Scientific Research Publishing

Advances in Nanoparticles, 2024, 13, 1-9
https://www.scirp.org/journal/anp
ISSN Online: 2169-0529
ISSN Print: 2169-0510

Research on the Development of Fibroin and Nano-Fiber from Silk Cocoons for Regenerated Tissue Engineering Applications by Electro-Spinning

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How to cite this paper: Hasan, Md.K. and Ding, X.B. (2024) Research on the Development of Fibroin and Nano-Fiber from Silk Cocoons for Regenerated Tissue Engineering Applications by Electro-Spinning. *Advances in Nanoparticles*, **13**, 1-9.
https://doi.org/10.4236/anp.2024.131001

Received: January 19, 2024
Accepted: February 20, 2024
Published: February 23, 2024

Abstract

In this paper, the main goal is to prepare silk fibroin nano-fiber, which is used for regenerated tissue applications. Silk scaffold nano-fibers made by electro-spinning technology can be used in regenerated tissue applications. The purpose of the research is to prepare a silk-fibroin nano-fiber solution for potential applications in tissue engineering. Using a degumming process, pure silk fibroin protein is extracted from silk cocoons. The protein solution for fibroin is purified, and the protein content is determined. The precise chemical composition, exact temperature, time, voltage, distance, ratio, and humidity all have a huge impact on degumming, solubility, and electro-spinning nano-fibers. The SEM investigates the morphology of silk fibroin nano-fibres at different magnifications. It also reveals the surface condition, fiber orientation, and fiber thickness of the silk fibroin nano-fiber. The results show that regenerated silk fibroin and nano-fiber can be used in silk fibroin scaffolds for various tissue engineering applications.

Keywords

Silk Fibroin, Scaffold, Electro-Spinning, NANO-FIBER, Tissue Engineering

1. Introduction

Recently, silk fibroin, a natural macromolecule of silkworms has been the focus of the attention of scientists for a long time. For biomedical sutures, silk fibroin, a fibrous protein from the Bombyx mori silkworm, has been used for a long time [1]. A new class of silk fibroin (SF) has newly proposed as an excellent candidate

for tissue regeneration and drug carriers. SF nano-fibers are investigating the physical and chemical properties of silk-fibroin composite scaffolds. For example, it has a long history of using sutures and artificial ligaments. Silk is a biological material that offers a wide range of mechanical and functional properties for biomedical applications in terms of mechanical stability and mechanical integrity. electro-spinning technology has recently been used to produce silk fibroin nano-fibers, which can be used in regenerative tissue applications [2]. Silk fibers form a group of proteins consisting of 18 amino acids, including glycine, alanine, and serine [3]. The use of three-dimensional tissue engineering scaffolds is advantageous because they provide space for immobilization, improve surface area, support large cell masses, and form specific structures [4] [5]. In vivo, degradation, a lack of active groups, and slow organic solvents prevent the use of synthetic polymers by adding cell growth factors. Recent studies have shown that silk fibroin is suitable for binding animal cells grown in vitro like collagen and is also essential for cell function [6] [7]. For example, Wu et al randomly wound SF fibers to form a net in which animal chondrocytes are three-dimensionally cultured, and their findings showed that SF can be used as a good scaffold for chondrocytes in three-dimensional culture. Silk fibroin fibers made from silkworms are hard at room temperature and in aqueous solutions, while synthetic materials with similar properties must be treated at high temperatures and with less environmentally friendly solvents. To satisfy a specific medicinal application, silk must be regenerated to a desired condition. Aqueous silk fibroin solutions are prepared by dissolving SF in soluble neutral salts such as calcium chloride or lithium bromide, for example [8]. Silk fibroin is soluble in high-ionic-strength aqueous solutions of chaotropic salts, which destabilize proteins in solution and improve their solubility [9]. Many researchers have tried to find suitable solvents to prepare silk fibroin solutions that can then be spun into fibers, but there is very little work in the literature on the complete processing of silk, process optimization, and silk solution properties. In this article, a reconstituted form of silk fibroin solution for tissue application is prepared and characterized.

