Silk fibroin from silk fibrous waste: characterization and electrospinning

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Abstract. In this work, the effect of fabrication parameters on silk fibroin (SF) nonwovens obtained by electrospinning were evaluated. Additionally, the relationship between secondary structures and thermal stability of the protein materials, with morphological characteristics of nonwovens obtained were analyzed. Silk fibroin in formic acid solution at 10% w/w was electrospun at 8 cm varying the ratio voltage/distance and flow rate. The nonwovens morphology was observed by Scanning Electron Microscopy (SEM) and fibers diameter were determined with ImageJ software. The changes in the secondary structure of silk fibroin before and after electrospinning were studied by Fourier Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC) to evaluate the effect of electrospinning process in the molecular structure of SF. Optimum morphology of SF nonwovens were obtained at R = 1 kV/cm and low flow rate, these process parameters were related to higher contents of crystalline structures in the materials. Results showed that SF nonwovens obtained under controlled process parameters hold great potential to be utilized in several applications such as tissue engineering.

1. Introduction
Silk fibroin (SF) is the predominant protein in the silk fibrous waste (SFW), which are generated during the manufacturing process of silk textiles. Colombian silk industry produces fibrous wastes from rejected cocoons, residual pieces of cocoons, fibers, yarns, and others, which represent about 30% of cocoon weight and cannot be utilized in the manufacture of textile products [1] [2]. For Colombian sericultors, the development of new value-added products is important in order to increase the sustainability of their productive chain, for example, by transforming SFW into SF. SF has been explored as a versatile biomaterial for the formation of films, porous scaffolds and nonwovens for various biomedical applications, due to its biocompatibility, slow degradability and robust mechanical properties [3][4].

In the electrospinning process, there are different kind of parameters related to solution parameters (polymer type, solution concentration, and solvent properties); processing parameters (voltage, distance between syringe and collector, and flow rate); and environmental parameters (temperature, atmosphere pressure, and relative humidity) [5]. An appropriated election of these parameters will defined the morphology and characteristics of the nonwovens for medical applications such as controlled drug release and active ingredients for use in tissue engineering [6].

SF is a protein with a secondary structure conformed by α-helices, β-sheets, β-turn and random coil, which evaluated by FTIR vibrate allow different frequencies associated with individual secondary
folding, depending on the differential pattern in H-bonding and geometric orientations of amide bonds [7]. The applications for SF are related to the formation of the highly periodic crystalline regions [8]. The β-sheets serve as physical cross-links in silk-based biomaterials, providing mechanical stability and water insolubility, parameters directly related to degradation rates [4]. The ratio of different secondary structures has been used to examine the molecular conformation of silk, which strongly affect the properties of the SF materials as films or nonwovens [9].

In this work, the effect of fabrication parameters of SF nonwovens by electrospinning were evaluated, and the relationship between secondary structures and thermal stability, with morphological characteristics of nonwovens obtained were analyzed. Results showed that SF nonwovens obtained under controlled process parameters hold great potential to be utilized in several applications such as tissue engineering.

2. Experimental Section

2.1 Preparation of SF films

Silk fibrous wastes (SFW) were degummed in aqueous solution of Na2CO3-0.5% w/v at boiling temperature during 30 min. Degummed SFW were filtered, rinsed with warm water, dried at 60 °C for 24 h, and dissolved using LiBr 9.3 M at 60 °C for 4 h. SF solution was filtered, dialyzed, centrifuged and filtered. After that, the solutions were casted and dried at 35 °C for 72 h to obtain SF films [3].

2.2 Rheological behavior of SF/FA solutions

SF films were cut into small pieces and dissolved in formic acid (FA) at 10 w/w% under constant stirring. The rheological behavior of the solution was studied with a Bholin Instrument CVO rheometer (Malvern, UK), using a cone-plate (4 °/40 mm) geometry, with a gap of 0.150 mm, at room temperature. Tests were performed in a shear rate range from 0.1 to 1000 1/s, and the viscosity of the solution was measured among this range.

