Materials Science and Engineering C 31 (2011) 997–1001

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Hydrogels from silk fibroin metastable solution: Formation and characterization from a biomaterial perspective

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ARTICLE INFO

Article history:
Received 9 November 2010
Received in revised form 24 January 2011
Accepted 17 February 2011
Available online 24 February 2011

Keywords:
Biomaterials
Biopolymers
Silk fibroin
Hydrogels

ABSTRACT

Silk fibroin (SF) hydrogels were obtained from the dialysis of a SF metastable solution. Temperature and calcium concentration in SF solution/hydrogel were measured, as critical variables for SF gelation phenomenon. Gelation time of SF solution was increased by decreasing the dialysis temperature, whereas the residual calcium concentration was higher when higher dialysis temperatures were applied. Hydrogels obtained at 20 °C were characterized after freeze-drying. SEM micrographs showed porous structures, of *ca.* 20 μm (in cross-sectional area) and 5 μm (on surface). XRD indicated the presence of a β-sheet structure that is formed during SF gelation. In hydrogel formation, SF molecules in solution are dehydrated and interact by intra and intermolecular hydrogen bonds, forming a stable hydrogel. DSC measurements showed the decomposition peak for SF at 290 °C, characteristic of SF β-sheet structure, which is in accordance with the XRD results and demonstrate its high thermal resistance. SF hydrogels were found not to be toxic to cells using *in vitro* cytotoxicity tests. Results indicate that silk fibroin hydrogels hold promise for use in the biomaterial field.

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1. Introduction

Silk fibroin (SF) has been used in textile industry for thousands of years and as biomedical sutures for centuries, but only in recent decades its potential as biomaterial has been studied [1–4]. It is known that SF has excellent mechanical properties, biocompatibility with a variety of cells, susceptibility to proteolytic degradation and a low inflammatory response [4]. However, to produce SF derived materials, solvents with high ionic strength are used to break down the strong hydrogen bonds within the β-sheet molecular structure of the silk fibers. These solvents usually contain high concentration of salts that are further removed by dialysis. Once the ionic force of the solvent decreases during dialysis, SF solution becomes metastable and may undergo a sol–gel transition.

The sol–gel transition of SF was previously studied by Matsumoto [5], who reported that changes in gelation depend on the protein concentration, temperature and pH. The hydrogel formation occurs because SF chains tend to aggregate, passing from an amorphous conformation (random coil) to a more stable structure (β-sheet). The formation of β-sheets stabilizes the hydrogel and is irreversible under physiological conditions. This hydrogel can only be degraded by enzymatic or oxidative processes [5,6].

Understanding the sol–gel transition and the variables that can influence this phenomenon is important to define the thermodynamic path and the final products that are desired. During dialysis, SF solution becomes metastable due to the lack of ions available to promote its solvation. Any perturbation in this metastable system can result in instant phase separation and hydrogel formation [7].

Several authors have reported the fabrication of SF hydrogels via different methods, such as freeze-thawing with water miscible organic solvent [8], salt leaching, gas foaming and freeze-drying [9]. It is also known that a dialyzed SF solution may naturally undergo gelation over time, which can be either desired or not depending on the final product of interest (*e.g.*, hydrogel or membranes). To better understand and control the sol–gel transition of SF metastable solution during dialysis, we investigated the gelation time and residual calcium concentration in a SF solution by varying the temperature of dialysis. We show that SF hydrogels can be prepared without requiring additional steps, such as salt leaching, to obtain porous and moldable structures. Hydrogels formed at 20 °C were physically and chemically characterized. A cytotoxicity test was performed as an initial test to verify the biocompatibility of SF hydrogels and, therefore, their potential for use as biomaterials.

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doi:10.1016/j.msec.2011.02.019

2. Materials and methods

2.1. Silk fibroin solution

Silk cocoons of *Bombyx mori* silkworm (Bratac-Brazil) were degummed three times by soaking in 1 g/L of Na_2CO_3 solution at 85 °C for 30 min to remove the sericin of the cocoons, and then rinsing in deionized water. The fibers were dried for 24 h at 40 °C and then dissolved for *ca.* 1.5 h in a ternary solvent of $CaCl_2$:CH_3CH_2OH:H_2O (1:2:8 molar) at 85 °C to a concentration of 0.1 g/mL [10].

