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Formation of silk fibroin hydrogel and evaluation of its drug release profile

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ABSTRACT: Silk hydrogels are interesting materials to be used as matrix in controlled drug delivery devices. However, methods to accelerate fibroin gelation and allow the drug incorporation during the hydrogel preparation are needed in literature. In this article we report the preparation of silk fibroin hydrogels with addition of several contents of ethanol, used to accelerate fibroin gelation kinetics, and we also evaluate the potential of these hydrogels to be used as matrices for drug delivery. Chemical and conformational properties did not change despite the amount of ethanol incorporated in the hydrogel. Hydrogels containing diclofenac sodium dissolved in ethanol showed a faster initial release of the drug than hydrogels with the drug dissolved in water but equilibrium was reached later. This indicates a more sustained drug delivery from hydrogels in which the model drug was dissolved in ethanol. Fibroin hydrogels confirm their promising use as biopolymeric matrices for controlled drug release. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41802.

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INTRODUCTION

In the last decades, polymeric systems for controlled drug release have been studied, mainly due to their ability to sustain drug release for long periods of time, enhanced target, low toxicity, easy manipulation/administration, and variety of forms (films, gels, capsules, tablets, creams, among others).[1] The release properties, the load-bearing capacity, the porosity, and the cell-matrix interactions should be considered for the development of a material to be used in controlled drug delivery and also as scaffold for tissue engineering. Also, the possibility to adjust the release profile of a polymeric system is important to achieve the therapeutic concentration of the drug.[2] Silk fibroin (SF) is a protein fiber whose level of crystallinity and molecular conformation can be controlled, making it a good candidate for controlled drug delivery device.

SF is the structural protein of silk fibers (*B. mori*) and is mainly composed by amino acids glycine, alanine, and serine. SF presents two main conformational states, called Silk I (random coils and α-helix) and Silk II (β-sheets).[3] SF conformation transition from Silk I to Silk II can be induced by exposure to heat,[4] humidity,[5,6] physical stretching,[7] or shear stress.[8] Also organic solvents,[9,10] such as methanol and ethanol can be used to induce Silk II conformation. These alcohols are hydrophilic and miscible in water. When in contact with SF, they dehydrate SF hydrophobic domains and allow chain-chain interactions, resulting in β-sheet stable structure.

Fibroin is a versatile natural polymer and can be processed into several forms such as nanofibers,[11] microspheres,[12] powders,[13] hydrogels,[14] and films.[9] SF has some advantages over other natural polymers for hydrogel formation, because its chains are capable to physically crosslink, forming intra- and intermolecular hydrophobic interactions and hydrogen bonds, without any chemical modifications. SF aqueous solution is metastable and SF molecules self-assemble naturally over time into stable crystalline β-sheet fibrils. This transition occurs naturally in SF aqueous solution because stabilized β-sheet is the most thermodynamically favorable condition, and is only dependent on gelation kinetic.[14] Also, the mechanical properties and degradation rate can be fine-tuned by adjusting SF hydrogel processing conditions and crystallinity.[2]

Because of these properties, SF hydrogels have been studied for use as cell culture matrices, encapsulation of drugs and cells, vehicles for the delivery of bioactive molecules, and in tissue engineering, as filling agent and transport vehicle for bioactive molecules and as scaffold to support adhesion, proliferation,

Table I. Composition and Nomenclature of Fibroin Hydrogels

Nomenclature	Volume of fibroin solution (0.05 g/mL)	Volume of ethanol solution (50 vol %)	Volume ratio fibroin/ethanol
SF100	20 mL	0	100/0
SF75	15 mL	5 mL	75/25
SF50	10 mL	10 mL	50/50
SF25	5 mL	15 mL	25/75

and differentiation of cells leading to tissue or organ regeneration.[15–21]

The aim of this study is (a) to prepare SF hydrogels with several contents of ethanol, in order to improve the gelation time and hydrogel properties and (b) to study the kinetic of drug release from the hydrogels.