2.3 Electrospinning of SF solutions

The electrospinning process was performed using a set up equipped with a syringe with a metallic needle 21G. The anode of a power supply was connected to the needle, and the cathode was connected to a flat metal plate to be used as static collector. The process was developed in a distance from the top of the needle to the collector of 8 cm, at voltages of 8 and 16 kV to obtain relations R (voltage/distance) of 1 kV/cm and 2 kV/cm respectively. Using flow rates of 0.05 and 0.1 ml/h.

2.4 Morphological characterization of nonwoven

The nonwoven morphology was characterized by a SEM JSM 6490-LV (JEOL, Japan). The mean diameters of the SEM images were determined for 100 measurements using the ImageJ software.

2.5 Chemical characterization (FTIR-ATR)

FTIR was performed using an IR Affinity-1S spectrophotometer (Shimadzu Corp., Japan), equipped with ATR (SPECAC). A total of 64 scans with a resolution of 4.0 cm⁻¹ were in 400 – 4000 cm⁻¹ range [10]. The relative content of secondary structures in silk fibroin materials (films and nonwovens) were determined using second derivative analysis of the infrared spectra covering the amide I region (1595 – 1705 cm⁻¹) by means of OriginPro software [10] [7] [4].
2.6 Thermal analysis of SF materials

DSC for SF samples were measured in a TA Instrument Q2000 DSC under nitrogen atmosphere, with gas flow of 50 ml min\(^{-1}\). The heating rate was 10 °C min\(^{-1}\) from 30 to 320 °C.

3. Results and Discussion

3.1 Rheology of SF/FA solution

Regarding the rheology of SF solution (see Figure 1), its behavior is very close to Newtonian flow. This approximated Newtonian behavior is due to the inability of the SF to aggregate in formic acid, allowing the solution to be subjected to a wide range of applied stresses, producing no major changes in the viscosity of the solution [11].

![Figure 1. Viscosity vs. shear rate for the silk fibroin in formic acid at 10% w/w](image)

3.2 Morphology of the SF fibers

Electrospinning conditions utilized in the production process are described in Table 1. Morphological features of the SF fibers are shown in Figure 2. SEM images of electrospun nonwovens obtained from SF/FA at 10% w/w and 8 cm were collected and analyzed with ImageJ software. The results indicate that nonwovens obtained with an operating value of \(R = 1\) kV/cm, produced fibers with less amount of defects and beads. However, these electrospinning conditions also led to fibers with apparent tendency to larger diameters and higher dimension dispersion in terms of diameters (higher standard deviations compared to \(R = 2\) kV/cm). It was also observed that at increasing flow rates while -remaining the rest of the parameters constant- fibers with higher amount of beads and irregularities were promoted. According to these results, more homogeneous morphology of SF nonwovens was obtained at \(R = 1\) kV/cm and flow rate = 0.05 ml/h.

| Distance to collector (kV) | R (kV/cm) | Flow rate (ml/h) | Name of the sample |
|---------------------------|-----------|------------------|--------------------|
| 8                         | 1         | 0.05             | R1_L               |
| 16                        | 2         | 0.05             | R2_L               |
| 8                         | 1         | 0.1              | R1_H               |
| 16                        | 2         | 0.1              | R2_H               |

Where: \(R = \) voltage/distance.
3.3 Chemical analysis (FTIR-ATR)

FTIR spectra of SF films and electrospun mats were collected in the range of 1700-1450 cm\(^{-1}\), being this the most informative region in the IR spectra of SF (amide I and II) [12]. The amide regions I and II showed characteristic peaks of the random coil and \(\alpha\)-helix conformation around 1643 cm\(^{-1}\) and 1539 cm\(^{-1}\), respectively [13]. These peaks coexisted with distinctive absorption signals around 1515 cm\(^{-1}\) (amide II), characteristic of \(\beta\)-sheet structures [14]. Results in Figure 3 suggest that crystalline and amorphous structures coexist in different proportion in the SF samples and a greater presence of \(\beta\)-sheet structures with defined signals in samples R1_L, R2_L and Film compared to R1_H and R2_H.

