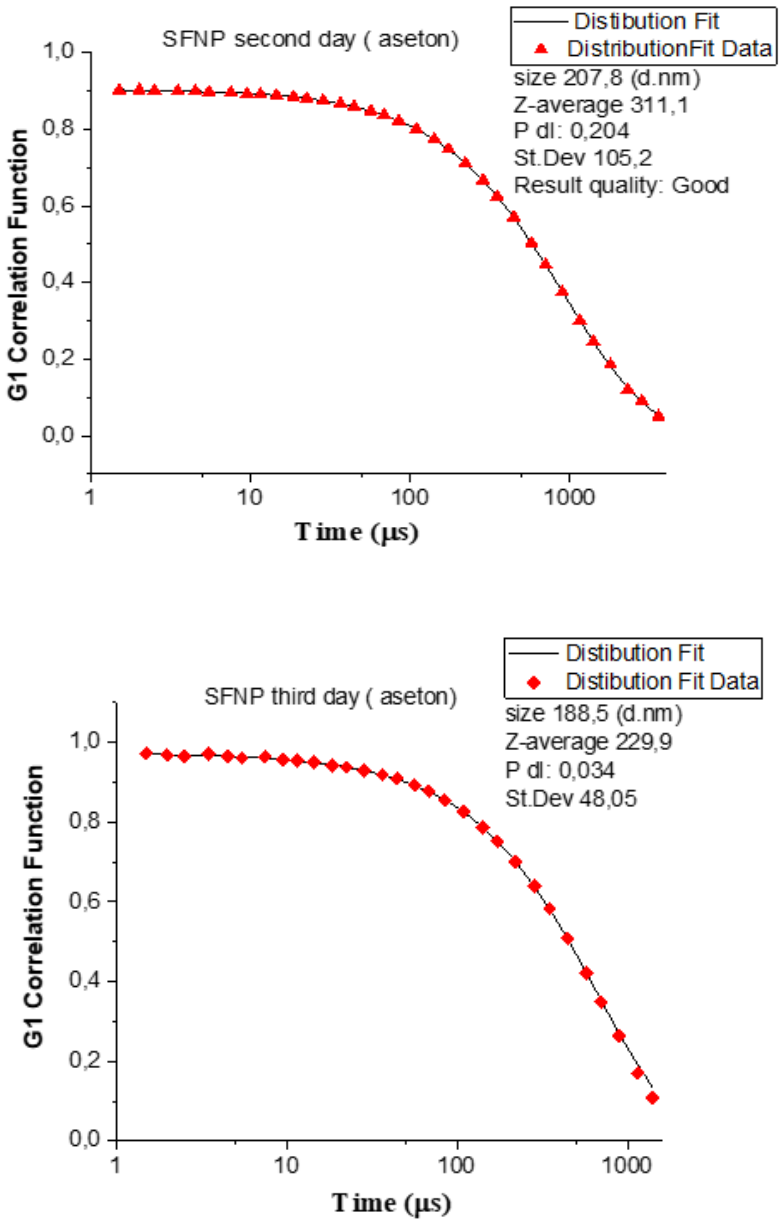


Fig 1. Fitting analysis of SFNPs in Dynamic Light Scattering.

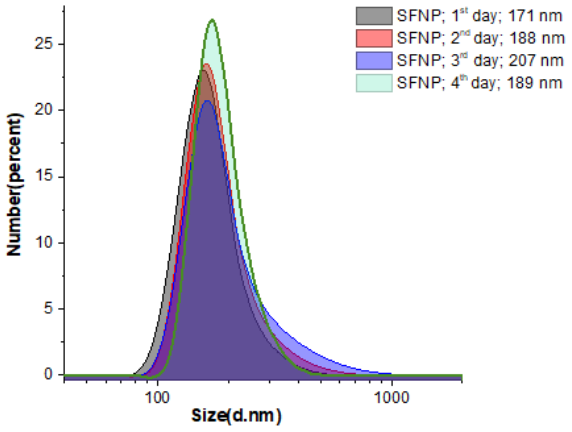


Fig. 2. Size distribution of SFNPs in Dynamic Light Scattering.

3.2. Secondary structure analysis

3.2.1. CD analysis

CD is one of the best measurements to characterize SF in solution. It is a powerful technique to evaluate any conformational changes in the structure of proteins [13]. The secondary structure of SF can be determined by CD spectroscopy in the 'far-UV' spectral region (190–250 nm).