MATERIAL AND METHODS

Preparation of Silk Fibroin Solution

Silk fibroin was extracted from *Bombyx mori* silkworm cocoons, gently supplied by Bratac (Bastos, SP, Brazil). For sericin removal, the cocoons were degummed three times with 1 g/L Na_2CO_3 aqueous solution at 85°C for 30 min. The fibroin fibers were dried at room temperature (ca. 25°C) for 48 h and dissolved in $CaCl_2$:CH_3CH_2OH:H_2O (1 : 2 : 8 molar ratio) at 85°C for 1 h and 30 min, to a final concentration of 0.10 g/mL.[22]

Preparation of Silk Fibroin Hydrogels

SF solution was dialyzed in distilled water for 3 days at 8°C, to remove the salts of the solvent, and had a final concentration of 0.05 g/mL. A 50 vol % ethanol solution was added in the SF solution in several volume ratios (fibroin/ethanol), as shown in Table I, resulting in SF final concentrations in the hydrogel of 0.05, 0.0375, 0.025, and 0.0125 g/mL, for SF100, SF75, SF50, and SF25, respectively. The mixed solutions (20 mL total) were put on molds (diameter 3.5 cm) and placed on a thermostatic bath at 37°C, until gelation occurred.

Characterization

Morphology of silk fibroin hydrogels was observed by scanning electron microscopy (SEM) using a LEO 440i, with accelerating voltage of 10 kV. The samples were frozen in liquid nitrogen, fractured, and then freeze-dried (Liobras, L101, Brazil) for 24 h.

The mechanical compression test was conducted in a texture analyzer TA.XT2 (Stable Microsystems SMD). We used a cylindrical probe of 0.5" in diameter and a penetration rate of 1 mm/s to penetrate 15 mm of the hydrogels.

Chemical composition of SF in the hydrogels was obtained by Fourier transformed infrared spectroscopy (FTIR) spectra with a MB 102 (Bomem) to verify structural changes induced by ethanol. The hydrogels were frozen in liquid nitrogen, freeze-dried, and analyzed in KBr.

Small-angle X-ray scattering (SAXS) was used to characterize the conformation and supramolecular organization of SF in the hydrogels. The SAXS data were collected using the beamline SAXS2, located at the Brazilian Synchrotron Light Laboratory (LNLS, Campinas, Brazil) using a 2D detector (Pilatus-Dectris). A wavelength of $\lambda = 1.51$ Å, and two-dimensional detector (MarCCD) were used. Data analysis was made using the SASfit® program.

Drug-Containing Hydrogel

Among all the fibroin hydrogels prepared in this work, we chose to analyze the incorporation and release of a model drug (diclofenac sodium) just on the SF50 hydrogel. This hydrogel had a considerable reduced time of gelation and also presented good integrity. Diclofenac sodium (Sigma Aldrich) was dissolved at a concentration of 10 mg/mL in (i) water or (ii) 50 vol % ethanol. For the preparation of drug-containing hydrogels, 10 mL of fibroin dialyzed solution (0.05 g/mL) was mixed with 10 mL of the drug solution (10 mg/mL diclofenac sodium dissolved in water or in 50 vol % ethanol), prior to hydrogel formation. The mixture was placed on molds of 3.5 cm in diameter and placed on a thermostatic bath at 37°C, until gelation occurred. SF hydrogels prepared with the drug dissolved in water are called SF-H_20, while hydrogels prepared with the drug dissolved in ethanol are called SF-EtOH.

Drug Release from Silk Fibroin Hydrogels

Pieces of 1.5 × 1.5 × 1.5 cm of fibroin hydrogels containing the drug model (diclofenac sodium) were immersed in 50 mL of phosphate buffer (PBS, Sigma Aldrich) at pH 7.4, followed by incubation at 37°C with constant shaking. Each sample contained 16 mg of drug and 85 mg of fibroin. Samples of 2 mL of solvent were collected periodically, replaced with fresh PBS (pH 7.4), and analyzed by UV-VIS spectrometry ($\lambda = 276$ nm). Each experiment was performed in triplicate.

