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Glycerin and Ethanol as Additives on Silk Fibroin Films: Insoluble and Malleable Films

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ABSTRACT: Silk fibroin (SF) films have been largely studied as biomaterials due to their biocompatibility and biodegradability. Casting a SF aqueous solution at room temperature is a common technique to produce SF films at relative low cost and processing time; however, their brittleness and solubility in water make them unsuitable for certain biomedical applications. In this study, the incorporation of additives, ethanol and glycerin, are presented as an alternative to both improve mechanical properties of SF films and decrease their solubility in water. SF films with additives were further characterized using scanning electron microscopy, X-ray diffraction, Fourier transformed infrared spectroscopy with attenuated total reflection, analysis of water solubility, mechanical test of traction, and *in vitro* cytotoxicity experiments. The results show that SF films containing additives are stable in water due to the effect of glycerin and ethanol, and do not require post-treatments. Furthermore, great improvements on elongation of the films were achieved, mainly in the presence of both additives. In addition, all films were not toxic to cells, which is a first indication of their biocompatibility. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: additives; biocompatibility; mechanical properties; biopolymers

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INTRODUCTION

Silk is a renewable natural resource that can be obtained at a relatively low cost and consists primarily of two protein components: fibroin and sericin. Silk fibroin (SF) is the structural protein of silk fibers and sericin is the water-soluble glue that binds the fibers together.[1] SF presents the necessary characteristics for biomedical applications, such as compatibility with several types of cells, high mechanical strength, high thermal stability, and microbial resistance.[1–3]

SF is a protein that is mainly composed by the amino acids glycine, alanine, and serine, forming highly organized antiparallel β-sheet crystal regions and semicrystalline regions that are responsible for silk's outstanding mechanical properties.[1,4–6] SF can assume distinct conformations; α-helix and random-coil conformations (also called silk I) and the β-sheet conformation (silk II). The silk I structure is the water-soluble state and, on exposure to heat, organic solvents or excitation, easily converts to a silk II structure. Silk II is the most stable structure, where SF chains are connected by hydrogen bonds between the adjacent segments of polypeptide chains.[6–8]

SF has been used as a matrix for biomedical applications including cell culture medium, enzyme-immobilizing membranes, drug carrier, and as an oral dosage gel.[4,5,9–12] The advantages of using SF are related to its unique combination of properties, such as high mechanical and microbial resistance, thermal stability, biocompatibility, enzymatic degradability, and the fact that it can be processed in several forms, such as powders, hydrogels, or films.[1,7]

SF films presents good oxygen and water vapor permeability in the wet state, similar to that of human skin, depending on the content of the silk I and silk II structures.[7] However, pure SF films are soluble in water when prepared at room conditions due to the dominating random-coil structures. Hence, important parameters of SF films that should be controlled for biomaterial application are the molecular conformation of SF and the elasticity and malleability of the films, since they are stiff when dried.

A common method used to modify SF secondary structure is the post-treatment of the films with dehydrating solvents such as methanol (MeOH) and ethanol (EtOH), which increases the

β-sheet content and diminishes the water solubility. Dehydration of SF in an alcohol promotes chain transformations from the random-coil to β-sheet conformation, resulting in the formation of interpenetrating network.[13–15] Gil et al.[14] used aqueous MeOH to induce SF crystallization in protein membranes prepared by mixing gelatin and SF. Nogueira et al.[15] obtained insoluble SF dense membranes by immersing them in 70 wt % EtOH to induce structural changes and reduce water solubility.

SF films prepared at room temperature are brittle and soluble in water, possibly compromising their application in certain biomedical areas. Therefore, the use of plasticizers becomes necessary to improve their mechanical properties and obtain more flexible films.[16–18] Glycerin has been previously used to improve SF mechanical properties by adding 10% glycerin solution during film formation.[8] Recently, Dai et al.[17] investigated the structural transition behavior of SF/PVA (polyvinyl alcohol) blends, after the addition of glycerin to the blends, and evaluated the changes in mechanical properties. Glycerin reduces the phase separation of SF/PVA blends by inducing intermolecular interactions between the two polymers and improving the elongation properties of the blends. Lu et al.[18] blended SF with glycerin in several concentrations and studied the interactions between them. The authors produced water-insoluble films in glycerin contents of above 20 wt %. In addition, the authors observed that it was possible to obtain more flexible films that supported fibroblast attachment and growth.