2. Materials and Methods

Materials

Silkworm cocoons are obtained from the Hangzhou Academy of Agriculture Science (Hangzhou, China). Ethanol (C_2H_5OH), calcium chloride, anhydrous ($CaCl_2$), and formic acid are purchased from Hangzhou Gaojing Fine Chemical Industry Co. Ltd. Soap is purchased from a normal shop.

3. Fibroin

3.1. Preparation for Making Fibroin

For making silk fibroin, we take 20 g of silk cocoons without dust, 20 g of soap, and 1000 ml of deionized water (**Figure 1**). Heat the water and keep the temperature at 98˚C. We mix the soap, and when the soap dissolves in water properly,

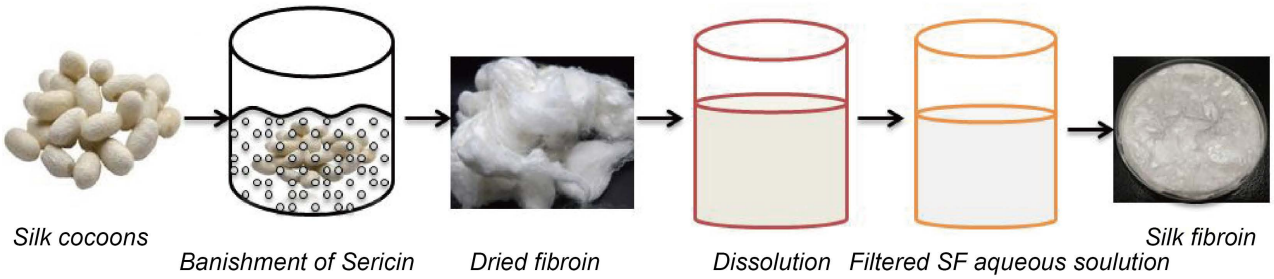


Figure 1. The process of making silk fibroin.

we add the silkworm cocoons and keep them for 60 minutes. 1 hour later, we stop heating and can see the fibre now, not the cocoons. We wash the fibre with deionized water and repeat this process. To get this silk fibre, we keep it in the dryer at 55˚C for 12 - 24 hours [1].

3.2. Dissolving

Ethanol (C_2H_5OH) = 44 ml
Calcium chloride (C_aCl_2) = 42.8 g
Water (H_2O) = 56 ml
Ratio: C_2H_5OH:C_aCl_2:H_2O = 2:1:8

In the beginning, we add 42.8 g of $CaCl_2$ and 56 ml of H_2O and wait until the heat is completely released (**Figure 1**). When the CaCl2 and H_2O mixture is cool, mix 44 ml of C_2H_5OH. After that, we measure 10 - 12 g of degum silk fibroin, mix it with that solution, and dissolve it at 75˚C for 2 h [1]. When the times have finished, take it out and wait for the room temperature to change, and then use three pieces of mashed fabric to filter the solution twice. Then keep that solution in a pipe poly and muffle that poly on both sides as if the air can't enter inside the poly. We kept that solution in the deionizer water, and every 12 hours later we changed that water and used new water. We continue this change for about 3 - 4 days. After that, we divide this solution into six equal parts and use the centrifuge machine at 8000 RPM for 8 - 10 minutes. This process is continued 3 - 4 times. Then we collect that solution on a small, round plastic plate and keep it in the deep fridge for about 24 - 36 hours. After that, we muffled the plate with thin poly and made some holes in it. We keep that solution plate in the vacuum machine for 48 hours. When the time has passed, we could use it for the next step.

4. Nano-Fiber

4.1. Preparation of Spinning Drop

After getting the silk fibroin, we make the electro-spinning solution. We take only 1.0 g of silk fibroin and dissolve it in 10 ml of formic acid. We keep this spinning solution for 12 hours under stirred magnetic force, and then we let it stand for 1 hour for deforming treatment and also until all the bubbles are released (**Figure 2**). Transfer it to a glass syringe; the row of excess bubbles is the exit,