2.2. Hydrogel formation

SF hydrogels were prepared by dialyzing SF solution at several temperatures. Residual calcium concentration was determined on SF solution during dialysis until the gelation point (SF solution became a monolith). The experiments were performed by filling a cellulose acetate tube (Viscofan 22 EU-20, USA) with 5 mL of SF solution and immersing it in 75 mL of ultra pure water. The solution was kept at a controlled temperature in a thermostatic bath at 10, 15, 20 and 25 °C. Five replicates were analyzed for each temperature. Released calcium concentration was determined every 24 h before replacing the dialysis bath water (Atomic Absorption Spectroscopy, PERKIN ELMER A ANALYST 100, EUA). Residual calcium concentration in SF solution and hydrogel was calculated by applying mass balance. Calcium concentration average values were calculated based on five replicates for each temperature.

2.3. Hydrogel characterization

Among all of our hydrogel samples, we chose to fully characterize hydrogels formed at 20 °C. Lower temperatures required long dialysis time what may not be suitable for large scale production. On the other hand, hydrogels formed at temperatures higher than 20 °C were fragile and did not withstand handling.

SF hydrogels prepared at 20 °C were frozen in liquid nitrogen and freeze-dried for 24 h at −54 °C and −760 mm Hg (Liobrás L101) and then characterized by scanning electron microscopy (SEM — JSM 5800LV, JEOL), X-ray diffraction (XRD — X'PERT PW3050 PHILIPS), differential scanning calorimetry (DSC — DSC 50 SHIMADZU) and cytotoxicity. XRD experiments were performed within a 10–60° 2θ range, step size of 0.02°, step time of 0.3 s and 0.06°/s speed. DSC measurements were obtained within a temperature range of 20–500 °C, at 10 °C/min and N_2 flow of 50 mL/min.

2.4. Cytotoxicity test

In vitro biocompatibility was performed according to ISO - 10993-5 (1999) using the Chinese hamster ovary cell line (CHO-k1). The cells were maintained in RPMI medium supplemented with antibiotics and antimycotic (100 units/mL penicillin, 100 μg/mL streptomycin and 0.025 μg/mL amphotericin), 2 mM glutamine, and 10% calf serum, at 37 °C in a humidified 5% CO_2 atmosphere until they reached confluence. For subculturing and for experiments, cells were harvested using 0.05% trypsin and 0.02% EDTA (ethylenediamine tetraacetic acid) in phosphate-buffered saline at pH 7.4. A colorimetric method, which uses a tetrazolium compound (MTS or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was used to determine the number of viable cells in proliferation. SF hydrogels were sterilized by gamma radiation (25 kGy) and immersed in RPMI medium at 37 °C for 48 h for extract preparation at a final concentration of 0.2 g/mL. The cytotoxicity test was performed in 96-well microplates seeded with 3000 cells per well and extract dilutions from 100 to 6.25%. The microplates were incubated for 72 h at 37 °C in a humidified 5% CO_2 atmosphere. The cell viability was measured by adding MTS/PMS (phenazine methosulphate) (20:1) solution and incubated for 2 h at 37 °C in a humidified 5% CO_2 incubator. The microplates were analyzed in a spectrophotometer at 495 nm. The test was compared with a negative control of 0.2 g/mL high-density-polyethylene (HDPE) and a positive control of 0.5% phenol in 0.9% saline solution. The Cytotoxicity Index for 50% of cell viability (CI_{50}) was graphically estimated.

2.5. Statistical analyses methodology

For residual calcium concentration analysis, five replicates were made at each temperature of dialysis. XRD and DSC analyses were performed on replicates. Cytotoxicity test was performed on four replicates (four wells for each extract concentration) and the error bar of cell viability percentage was calculated for each extract concentration, from the standard deviation of values from the four replicates, which were read in the spectrophotometer in replicates. The computer packages used for statistical analyses were Origin® and STATISTICA®.

3. Results

3.1. Hydrogel formation

The kinetic curve for calcium release from SF solution during dialysis is shown in Fig. 1. The dialysis was conducted until the SF solution turned into a monolithic hydrogel. Table 1 exhibits the values calculated for the average and standard deviation of the calcium concentration, correlating the time of gelation and temperature. Statistical analyses indicated that the average residual calcium concentrations were not statistically different between samples dialyzed at 10 and 15 °C and between samples dialyzed at 20 and 25 °C. Results indicate that the time for sol–gel transition is influenced mainly by the temperature at which dialysis was conducted.