The main objective of our study was to evaluate the combination of glycerin and ethanol in the properties of SF films. The proposed method to obtain these films is by mixing aqueous EtOH solution with SF aqueous solution, with no need for post-treatment. To improve the mechanical properties of SF films, we studied the addition of glycerin. Glycerin was chosen because of its aqueous solubility and compatibility with SF, besides the fact that it presents the same hydroxyl groups as SF.[8]

EXPERIMENTAL

Preparation of SF Solution

SF was extracted from the cocoons of the *Bombyx mori* silkworm, supplied by Fiação Bratac (São Paulo, Brazil). For removal of sericin, the cocoons of *B. mori* were soaked three times with 0.1% (w/v) Na_2CO_3 aqueous solution in a thermostatic bath at 85°C for 30 min. A final wash was carried out with distilled water for 30 min.[4] The SF fibers were dried at room temperature for 48 h. Degummed SF fibers were dissolved in a ternary solvent of $CaCl_2$: CH_3CH_2OH : H_2O (1 : 2 : 8 mole ratio) at 85°C for 1 h and 30 min to a concentration of 5% (w/v).

Preparation of SF Films

SF salt solution was dialyzed in distilled water for 3 days at 8°C to remove the salts of the solvent and obtain an all aqueous SF solution. The dialysis water was changed every 24 h and the final solution had a SF concentration of 2.5 wt %.

The dialyzed SF solution received aqueous solutions of ethanol and/or glycerin to obtain more flexible and insoluble films. Table I shows the seven different types of SF films studied: pure SF, SF with 30 wt % ethanol aqueous solution, SF with 1 or 3 wt % glycerin aqueous solution and finally SF with 30 wt % ethanol aqueous solution and 1, 3 or 5 wt% glycerin aqueous solution. The mixed solutions were cast into Petri dishes and dried at room temperature. All the films with additives had a volume percentage of SF aqueous solution of 50%. For the films with incorporation of ethanol and glycerin, the ratio of glycerin solution : ethanol solution was also 50 : 50 (v/v). For example, to obtain the SF-Gly3-Et film, 5 mL of SF aqueous solution, 2.5 mL of 3 wt % glycerin aqueous solution and 2.5 mL of 30 wt % ethanol aqueous solution were mixed. The films were not subjected to any kind of post-treatment.

Table I. Nomenclature of SF Films

Films	Nomenclature
SF	SF
SF: Ethanol 30% (w/v)	SF-Et
SF: Glycerin 1% (w/v)	SF-Gly1
SF: Glycerin 3% (w/v)	SF-Gly3
SF: Glycerin 1% (w/v) : Ethanol 30% (w/v)	SF-Gly1-Et
SF: Glycerin 3% (w/v) : Ethanol 30% (w/v)	SF-Gly3-Et
SF: Glycerin 5% (w/v) : Ethanol 30% (w/v)	SF-Gly5-Et

Characterization

Scanning Electron Microscopy. The morphology of the SF films, with or without additives, was observed by scanning electron microscopy (SEM) using a LEO 440i on samples coated with a gold layer, with an accelerating voltage of 10 kV. The samples were frozen in liquid nitrogen, fractured, and then freeze-dried (Liobras, L101, Brazil) for 24 h before SEM observation.

Solubility Test. SF films with a previously determined moisture content (w_0) were cut into pieces of 1 × 1 cm^2, weighed (m_0), immersed in 10 mL of distilled water, and kept under slow shaking (150 rpm) at 37°C for 24 h. After the incubation period, the films were removed from water, dried for 24 h at 105°C and weighed (m_f). The percentage of soluble mass was determined according to eq. (1).

$$\text{Soluble mass}(\%) = \frac{m_0(1 - w_0) - m_f}{m_0(1 - w_0)} \quad (1)$$

Complementary, pieces of 2 × 2 cm^2 of the films were immersed in 50 mL of distilled water for 7 days and visually evaluated for their physical integrity on the seventh day, to verify whether longer periods of immersion in water could change the integrity and water stability of films.

X-Ray Diffraction. The crystalline structure of the films was analyzed by X-ray diffraction (XRD) with a X'PERT PW3050 Philips, using a monochromatic Cu-Kα radiation (λ = 1,54 Å) in the 2θ range of 10° to 35° and scanning rate of 0.6°/min.