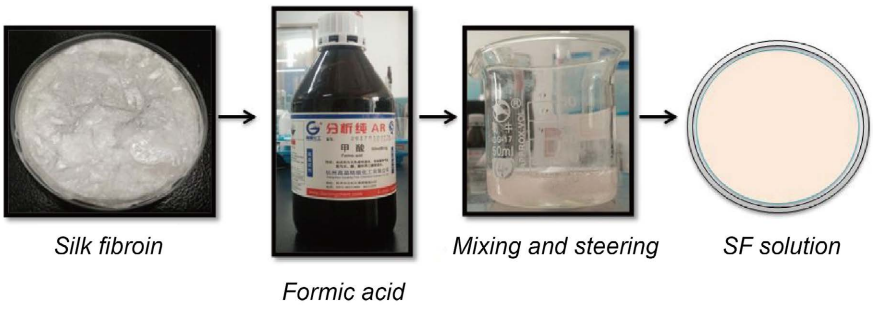


Figure 2. The process of making silk fibroin solution.

and then is used for the electro-spinning drop.

4.2. Preparation of Silk Fibroin Nano-Fibers

The above spinning solution is transferred into a glass syringe equipped with a stainless steel capillary nozzle, and this nozzle is connected to a high-voltage power supply (**Figure 3**). In this experiment, the voltage is set to 14 kV, the feed rate of the solution is 0.20 ml/h, the distance between the syringe needle and the receiving foil plate is adjusted to 14 cm, the spinning temperature is kept between 20˚ and 30˚C, and the humidity is 55% [2].

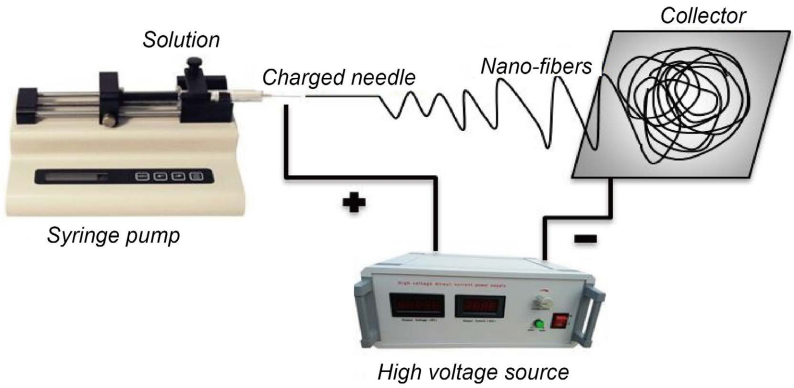


Figure 3. The process of making electro-spinning nano-fiber.

5. Experimental Part

5.1. Degumming Process of Silk Cocoons

The process for the removal of sericin in chemical degumming is a mixture of different effects, such as dispersion or solubilization and hydrolysis of different sericin polypeptides [10]. As heavy alkaline compounds are added to the degumming bath, hydrolysis is the dominant mode of action. As a result, appropriate protocols for monitoring process parameters such as temperature, time, and soap are implemented to achieve successful sericin removal without causing the hydrolytic deterioration of fibers, which is readily triggered by the presence of harsh chemicals in the treatment bath. Every analysis begins with a 20-gram sample of silk cocoon fiber.

In this experiment, we use 20 g of silk cocoons without dust, 20 g of soap, and 1000 ml of deionized water, and the water temperature of 98˚C is kept constant. We mix the soap, and when the soap dissolves in water properly, we add the silkworm cocoons and keep them for 60 minutes. Due to the solution and the temperature, sericin is lost from the silk fiber. After 1 hour, we stop heating, and we see its fiber now, not cocoons. We wash the fiber with deionized water and repeat this process (**Figure 4**). When we get this silk fiber, we keep it in the dryer at 55˚C for 12 - 24 hours [1].

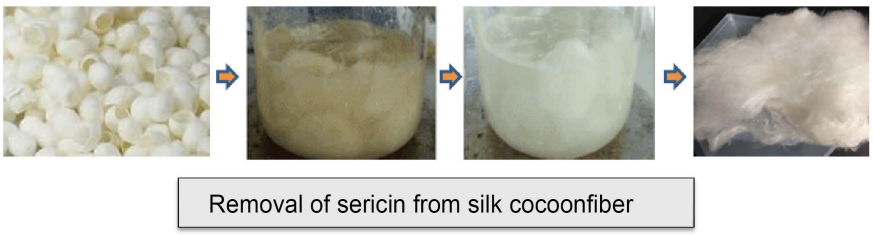


Figure 4. The process of removing sericin from silk cocoons.