In figure 3, SF solution displayed a single negative band at around 195 nm, characteristic of random coil conformation, whereas SFNP showed a characteristic β-sheet structure with positive peak at 195 nm and negative at 220 nm respectively.

Conformational changes of proteins can be assessed using Fourier-transform infrared spectroscopy (FTIR) as well. Amide I vibrational band is the most sensitive spectral region used to study protein secondary structure. Figure 4 illustrates infrared spectra between 1560-1720 cm^{-1} for SF solution. Amide 1 band of the SF solution estimated secondary structure of the silk. Beta sheet formation was clearly seen under acetone treatment of silk nanoparticles. Assignments of the components shown in Table 1.

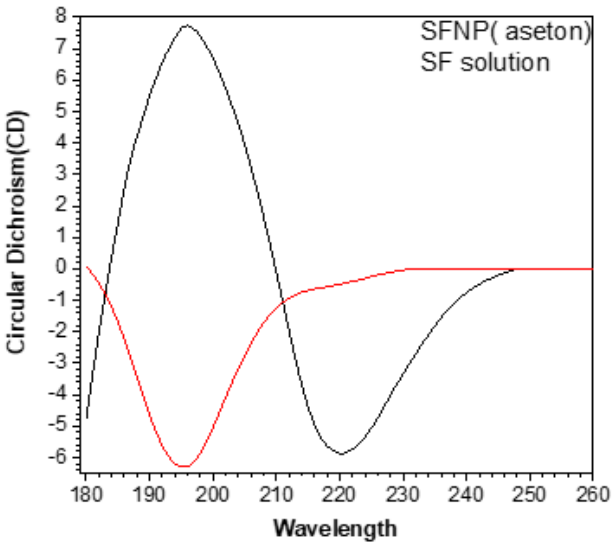


Fig. 3. Far-UV CD spectra of SF. Black and red lines represent SFNP and SF solutions, respectively.

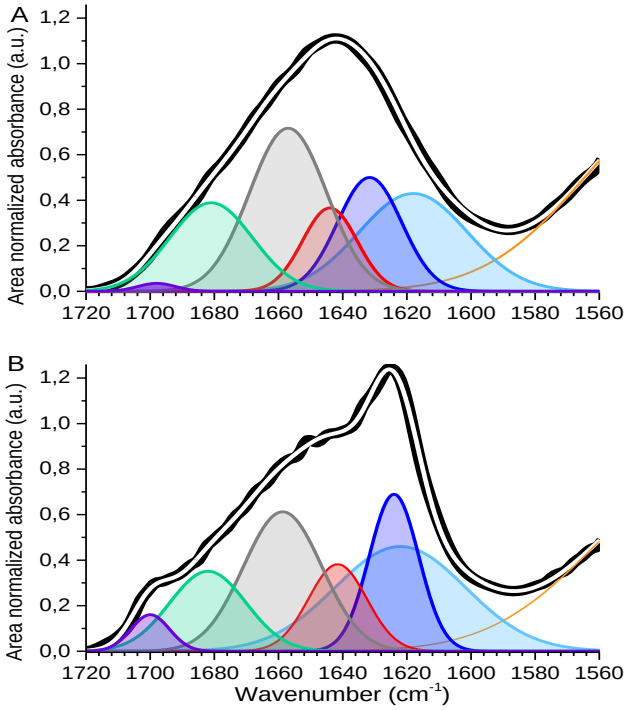


Fig. 4. Infrared spectra of SF.

Table 1.

Estimation of secondary structure elements for various SF films from deconvolution of Amide I bands of FTIR spectra.

	1700cm^{-1} Intermolecular β-sheet (antiparallel)	1683cm^{-1} β-turn	1658cm^{-1} Random coil	1641cm^{-1} α-helix	1622cm^{-1} Intramolecular β-sheet	1615cm^{-1} Intermolecular β-sheet (aggregate)	1622cm^{-1} Side chain	Total β-structure
SF films	0,7	17,2	29,4	10,7	17,1	24,9	0	59.9
SFNP	3,1	13,6	24,2	11,2	17,3	30,5	0	64.5

All data show that we have silk fibroin aggregates, however, not all β-sheet aggregates are amyloids. Therefore, we used Congo Red staining assay to find out whether aggregates are amyloid or not.