Fourier Transformed Infrared Spectroscopy with Attenuated Total Reflection. Molecular conformation of SF in the films was obtained by Fourier transformed infrared (FTIR) spectroscopy

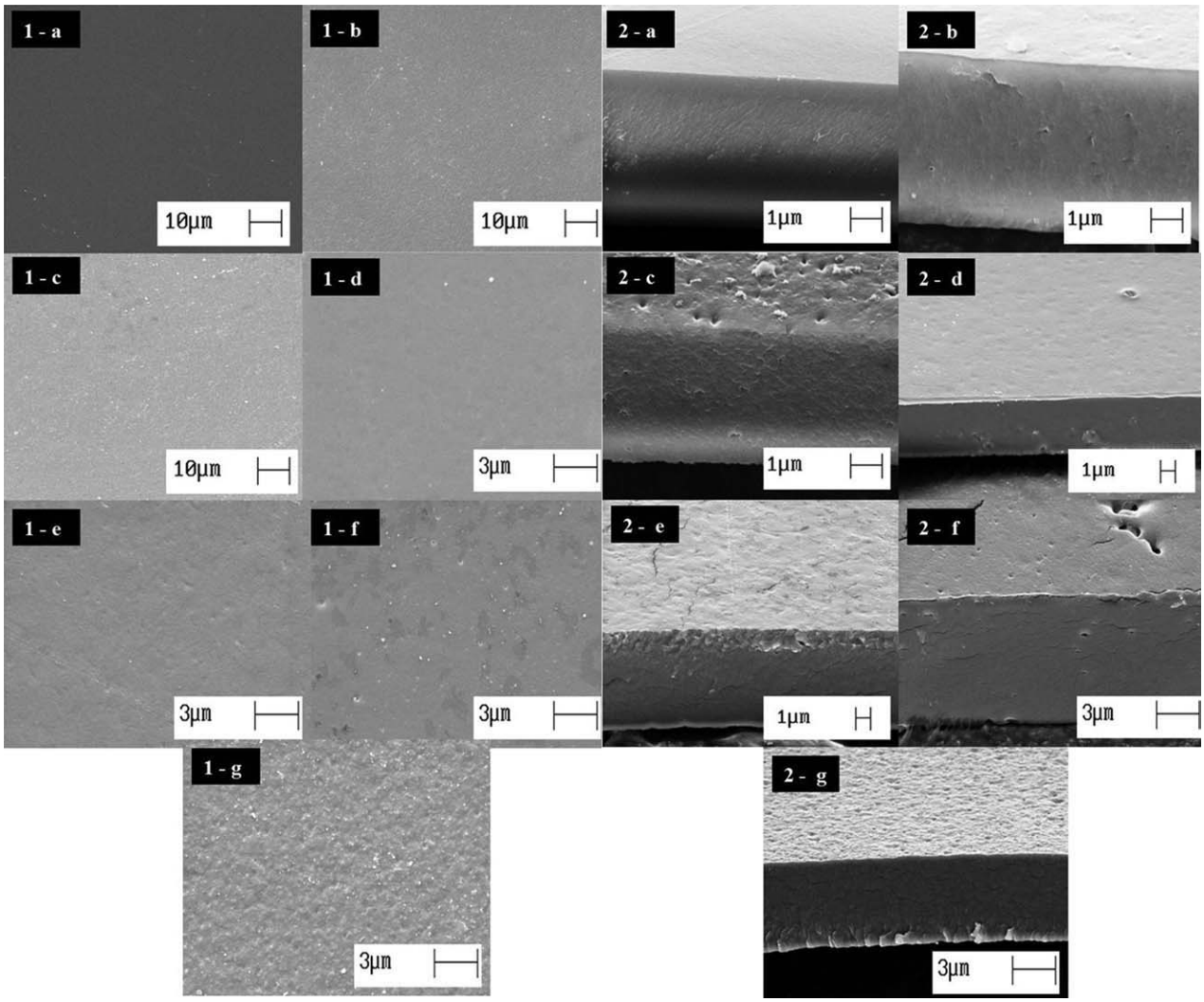

Figure 1. SEM micrographs of surface (1) and fracture (2) of the films of pure SF (a), SF-Et (b), SF-Gly1 (c), SF-Gly3 (d), SF-Gly1-Et (e), SF-Gly3-Et (f), and SF-Gly5-Et (g).

with a MB 102 (Bomem) equipped with a ZnSe ATR cell to verify structural changes induced by glycerin and ethanol.

Mechanical Properties. The tensile tests were performed according to ASTM D882 (2002) using a TA.XT2 texture analyzer (Stable Microsystems SMD). The films (7 × 2.5 cm) were stored under standard conditions (25°C, RH 50%) for 48 h before the tests. A crosshead speed of 10 mm/s was used. The average values of tensile strength were obtained from eight specimens. The Tukey-Kramer test was used to analyze the results, and differences between treatments were considered statistically significant for $P < 0.05$.