In this figure, we can see the time is increasing and on the other hand, the degumming percentage is increased (**Figure 5**). When the time is increased to 0, 10, 20, 30, 40, 50, and 60 minutes then the degumming percentage reaches 0, 5, 20, 50, 75, 90, and 100 percent. Both indicate a positive changing side.

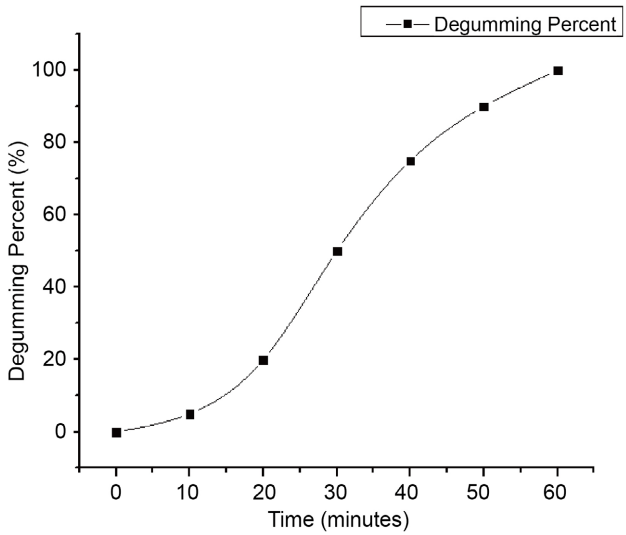


Figure 5. Effect of degumming time and degumming percentage.

5.2. Dissolving and Fibroin Making Process of Silk Fiber

In this study, we select a ratio of: C_2H_5OH: $CaCl_2$: H_2O = 2:1:8, add 42.8 g of $CaCl_2$ and 56 ml of H_2O, and wait till the heat is completely released. When the $CaCl_2$ and H_2O mixture cools, mix 44 ml of C_2H_5OH. After that, we take 10 - 12 grams of degummed silk fibroin, mix it with that solution, and dissolve it at 75˚C for 120 minutes [1].

After 120 minutes later, we take that solution out and wait for the room temperature condition, and then use three pieces of mash fabric to filter the solution twice. We keep that solution in the deionized water, and every 12 hours later we change the deionized water. We continue this change for about 3 - 4 days. After 3 - 4 days later, we take that solution, divide it into 6 parts equally, and put that solution into the centrifuge machine at 8000 RPM for 8 - 10 minutes. We use this centrifuge machine 3 - 4 times to do this same process. Then we collect that solution into a small round plastic plate and keep this solution in the deep fridge for about 24 - 36 hours. After that, we muffled the plate with thin poly and made some holes in it. We keep this solution plate on the vacuum machine for 48 hours (Figure 6). When the process is finished, we get silk fibroin.

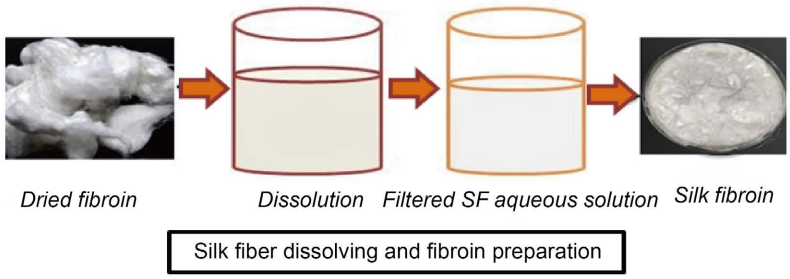


Figure 6. Dissolving process of silk fibers and fibroin-making process.

In this figure, we can see the time is increasing and on the other hand, the dissolving percentage is increasing (Figure 7). When the time is increased by 0, 20, 40, 60, 80, 100, and 120 minutes then the dissolving percentage reaches 0, 10, 30, 55, 75, 90, and 100 percent. The gradually dissolving percentage increases over time, and both sides show positive changes when we repeat the silk cocoon degumming process.