![Figure 3. ATR-FTIR spectra of SF samples](image-url)
In order to determine the proportion of crystalline and amorphous structures, and highlight each element in the overlapped secondary structure within the amide I band, the second derivative analysis was applied, as described in the literature [10] [7] [4]. Table 2 shows the changes in relative content of secondary structures in nonwovens compared to the film; this implies an effect of the electrospinning process and their parameters in the structure of the fibroin. The amount of crystalline structures for the nonwovens, mainly represented by β-sheet, increase in the following order: R1_L > R2_L > R1_H > R2_H. The amount of amorphous structures increase in opposite way. It demonstrated that electrospun mats obtained at R = 1 kV/cm contain a higher amount of β-sheet conformations than those obtained at R = 2 kV/cm. Additionally, the nonwovens produced at lower flows, showed higher crystallinity. These results are consistent with the required process parameters to obtain nonwovens yielding an overall better morphology.

**Table 2. Relative content of secondary structure of SF samples**

| Assignment (%) | Film | R1_L | R1_H | R2_L | R2_H |
|----------------|------|------|------|------|------|
| β-sheet        | 38.1 | 42.9 | 30.3 | 35.7 | 25.5 |
| β-turn         | 1.4  | 1.0  | 0.4  | 0.4  | 0.1  |
| Crystalline structures | 39.5 | 43.9 | 30.7 | 36.1 | 25.6 |
| Random Coil    | 33.9 | 10.5 | 40.0 | 12.7 | 38.0 |
| α-helix        | 12.7 | 27.0 | 14.4 | 41.3 | 30.6 |
| Turns and bends | 13.9 | 18.6 | 14.9 | 9.9  | 5.8  |
| Amorphous structures | 60.5 | 56.1 | 69.3 | 63.9 | 74.4 |

3.4 Thermal analysis of SF materials

Figure 4 shows the thermograms obtained from the nonwovens samples. All of them exhibited an endothermic peak around 100 °C associated with the evaporation of bound water. In the case of films analysis, this peak is not so pronounced and appears in a higher temperature, suggesting better interaction of the SF with water, when this one is in the form of nonwovens rather than in the form of films, due to a remarkable higher surface area [15]. SF films show a crystallization peak around 220 °C which is absent in the case of SF nonwovens. At the crystallization transition, unstable non-crystal structures can be transformed to β-sheet, similar findings were reported by Q. Lu, et al [4]. After the crystallization transition, the film started to degrade, showing an endothermic peak at around 277 °C. The degradation temperature for nonwovens are around 282 °C, exhibiting greater thermal stability related to the amount of amorphous structures found in the FTIR analysis.
4. Conclusion

Optimum conditions for defects-free SF electrospun nonwoven, using formic acid as solvent, were obtained. Quantitative analysis (chemical and thermal) of the secondary structure in SF nonwovens indicated an increase of β-sheet structures and better morphological properties at decreasing \( R \) and flow rate in electrospinning process. These nonwovens has potential application in fields such as pharmacy (wound dressing and active ingredients carrier) and biomedicine [16].