Cytotoxicity Test. As pure SF film is soluble in water, a treatment in 70 vol % ethanol, for 20 min, was carried out with this film. This procedure enabled the cytotoxicity test to be performed in this film. It is important to emphasize that the treatment with ethanol was applied only for the cytotoxicity test and only in the pure SF film.

All films were sterilized by autoclave. The tests were performed according to ISO 10993-5 (1999). A lineage of Chinese hamster ovary cell, CHO-k1 was used. After sterilization, the films were immersed in RPMI 1640 (Gibco – 23400-021) culture medium in a proportion of 1 cm^2/mL and left in the incubator at 37°C for 48 h to fulfill the extraction conditions. The extracts were filtered with cellulose acetate membrane with a pore size of 0.45 μm and serial dilutions of extracts were prepared from 100 to 6.25% in sterile culture RPMI. Phenol solution (0.5%) was used as a positive control and 0.2 g/mL high-density polyethylene as a negative control.

The suspension of CHO-k1 cells and the dilutions of extracts were added to a 96-well microplate and kept in an incubator with 5% CO_2 at 37° C for 72 h. To evaluate cell viability, a solution of MTS (supravital dye tetrazolium compound)/PMS (electron coupling agent) was added, followed by incubation for 2 h. The microplates were analyzed with a microplate spectrophotometer at 490 nm to identify the 50% cytotoxicity index (CI_{50}).

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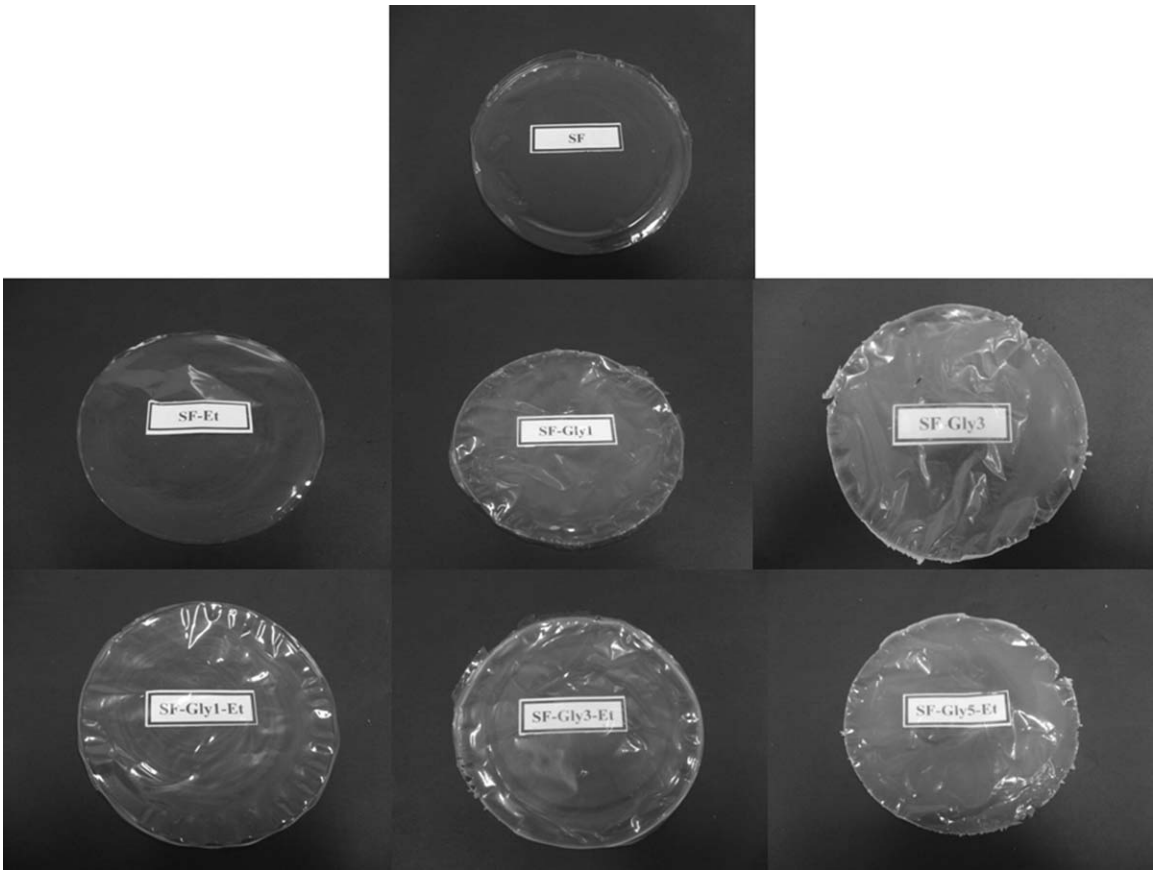

Figure 2. Photographs of SF films with and without additives.