Under a cross- polarized microscope, the green birefringence dots were seen in the SFNP solution with CR. According to this finding, attached CR molecules are arranged in an amyloid-like manner, with alignment along the fibre axis. The birefringence is visible in other protein aggregates when bound CR molecules are arranged arbitrarily. In figure 6 amyloid fibrils were seen during birefringence with CR.

It has been shown that CR is dichroic and, as a result, the change in intensity of red can be easy to see in smears of Congo red [14].

UV-VIS spectroscopy was also used to examine the CR-SFNP complex and to show the specific binding of CR with silk fibroin nanoparticles. Upon for the figure 7 increasing concentration of silk fibroin indicates red shift of the absorbance band of Congo Red dye.

As CR binds to SFNP, the resulting CR-SFNP complex changes the absorbance band according to the CR concentration. As concentrations changed, the isosbestic point shifted, leaving only two states of CR: bound and free. There is no intermediary structure in the transition from the random coil to the beta-sheet structure, according to the observed isodichroic point.

Fig. 5. Birefringence image of SFNP-CR complexes.

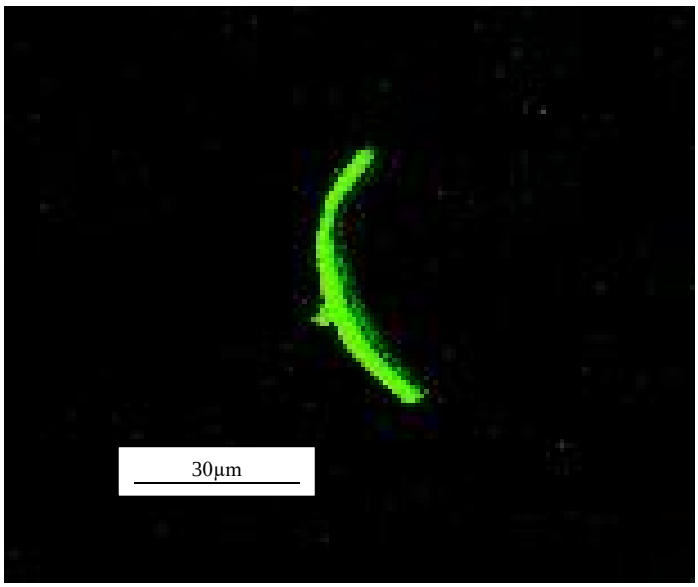


Fig. 6. Birefringence image of SFNP-CR complexes with amyloid fibril.

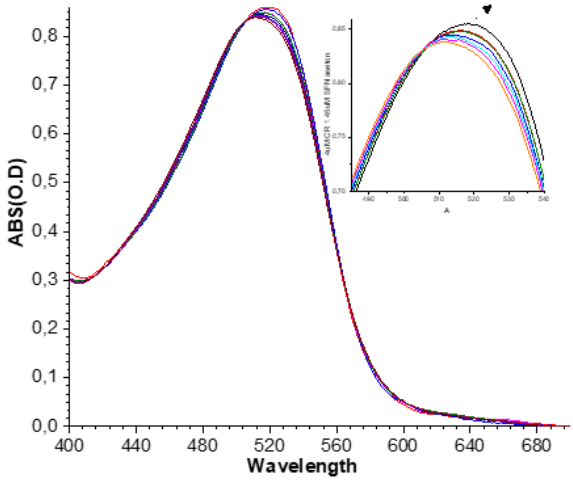


Fig. 7. UV-VIS spectra of CR titrated with increasing concentrations of SF in range of 2 µM- 20 µM.

CONCLUSION

In this research we aimed to achieve the stability of SFNPs by processing them with GA solution. As a result of this procedure, the produced silk nanoparticles showed no signs of clumping even after several days with a constant molecular size of 189±15 nm on average which was ideal for drug delivery and other therapeutic targets. We further presented proof on the basis of CD and FTIR that conformational changes occurred in secondary structure SF nanoparticles where random coil transformed into β-sheet. With CR binding assay we confirmed that β-sheet aggregates were amyloids.

This novel fabrication technique not only increase the productivity of silk-based nanoparticles but also promise to extend the duration of storage for various medical uses.

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