The SF solution, before dialysis, is salt supersaturated and can be stored for several months without undergoing gelation due to the strong ionic force that promotes SF fibers solvation. However, during dialysis, the salt ions diffuse from SF solution into the dialysis water and, as a result, the ionic force decreases allowing more interaction among SF molecules. When the dialysis temperature increases, the time required for the SF solution to gelify decreases. Molecule aggregation and gelation rate increase with the increase of temperature, due to the increase in hydrophobic interactions among SF chains [5,7]. This phenomenon has also been observed by other researchers [5,6], who related the gelation of dialyzed SF solution as a kinetic process

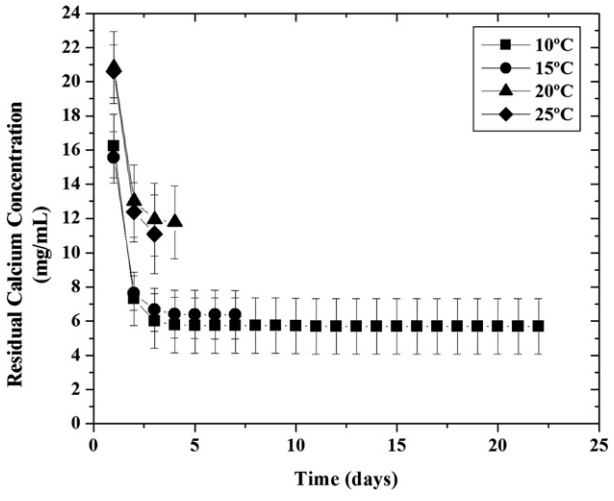

Fig. 1. Residual calcium concentration in silk fibroin solution during its dialysis until complete gelation is reached.

Table 1
Influence of dialysis temperature on residual calcium concentration and on the time of gelation of fibroin silk solution.

Temperature (°C)	Residual calcium concentration[a] (mg/mL)	Time (days)
10	5.70 ± 1.63	22
15	6.39 ± 1.42	7
20	11.77 ± 2.13	4
25	11.09 ± 2.29	3

[a] Average of 5 replicates ± standard deviation.

influenced by several parameters, such as temperature, pH or salt concentration [11].

The residual calcium concentration in SF hydrogel is higher for samples dialyzed at higher temperatures. The decrease in calcium ion diffusion through the dialysis membrane could be explained by a higher interaction between SF molecules and solvent ions that leads to a competition between water from the dialysis bath and SF molecules for interactions with solvent ions. Therefore, at low temperatures, the interactions between SF molecules and solvent ions are weaker, favoring ion diffusion. Another hypothesis is that the dialysis membrane properties are affected by temperature changes, thus, at different temperatures, the ion permeability rate through the membrane is also different. Additionally, it is worthy to consider that at higher temperatures the hydrogel formation is quicker and acts as a mechanical barrier to the passage of ions, which justifies the higher residual calcium concentration on the hydrogels. At lower temperatures, the ion permeability is probably maintained for longer time, until gelation occurs, and thus the residual calcium concentration is decreased.

Interactions between solvent ions and SF molecules can favor solution gelation. The initial calcium concentration in the SF solution is approximately 156 mg/mL. At this concentration, the ionic strength of the solvent is enough to keep the solution stable and avoid SF gelation even at high temperatures and long storage time. After the first day of dialysis, calcium concentration drops to 16 or 21 mg/mL, for 10 and 15 °C or 20 and 25 °C, respectively, and the solution becomes metastable, *e.g.*, the solution is prone to undergo a change of state or phase separation [5,12].

Time and temperature of dialysis are key parameters that define the properties of materials derived from SF. During membrane formation, for example, the SF metastable solution can gelify due to the perturbation in the system associated with SF casting in the Petri dish. The casted hydrogel dries and forms a membrane; however, this membrane is extremely fragile and does not resist handling. On the other hand, SF membranes obtained from drying a SF solution without undergoing gelation are rigid and withstand handling. In this context, the knowledge of the kinetics of dialysis is very important, since it determines the point at which the dialysis should be interrupted to prevent or induce gelation.

3.2. Hydrogel morphology

Hydrogels obtained during dialysis of SF solution are shown in Fig. 2(a). The samples presented a cylindrical shape molded by dialysis tube. If desirable, SF hydrogels can be molded into other shapes by inducing gelation of SF solution in appropriate molds [6,13]. The SEM micrograph of the cross section of SF hydrogel is presented in Fig. 2(b). Freeze-dried SF hydrogel presents a porous structure, with pore sizes of around 20 μm in its bulk and pore sizes of around 5 μm on its surface. The goal of this study was to analyze hydrogels formed spontaneously during the dialysis of SF solution; therefore, no treatments were performed to control their porosity. SF hydrogel, produced by the described method, is not adequate for application as a cell scaffold, since the pores for this purpose should be interconnected with a size of at least 100 μm [9,14]. However, the porosity can be controlled by fine tuning the freezing and freeze-drying parameters or by adding porogenic agents (such as salt crystals) and following procedures that are described in the literature [8,9].