RESULTS AND DISCUSSION

Morphology

The SEM micrographs of SF films, with or without glycerin and ethanol, are shown in Figure 1. In general, all the samples presented smooth surfaces and homogeneous and regular fracture characteristics of dense polymeric materials. However, following the addition of glycerin, the films' surface presented a certain irregularity. Ethanol did not cause any irregularity of the surface or fracture, as can be seen by comparing Figures 1-a and 1-b. Thus, we conclude that the increase in the irregularity of the surface of the films is related to the increase in glycerin concentration, as shown in Figures 1-g and 2-g. Phase separation was not detected on the SEM scale, indicating that glycerin and ethanol are efficient and compatible additives to be blended in SF solution.

Solubility Test and Characteristics of the Films

Before performing the solubility test, a visual characterization of the films regarding their flexibility and transparency was performed. Figure 2 presents the films after solvent evaporation. SF films without additives were transparent, brittle and presented low flexibility. Upon the addition of ethanol, the films were more flexible and transparent. When glycerin was added to the dialyzed SF solution, the films were opaque and more flexible than the films of pure SF and SF-Et. The films with glycerin and ethanol exhibit plastic characteristics (plasticity increased with the concentration of glycerin), opacity, and insolubility in water.

Table II shows the results of the solubility test after 24 h of immersion in 10 mL of distilled water. Only pure SF film was completely soluble in water. The SF-Et and SF-Gly1-Et films were completely insoluble in water. The other films containing glycerin (SF-Gly1 and SF-Gly3) and glycerin and ethanol (SF-Gly3-Et and SF-Gly5-Et) showed varying solubility from 13 to 30% of soluble mass. This partial solubility may be related to

Table II. Results of Water Solubility Test of SF Films

Nomenclature	Soluble mass[a] (%)	Stability in water
SF	100.0	Completely soluble
SF-Et	0.0	Stable
SF-Gly1	29.8 ± 6.3	Stable
SF-Gly3	13.6 ± 3.5	Stable
SF-Gly1-Et	0.0	Stable
SF-Gly3-Et	26.9 ± 5.6	Stable
SF-Gly5-Et	28.9 ± 3.8	Stable

Soluble mass of SF films was analyzed after 24 h of immersion in water and stability of the films was determined after 7 days of immersion in water.
[a]Average ± average deviation ($n = 3$).

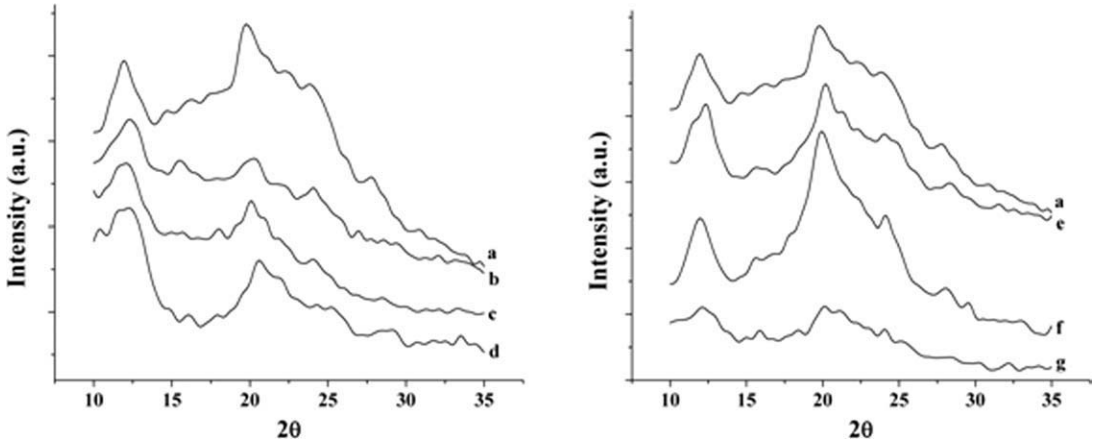

Figure 3. XRD of films of pure SF (a), SF-Et (b), SF-Gly1 (c), SF-Gly3 (d), SF-Gly1-Et (e), SF-Gly3-Et (f), and SF-Gly5-Et (g).

glycerin leaching from the film. Glycerin is a common plasticizer and acts by increasing the intermolecular space, providing better elasticity. Glycerin is highly soluble in water and can be leached from the films due to the immersion in water, which justifies the decrease in the mass of the films. In addition, all SF films, with exception of the pure SF film, maintained their structural integrity and presented good handling properties after the solubility tests (24 h and 7 days). In the solubility test, SF-Gly3 had lower soluble mass than SF-Gly1 even though higher content of glycerin was added. As already observed by Lu et al.,[18] increasing glycerin content in SF films result in decreased SF water solubility. Thus, there are two phenomena that affect the solubility of SF films in water: the first one would be that glycerin can be leached from the films; the second one is the fact that glycerin content stabilizes SF film, resulting in low SF dissolution.