RESULTS AND DISCUSSION

Morphological Characterization

In the first part of this work, we investigated the formation of fibroin hydrogels by varying the content of ethanol that was mixed with the fibroin dialyzed solution. For that, we monitor the gelation time by visual inspection of the samples. Hydrogels SF100, SF75, SF50, and SF25 were formed after 72 h, 7 h, 27 min, and 8 min, respectively. Increasing ethanol content considerably decreased the gelation time of SF hydrogels. Ethanol dehydrates SF molecules, increasing intra and intermolecular bonds, providing the formation of silk II structures, which stabilizes the hydrogel.

In Figure 1, we present the photographs of the hydrogels. SF100 hydrogel is more rigid than the other hydrogels, but it differs from the others because it is not uniform and crumbles easily. SF25 hydrogel is the most fragile, and difficult to be removed from the mold. There is no difference between the hydrogels SF75 and SF50 in terms of visual inspection and handling. Both have a good consistency, do not break and can be easily removed from the mold.

The microscopic morphology of the hydrogels was observed by scanning electron microscopy (Figure 2). We observe more defined and smaller pores by increasing ethanol content in the hydrogels, which may be related to the induction of a faster

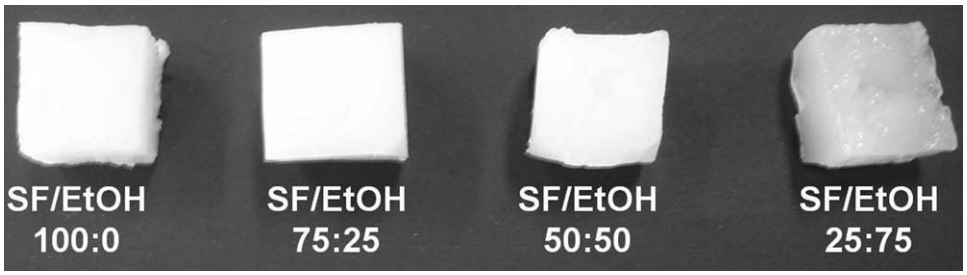

Figure 1. Photographs of the hydrogels.

gelation. Despite being smaller, a higher quantity of pores and the formation of an interconnected network are observed by increasing ethanol content. However, SF25 hydrogel had not well defined pores, which is a result of the high amount of ethanol. Before lyophilization, the SF25 hydrogels were less consistent than the others probably due to the low SF content in its formulation.

Mechanical Characterization

We can see in Table II that the compressive strength decreases by increasing the amount of ethanol in the hydrogels. This is related to the lower content of SF in the hydrogels with ethanol. We choose to maintain the total volume of sample for the tests, and this resulted in a lower SF weight content than SF100 sample. As SF is the main responsible for the mechanical resistance of the hydrogel, a decrease in the resistance by increasing ethanol content in the samples is expected. The percentage of compressive strength reduction was 58.9, 78.3, and 97.3% for SF75, SF50, and SF25, respectively, when compared with SF100 hydrogel.

Despite the fact that the addition of ethanol did not promote improvements in the mechanical properties, hydrogels were obtained quicker and with better structural integrity than the hydrogels of pure silk fibroin (SF100).