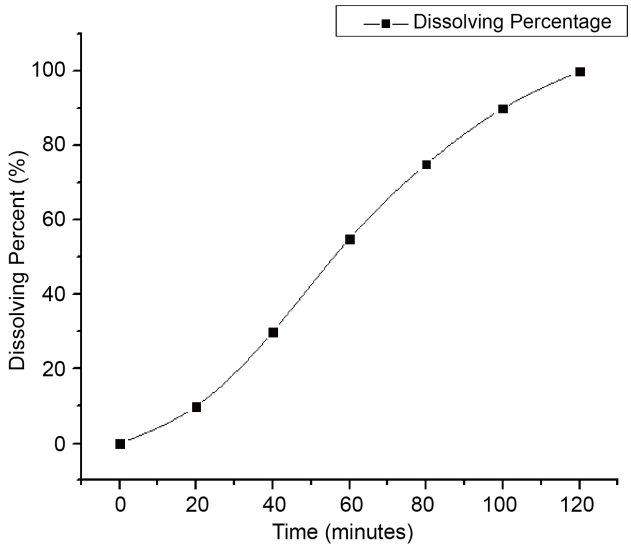


Figure 7. Effect of time and dissolving percentage.

5.3. Preparation of Regenerated Silk Fibroin Solution and Nano-Fibers

After getting the silk fibroin, we are ready to make the electro-spinning solution. We take 1.0 g of silk fibroin, dissolve it in 10 ml of formic acid, and we keep this solution for 12 hours under a stirring magnetic force. 12 hours later, we let stand for 1 hour for deforming treatment and also for releasing all the bubbles. We transfer the solution into the glass syringe and make sure all the excess bubbles are released. The glass syringe is equipped with a stainless steel capillary nozzle, and this nozzle is connected to a high-voltage power supply. In this experiment, the voltage applied is set to 14 kV, the feed rate of the solution is 0.20 ml/h, the distance between the syringe needle and the receiving foil plate is adjusted to 14 cm, the spinning temperature is kept between 20 and 30˚C, and the humidity is 55% [2]. A few hours later, we get the nano-fiber for regeneration (**Figure 8**).

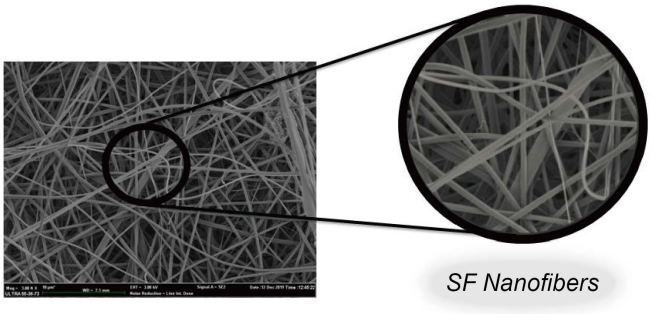
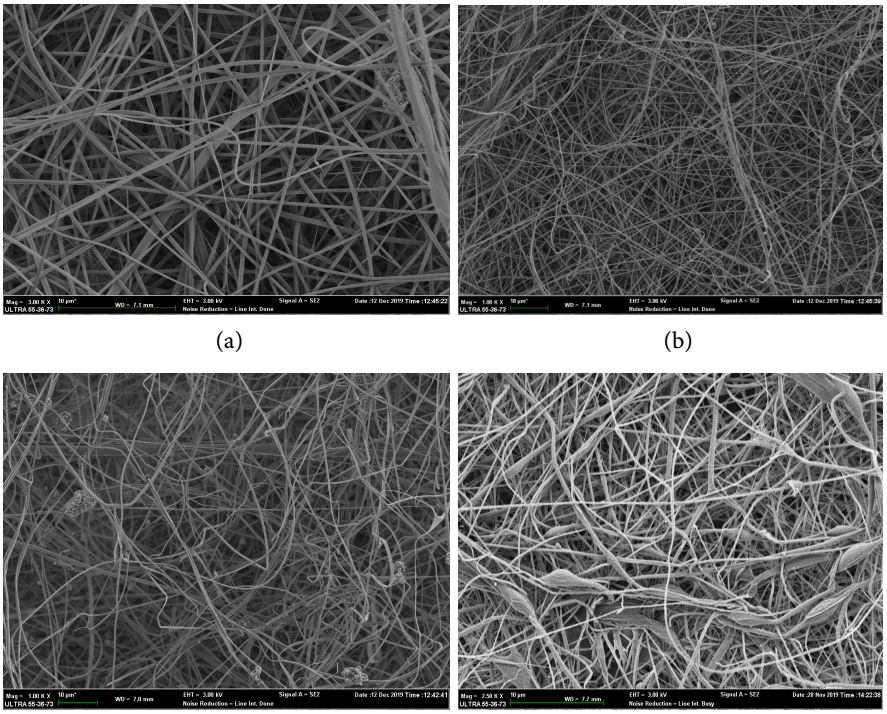


Figure 8. Sample of produced Nano-fiber.