In films containing both glycerin and ethanol (SF-Gly-Et), the glycerin content is 50% (vol) lower than SF-Gly films. This fact explains why their soluble mass percentage is similar to that found in SF-Gly1 film. In these films, the presence of ethanol probably helps to stabilize the film. Ethanol is known to be capable of inducing a conformational change in SF chains. When in contact with organic solvents, such as ethanol, SF chains are dehydrated and rearrange into a more stable conformation, the β-sheet, also called silk II. It is possible that the decrease in the solubility of the films might be related to the addition of ethanol and/or glycerin on SF, which could induce a structural transition to a more stable and water-insoluble form. To verify the structure of the films, XRD and FTIR-attenuated total reflection (ATR) analyses were carried out.

XRD

X-ray diffractograms are shown in Figure 3. All samples depicted a halo around 20°, characteristic of the β-sheet conformation (silk II) and another halo around 12°, characteristic of the α-helix conformation (silk I). The result is consistent with that presented in the literature, affirming the coexistence of the α-helix and β-sheet conformations in the dense films of SF.[10]

The diffractograms obtained with additives on the SF films suggest that, with the addition of glycerin and ethanol, the intensity of the halo of the α-helix structure is decreased, while the halo of the β-sheet is more evident, especially for the SF-Gly3-Et film, indicating that the combination of ethanol and glycerin improves SF crystallinity. The films of SF with additives are insoluble in water, unlike the films prepared without additives, suggesting that a mechanism of stabilization of SF molecules may take place, probably induced by an increase in crystallinity.

FTIR-ATR

Table III presents the values for amides I, II, and III of the films obtained by the FTIR spectra presented in Figure 4. From the FTIR spectra of the pure SF film, it may be observed that, although no post-treatments were performed in the analyzed sample, the values of the bands of amide I and II are mainly in the range of the β-sheet conformation, while the amide III band is located in the α-helix conformation range.[2,17]

By comparing the values of the bands of amide I, II and III for the SF-Et film, the secondary conformation of SF is predominantly silk I; however, the silk II structure is also observed. SF films with glycerin (SF-Gly1, SF-Gly3, SF-Gly1-Et, SF-Gly3-Et, SF-Gly5-Et) demonstrated the presence of two peaks with the same intensity at 920 and 1030 cm^{-1}, related to insaturation at the α-carbon of glycerin. This vibration refers to the axial deformation of the CO bond of glycerin. Another characteristic peak at around 3280 cm^{-1} is observed in the spectra of the SF films

Table III. Comparison between Peaks of Amides I, II, and III of the Films

Film	Amide I (cm^{-1})	Amide II (cm^{-1})	Amide III (cm^{-1})
SF	1638	1519	1232
SF-Et	1646	1529	1238
SF-Gly1	1646	1536	1238
SF-Gly3	1634	1519	1234
SF-Gly1-Et	1634	1519	1238
SF-Gly3-Et	1638	1519	1237
SF-Gly5-Et	1646	1524	1234
Literature[2]	1630	1515	1230