Chemical Characterization

FTIR was used to investigate fibroin composition after gelation, in the presence of several contents of ethanol. Fibroin composition can be determined by the location of absorption bands related to Amide I, Amide II, and Amide III groups. FTIR absorption bands located at 1630 (Amide I), 1530 (Amide II), and 1265 cm^{-1} (Amide III) are associated to Silk II (β-sheet) conformation, while absorption bands at 1660 (Amide I), 1540 (Amide II), and 1235 cm^{-1} (Amide III) are related to Silk I (random coil and α-helix) conformation.[23] According to Figure 3, SF hydrogels had absorption bands at 1628, 1533, 1261, and 1232 cm^{-1}, corresponding to the coexistence of Silk I and Silk II conformation, with predominance of Silk II, more stable. Similar spectra was observed for all hydrogels, indicating that

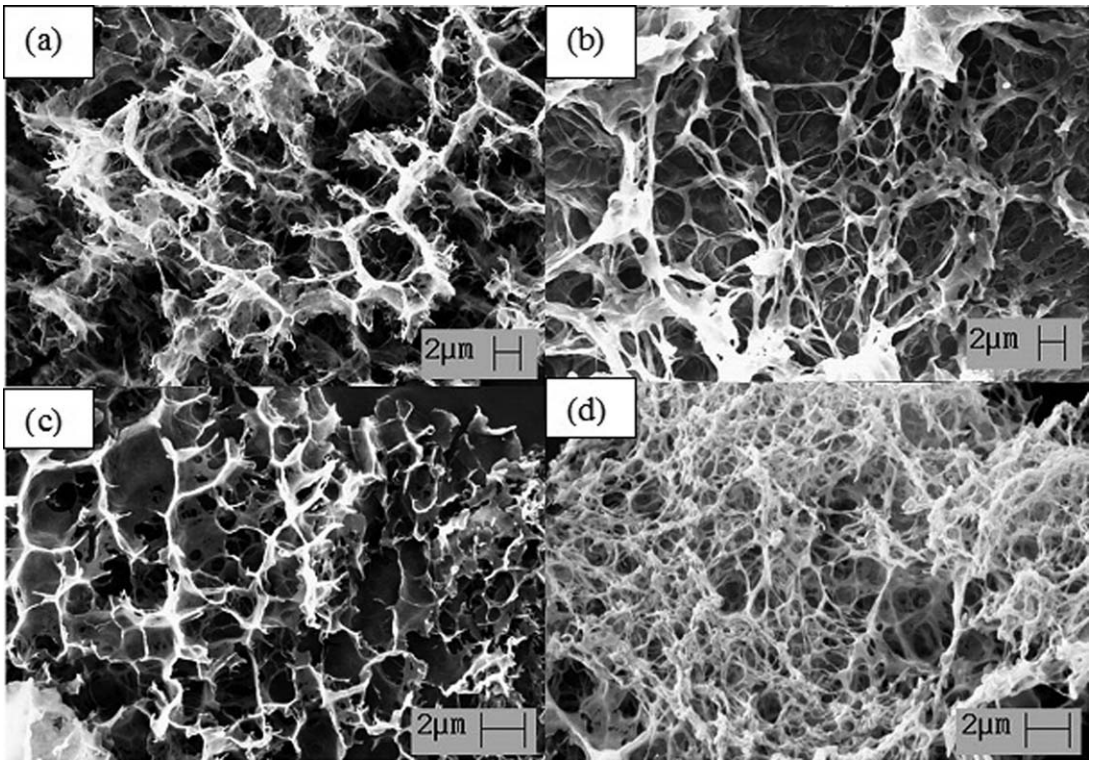

Figure 2. Micrographs of fracture surfaces of fibroin hydrogels SF100 (a), SF75 (b), SF50 (c), and SF25 (d).

Table II. Results of Compression Test of Fibroin Hydrogels After 15 mm Penetration

	Compressive strength (kPa)
SF100	48.91 ± 4.53
SF75	20.07 ± 4.37
SF50	10.63 ± 0.3
SF25	1.30 ± 0.1

the addition of ethanol did not cause composition changes in the final material, but just accelerates the gelation process.

From the SAXS data we can analyze the final molecular conformation of the hydrogels. Figure 4 shows the curves of scattering intensity I (*Q*) versus scattering vector (*Q*). It is evident that there are no peaks in the SAXS curves, showing that there is no change in the distance between the spreaders objects. This behavior suggests that with the addition of ethanol, the fibroin showed no disruption of its aggregates to form a new structure.