5.4. Scanning Electron Microscopy of Silk NANO-Fiber

When we start the electro-spinning process by using the flowing feed ratio, voltage, distance, temperature, and humidity, we get the nano-fibers. Which we can use for regenerated tissue engineering applications. We tested these nano-fibers by SEM (scanning electron micrographs). The results of scanning electron micrographs and illustrations of silk fibroin nano-fiber morphologies are given below.

The SEM observation of silk fibers obtained by different amounts of silk fibroin, different amounts of silk fibroin solution feed ratio, different distances, different temperatures, and different humidity is shown in **Figure 9**. It confirms the presence of silk nano-fiber on the surface, which we can use in regenerated applications. The presence of uniform silk nano-fibers is greater when we have used 1.0 g and 1.20 g of silk fibroin and 10 ml of formic acid at 14 kV, a feed ratio of 0.20 ml/h, a distance of 14 cm, a temperature between 20˚C and 30˚C, and a humidity of 55%, as shown in **Figure 9(a)** and **Figure 9(b)**. On the other side, we got uniform silk nano-fiber and knots on different surfaces, as shown in **Figure 9(c)** and **Figure 9(d)**. The closer SEM examination of silk nano-fiber showed that the surface is clean (**Figure 9(a)** and **Figure 9(b)**). On the other hand, we got knots on the surfaces (**Figure 9(b)** and **Figure 9(c)**). It is interesting to note



(a) (b)

(c) (d)

Figure 9. shows scanning electron micrographs illustrating differently regenerated silk fibroin nano-fiber morphologies. (a) electro-spinning by 1.0g silk fibroin and 10 ml formic acid, 14 kV, feed ratio 0.20 ml/h, distance 14 cm, temperature between 20˚C and 30˚C, and humidity 55%. (b) electro-spinning by 1.20 g silk fibroin and 10 ml formic acid, 14 kV, feed ratio 0.20 ml/h, distance 14 cm, temperature between 20˚C and 25˚C, and humidity of 55%. (c) electro-spinning by 1.5 g silk fibroin and 10 ml formic acid, 14 kV, feed ratio 0.18 ml/h, distance 14 cm, temperature between 20° and 30˚C, and humidity of 55%. (d) electro-spinning by 1.7 g silk fibroin and 10 ml formic acid, 14 kV, feed ratio 0.19 ml/h, distance 14 cm, temperature between 20˚ and 25˚C, and humidity of 55%.

that when we change the amount of fibroin, feed ratio, temperature, and humidity, we get different types of regenerated silk nano-fiber. The silk nano-fiber diameter obtained is 10 μm or 10,000 nm.

6. Conclusion

In this paper, the regenerated silk nano-fiber is investigated. This work is done to make the better-regenerated silk nano-fiber by using a silk fibroin solution. To prepare the electro-spinning solution, we cross many steps, such as the degumming process, dissolving process, silk fibroin preparation, and fibroin dissolving. In most cases, the time has increased, and the percentage has also increased proportionally. We found the better-regenerated silk nano-fiber quality when we used 1.0 g of silk fibroin, 10 ml of formic acid, a spinning drop feed ratio of 20 ml/h, a voltage of 14 kV, a distance of 14 cm, a temperature of 20˚C - 30˚C, and a humidity of 55%. Most of the time, we got knots into the silk nano-fiber surface. These knots are confirmed by the SEM test. It is impossible to see the single nano-fiber and the nano-fiber knots. Based on the SEM test results,

it is confirmed that the quality of the silk nano-fiber is good and we can use this silk nano-fiber for regenerated tissue engineering applications.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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