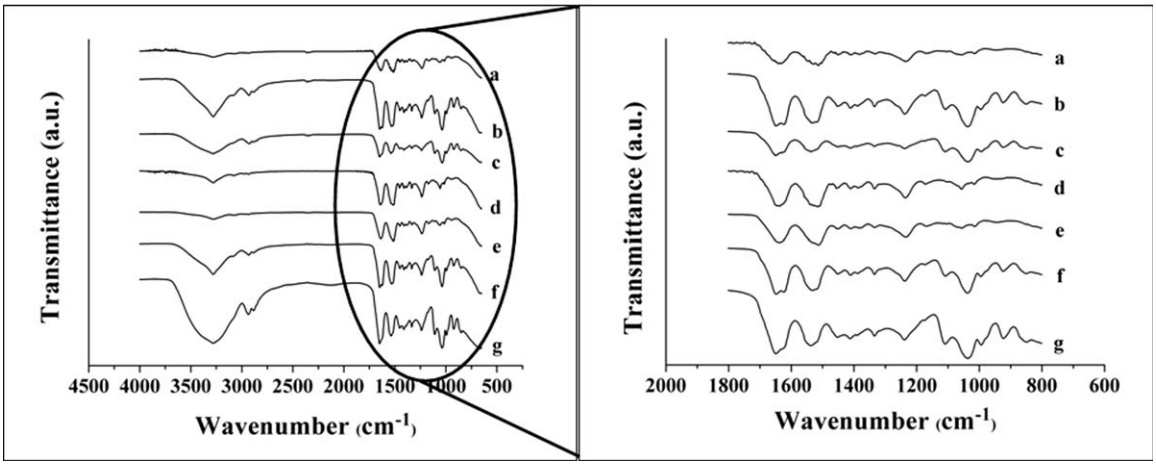

Figure 4. FTIR-ATR spectra of the films of pure SF (a), SF-Et (b), SF-Gly1 (c), SF-Gly3 (d), SF-Gly1-Et (e), SF-Gly3-Et (f), and SF-Gly5-Et (g).

containing glycerin, related to the axial deformation of the OH bond and indicating interactions between fibroin and glycerin.[11]

From the analysis of the infrared spectra, we conclude that there is a coexistence of the α-helix and β-sheet structures in all samples, with a predominance of β-sheet in the bands of amide I and II. The pure SF films are soluble in water, whereas samples with glycerin and ethanol are not soluble in water, which is a strong indication of the occurrence of a more stable structure in the SF. As also observed by Lu et al.,[18] glycerin molecules interact with SF chains, altering its hydrophobic hydration and inducing SF structural transitions to more stable forms. The presence of glycerin prevents β-sheets formation; however, formation of stable silk I structures takes place justifying the insolubility of SF films containing additives (glycerin or ethanol) in water.

Mechanical Tests

The effect of additives on the mechanical properties of SF films was investigated. Table IV presents the results of % elongation and strength at the break of the films. With the exception of the SF-Et film, the additives provided a significant increase in the percentage of elongation of the films when compared with pure SF films. This result was expected, as glycerin was added to act as a plasticizer, increasing the intermolecular space between SF chains and, consequently, the elasticity. Table IV also shows that, in general, with the addition of glycerin and ethanol, the strength at break is lower than the SF film. The strength at break of films with only glycerin (SF-Gly1 and SF-Gly3) is approximately 8 and 10 times lower than that of the SF film, respectively. This result is expected, as increasing elongation at break results in decreased strength at break.

The properties of the SF and SF-Et films are similar. Thus, ethanol did not contribute to the change in the mechanical properties of the films, but contributes to the stability of these films in water. In films with glycerin (SF-Gly1 and SF-Gly3), increasing the concentration of glycerin decreased the strength at break and the elongation. According to the literature,[17,18] increasing the glycerin concentration in films results in greater elongation and lower strength at break; however, this trend was not observed in our study, indicating that there is a maximum content of glycerin that results in increased elongation.

Mechanical properties of SF-PVA blended films were studied by Dai et al.,[17] who also observed that glycerin improved the malleability of the films due to the interaction of hydroxyl groups of glycerin with the molecular chain of SF, promoting a greater spacing between SF molecules. Thus, the movement of the molecular chain is facilitated to increase the elasticity of the films.[17] For a PVA/SF blend film, containing 5 wt % glycerin, the strength at break was 426 kg/cm^2 (approximately 41.77 MPa) and the elongation at break was 53%. In our study, we obtained higher elongation values for SF films with glycerin and with glycerin and ethanol. The SF-Gly1 film presented an elongation of 64.5%, 18% higher than the elongation observed for the PVA/SF blend film with 5 wt % glycerin. However, the strength at break for all types of films obtained in our study were lower than that observed by Dai et al.[17], and this fact can be related to the presence of PVA, which increased the strength of the films. In addition, the elongation value of SF-Gly1 film was similar to the value found for SF/glycerin blend film with 30 wt % glycerin (59.7%) studied by Lu et al.[18] In the latter, the ultimate strength was 11.6 MPa, whilst we have achieved 5.9 MPa. This difference can probably be explained because those authors used a 6% SF solution and we have used a 2% SF solution. On the

Table IV. Mechanical Properties of SF Films with or without Additives

Nomenclature	Elongation (%)	Ultimate strength (MPa)
SF	2.07 ± 0.65^a	53.22 ± 9.06
SF-Et	1.86 ± 0.79^a	35.19 ± 7.22
SF-Gly1	64.47 ± 23.49^b	5.91 ± 0.83^a
SF-Gly3	50.07 ± 4.24^b	4.47 ± 2.65^a
SF-Gly1-Et	105.73 ± 16.35^c	14.60 ± 2.29
SF-Gly3-Et	97.62 ± 20.04^{c,d}	37.33 ± 6.75
SF-Gly5-Et	76.97 ± 14.72^d	3.73 ± 0.29

Same letter indicate no statistically significant difference ($P < 0.05$).