For monodispersed systems, where the particles are randomly distributed with uncorrelated positions and orientations, I (*Q*) can be related to the molecular weight and radius of gyration of the molecule by the Guinier equation. The Guinier plot, exhibits a linear region for monodisperse systems. By contrast, the data showed in Figure 5 revealed that the region of Guinier is not linear. This condition may involve a polydisperse system for fibroin, showing that its molecules have different sizes and masses,[24] as already expected.

The folding of the spreader particle can be obtained from the scattering data. For this analysis graphics of Kratky are used. From these graphs we can verify if the spreader particle is compact, unfolded or completely unfolded.[25] Figure 6 presents the graph of Kratky for the samples SF100, SF75, SF50, SF25. We can observe that all hydrogels showed the same behavior: compact, showing that the percentage of ethanol added did not affect the conformation of fibroin.

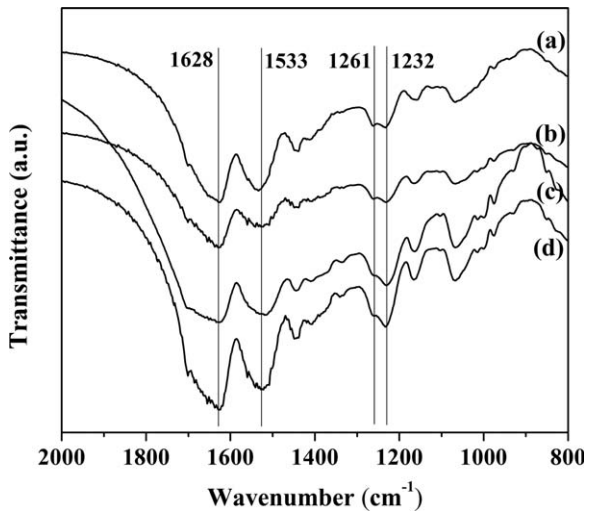

Figure 3. FTIR spectra of silk fibroin hydrogels SF100 (a), SF75 (b), SF50 (c), and SF25 (d).

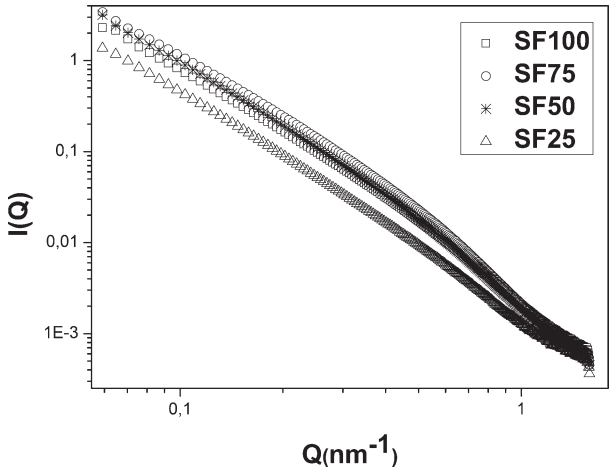

Figure 4. Scattering function I (*Q*) as a function of the scattering vector *Q*, for SF100, SF75, SF50, SF25.

If we consider that fibroin dialyzed solution is metastable and fibroin gelation is a kinetic process, it can be concluded that the addition of ethanol in the dialyzed solution will just accelerate the gelation kinetics (by dehydration of SF molecules and formation of intra- and inter-molecular H-bonds) and will not change the composition or conformation of the final hydrogel. The fibroin hydrogel is already a thermodynamic stable structure and the addition of ethanol will not change the final conformational state of fibroin, but just accelerate the kinetic of hydrogel formation.