Bibliography

[1] A. Restrepo-Osorio, C. Alvarez-López, D. Peláez, and O. Rojas, “Comparación de la fibroína de seda obtenida a partir de capullos y de subproductos fibrosos,” in VII Congreso Internacional de Materiales CIM 2013, 2013.
[2] K. Murugesh Babu, Silk : processing, properties and applications. Woodhead Publishing in association with the Textile Institute, 2013.
[3] D. N. Rockwood, R. C. Preda, T. Yücel, X. Wang, M. L. Lovett, and D. L. Kaplan, “Materials fabrication from Bombyx mori silk fibroin.,” Nat. Protoc., vol. 6, no. 10, pp. 1612–31, Oct. 2011.
[4] Q. Lu, X. Hu, X. Wang, J. A. Kluge, S. Lu, P. Cebe, and D. L. Kaplan, “Water-insoluble silk films with silk I structure,” Acta Biomater., vol. 6, no. 4, pp. 1380–1387, 2010.
[5] S. Ö. Gönen, M. Erol Taygun, A. Aktürk, and S. Küçükbayrak, “Fabrication of nanocomposite mat through incorporating bioactive glass particles into gelatin/poly(e-caprolactone) nanofibers by using Box–Behnken design,” Mater. Sci. Eng. C, vol. 67, pp. 684–693, Oct. 2016.
[6] P. C. Caracciolo, V. Thomas, Y. K. Vohra, F. Buffa, and G. A. Abraham, “Electrospinning of biodegradable poly(ester urethane)s and poly(ester urethane urea)s for soft tissue-engineering applications,” J. Mater. Sci. Mater. Med., vol. 20, no. 10, pp. 2129–2137, Oct. 2009.
[7] H. Yang, S. Yang, J. Kong, A. Dong, and S. Yu, “Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy.,” Nat. Protoc., vol. 10, no. 3, pp. 382–96, 2015.
[8] X. Hu, D. Kaplan, and P. Cebe, “Determining Beta-Sheet Crystallinity in Fibrous Proteins by Thermal Analysis and Infrared Spectroscopy,” Macromolecules, vol. 39, no. 18, pp. 6161–6170, Sep. 2006.
[9] B. K. Park and I. C. Um, “Effects of electric field on the maximum electro-spinning rate of silk fibroin solutions,” Int. J. Biol. Macromol., vol. 95, pp. 8–13, Feb. 2017.
[10] B. Marelli, M. A. Brenckle, D. L. Kaplan, and F. G. Omenetto, “Silk Fibroin as Edible Coating for Perishable Food Preservation.,” Sci. Rep., vol. 6, no. April, p. 25263, 2016.
[11] H. J. Cho, C. S. Ki, H. Oh, K. H. Lee, and I. C. Um, “Molecular weight distribution and solution properties of silk fibroins with different dissolution conditions,” Int. J. Biol. Macromol., vol. 51, no. 3, pp. 336–341, 2012.
[12] S. D. Aznar-Cervantes, A. A. Lozano-Pérez, M. García Montalbán, G. Villora, D. Vicente-Cervantes, and J. L. Cenis, “Importance of refrigeration time in the electrospinning of silk fibroin aqueous solutions,” J. Mater. Sci., vol. 50, no. 14, pp. 4879–4887, Jul. 2015.
[13] F. Zhang, B. Q. Zuo, and L. Bai, “Study on the structure of SF fiber mats electrospin with HFIP and FA and cells behavior,” J. Mater. Sci., vol. 44, no. 20, pp. 5652–5687, Oct. 2009.
[14] M. Wang, H. J. Jin, D. L. Kaplan, and G. C. Rutledge, “Mechanical properties of electrospun silk fibers,” Macromolecules, vol. 37, no. 18, pp. 6856–6864, Sep. 2004.
[15] Q. Zhang, S.-Q. Yan, and M.-Z. Li, “Porous Materials Based on Bombyx Mori Silk Fibroin,” J. Fiber Bioeng. Informatics, vol. 3, no. 1, pp. 1–8, 2010.
[16] S. Abdelhady, K. M. Honsy, and M. Kurakula, “Electro Spun-Nanofibrous Mats: A Modern Wound Dressing Matrix with a Potential of Drug Delivery and Therapeutics,” J. Eng. Fiber. Fabr., vol. 10, no. 4, pp. 179–193, 2015.