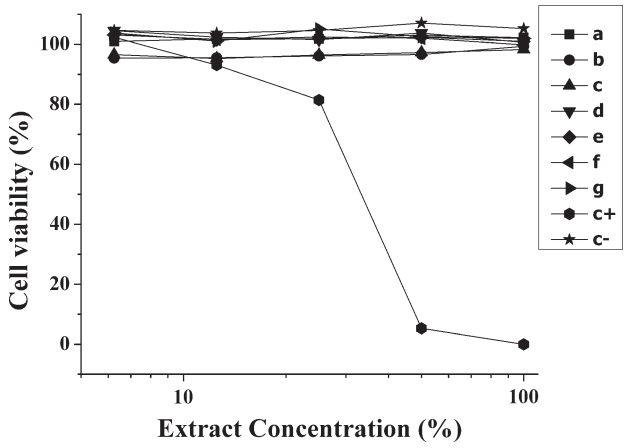

Figure 5. Cell viability of pure SF (a), SF-Et (b), SF-Gly1 (c), SF-Gly3 (d), SF-Gly1-Et (e), SF-Gly3-Et (f), and SF-Gly5-Et (g), determined by *in vitro* cytotoxicity test. Positive control (c+) is 0.5% v/v phenol solution and negative control (c−) is high density polyethylene.

other hand, in SF-Gly1-Et film, we achieved the same elongation as SF/glycerin blend with 40% glycerin (106%), but with the ultimate strength being 2.3 times higher, which indicates that ethanol plays a crucial role in improving strength of SF films.

SF films with glycerin and ethanol presented significant improvements in elongation properties, compared to the pure SF film. By increasing glycerin content, elongation decreases, while strength at break follows the order SF-Gly3-Et > SF-Gly1-Et > SF-Gly5-Et. These results are in accordance with the XRD results, where SF-Gly3-Et presented a more pronounced β-sheet halo than SF-Gly1-Et, while SF-Gly5-Et showed the less defined halos of all the films analyzed. This indicates that an increase in structural organization (detected by XRD) leads to an increase in the mechanical resistance of the SF films.

It is known that glycerin has plasticizing effect and provides mobility to the SF chains, increasing the film elasticity. On the other hand, ethanol promotes dehydration of SF chains, increasing their intermolecular bonds and producing a stiffer material. Thus, the addition of ethanol and glycerin may promote a competition between the plasticizing effect of glycerin and the chain dehydration effect of ethanol, influencing the mechanical properties. As shown in Table IV, the results for the SF-Gly5-Et are much lower when compared to SF-Gly3-Et and SF-Gly1-Et. For any plasticizer, there is an optimum amount, which results in a better plasticizing effect, and the same trend is observed in our study, where saturation seems to be reached for SF-Gly5-Et films, achieving the best results for SF-Gly3-Et. The same tendency was observed by XRD, where SF-Gly3-Et film showed the most defined and intense halos for the β-sheet and also for the α-helix structure. It seems that the influence of glycerin is more pronounced than the influence of ethanol, restraining the formation of the β-sheet structure, but forming stable silk I structures, resulting in low values of strength at break, but higher elasticity and water stability.

Biocompatibility—Cytotoxicity Test

Cytotoxicity was assayed as the viability of CHO-k1 cells in dilutions of SF film extracts, as shown in Figure 5. None of the samples were toxic to cells at the extract concentrations analyzed. Thus, SF films present great potential for use as biomaterials. In addition, the films present insolubility in water and exhibit cytocompatibility, characteristics required for *in vivo* applications.

CONCLUSIONS

Insoluble, malleable, and biocompatible SF films can be prepared via the conventional solvent-casting technique by simply adding glycerin or ethanol or both in the dialyzed SF solution. The advantage of this process is the ability to fabricate stable SF films suitable for biomedical applications with low cost, low processing time, and at ambient conditions. The addition of ethanol and glycerin induced the formation of films that were insoluble in water and presented better characteristics than the SF films conventionally post-treated by organic solvents. There was a very significant improvement in the elongation values of SF films with additives, when compared to the pure SF films, due to the combined effect of glycerin and ethanol. Moreover, the films were not toxic to cells, indicating that they are materials with the potential to be applied as biomaterials; however, further studies of biocompatibility should be performed.

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