During dialysis, the salts that solvate fibroin molecules are removed from solution and are replaced by water. Fibroin is predominantly hydrophobic and fibroin aqueous solution is metastable. In aqueous solution fibroin molecules start to aggregate and hydrophobic and hydrogen interactions may occur. This is a kinetic process that will result in a highly stable and organized fibroin hydrogel. The gelation of fibroin is a natural process, but the hydrogel formation can be accelerated by factors such as pH, temperature, and salt concentration.[26,27] In this work, we proved that fibroin gelation kinetics can also be accelerated by addition of ethanol in the fibroin dialyzed

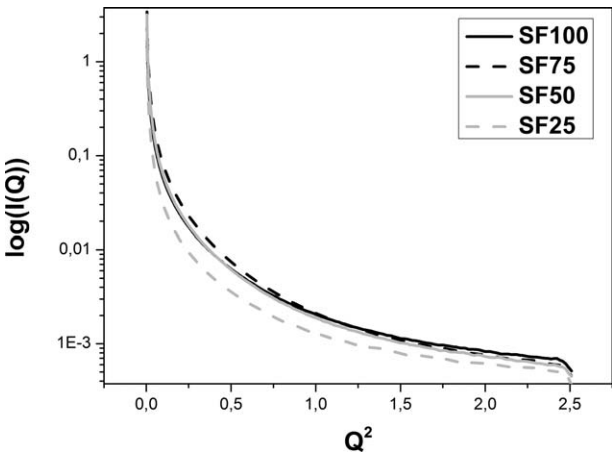

Figure 5. Guinier plot for SF100, SF75, SF50, SF25.

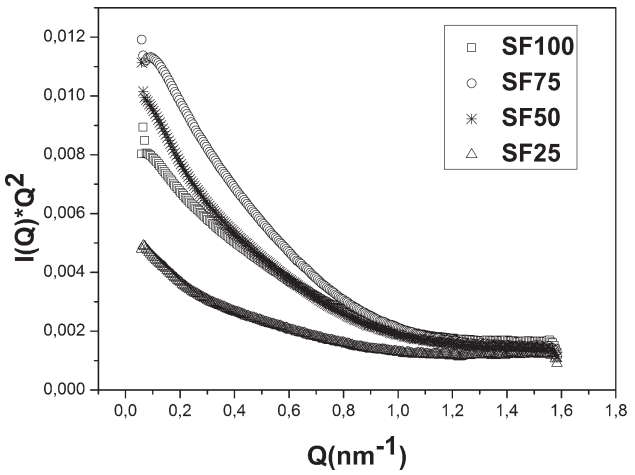

Figure 6. Graph of Kratky. Comparison of Kratky profiles for SF100, SF75, SF50, SF25.

solution, reducing the gelation time from 3 days to ca. 10 min, without altering the fibroin hydrogel chemical characteristics.

Drug-Loaded Hydrogels

Drug-loaded fibroin hydrogels had the same visual aspect of the fibroin hydrogels without the drug. Also, the time necessary for hydrogel formation with the drug dissolved in water (SF-H_2O) was ~3 days, the same time observed for pure fibroin hydrogels SF100 (without addition of ethanol). By contrast, when drug was dissolved in ethanol (SF-EtOH), the gelation time decrease to ~10 min, confirming the effect of ethanol in accelerating fibroin gelation kinetics.

The release behavior of the model drug (diclofenac sodium) from fibroin hydrogels in PBS (pH 7.4) is shown in Figure 7. The effect of water and ethanol, used to dissolve the diclofenac sodium, on the release profile from fibroin hydrogel can also be observed.

Diclofenac sodium release to PBS has a high rate in the first hours (ca. 33% and 48% after 3 h for the SF-H_2O and SF-EtOH, respectively), due to the gradient concentration between the drug incorporated in the hydrogel and the PBS. A similar profile of drug release was obtained for the two studied systems, but initial drug release in the hydrogel SF-EtOH was almost two times faster than hydrogel SF-H_2O. The drug release experiments were performed at the same conditions, thus, probably when ethanol was used to dissolve the drug, a faster initial drug release is induced in the fibroin hydrogel. Drug release seams to reach equilibrium after 5 h for the SF-H_2O hydrogel and after 10 h for the SF-EtOH hydrogel, indicating that a more sustained release was achieved in the SF-EtOH hydrogels. This could be explained by the fact that ethanol is used to induce a faster hydrogel formation, with well-organized β-sheets structures, similar to a crosslinking process, resulting in a more controlled drug release.

Moreover, the big error bars in the percentage of drug release are attributed to the use of different samples, in triplicate. As explained before, fibroin solution is metastable and, even though the exactly same conditions were used for all hydrogels preparation, small changes on solution manipulation may cause alterations in the final hydrogel, resulting in different results of drug release.

Diclofenac sodium was used as a drug model in this study. Studies in the literature have already investigated the release profile of diclofenac sodium from several types of matrices, such as wax granules,[28] zeolites composites,[29] interpenetrating networks of biological macromolecules,[30] Eudragit matrices,[31] natural rubber latex,[32] gelatin magnetic microspheres,[33] among others. Rujiravanit *et al.*[34] studied blend films of chitosan/SF crosslinked with glutaraldehyde as matrices to release theophylline, diclofenac sodium, amoxicillin trihydrate, and salicylic acid in buffer solution at several pHs. The results show a percentage of diclofenac sodium release to the buffer at neutral pH around 65% for the chitosan/SF blends containing 80% of chitosan and equilibrium seems to be reached after 10 min. Our results of diclofenac sodium release for the SF-EtOH hydrogels were similar to the results of Rujiravanit *et al.*[34] (around 60% of release), where ethanol could be considered as a crosslinking agent; however, the SF-EtOH hydrogels developed in our study present a more sustained release, reaching equilibrium after ~10 h, demonstrating that SF hydrogels could be a good matrix for sustained drug delivery.

It is known that the release rate of drugs from fibroin materials can be controlled by controlling β-sheet content and crystallinity.[35] However, external factor, such as pH and temperature can also affect the drug release and the polymer matrix degradation. In this work we prove that fibroin gelation kinetics can be accelerated by incorporation of ethanol within fibroin solution and that the drug can be dissolved directly in ethanol, showing simultaneously faster gelation time and more sustained drug release. The possibility to control the drug release rate by controlling fibroin chemical properties will be studied on future work and opens new perspectives on drug delivery and tissue engineering fields.

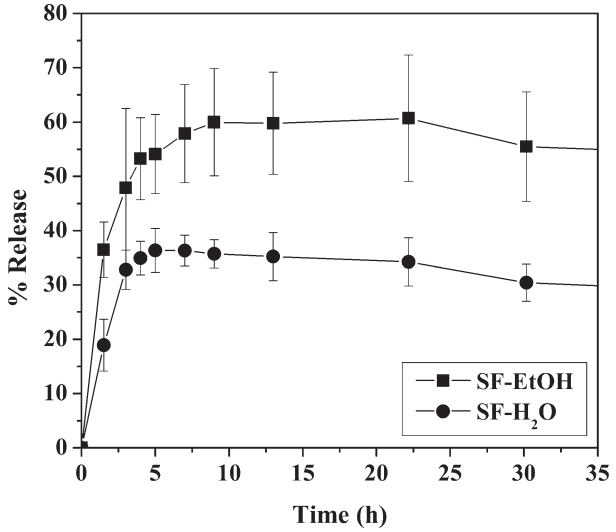

Figure 7. Effect of water and ethanol used to dissolve the drug on the drug release profile from fibroin hydrogels in PBS (pH 7.4).

CONCLUSIONS

Ethanol accelerates gelation kinetics, by dehydration of fibroin molecules, and it does not affect the conformation of fibroin hydrogels. Hydrogels containing the drug dissolved in ethanol presented more sustained drug release than hydrogels with the drug dissolved in water. Silk fibroin hydrogels are potential candidates for controlled drug delivery. Studies on controlling fibroin crystallinity and conformation and evaluating their influence on controlled release are subject of ongoing work.

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