3.3. XRD

The XRD spectrum of the freeze-dried SF hydrogel is shown in Fig. 3. A peak at $2\theta = 21°$ can be observed, indicating that the secondary conformation of the hydrogel is a β-sheet [7,10]. This result is in agreement with previous reports that showed the predominance of β-sheet secondary conformation on SF hydrogels, with smaller quantity of α-helix or random coil conformation [7,10,15]. In the

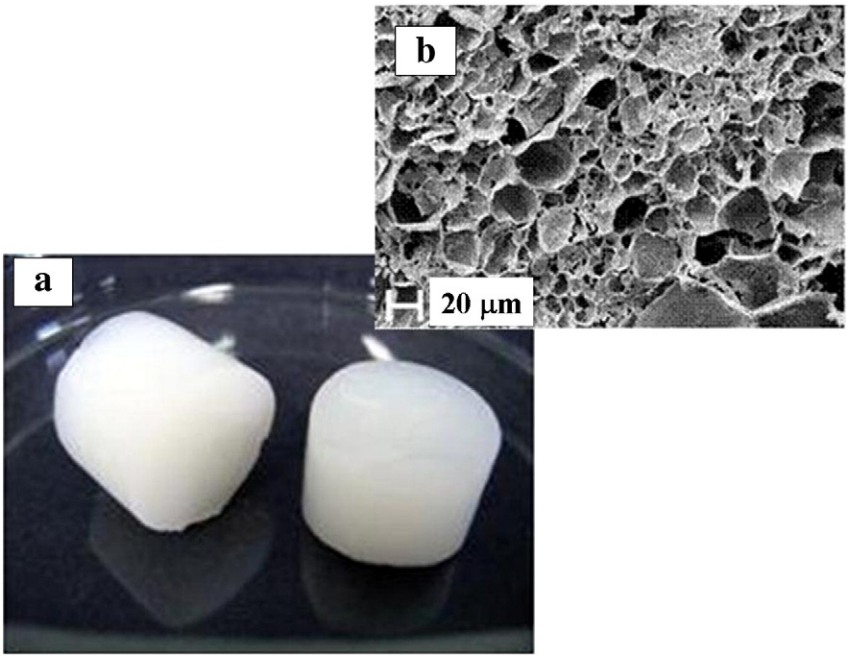

Fig. 2. Photograph (a) and SEM micrograph of (b) of a hydrogel cross section, formed during SF solution dialysis at 20 °C (magnification = 500×).

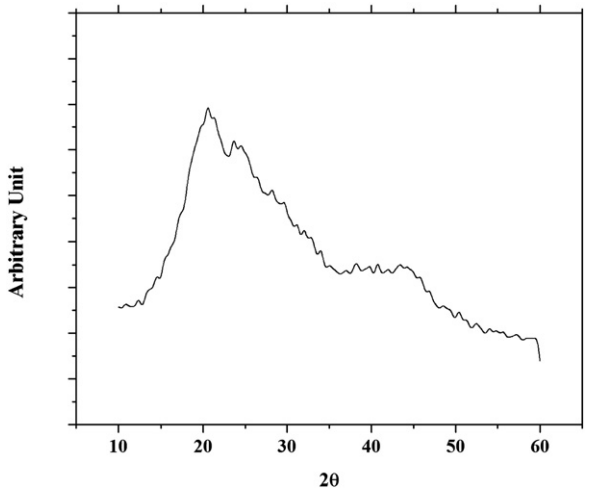

Fig. 3. X-ray diffraction of freeze-dried silk fibroin hydrogel formed at 20 °C.

spectrum showed in Fig. 3 it was not possible to clearly visualize the regions related to the α-helix and random coil structures, which have peaks at 12, 19 or 28°. These structures might be presented in lower concentration in the SF hydrogels and their peaks might be overlapping with the broad peak centered at 21°.

The β-sheet conformation is formed during SF solution gelation, as a result of SF molecular chain dehydration and their intra and intermolecular hydrogen bond formations. In solution, SF molecules are predominantly organized into the α-helix structure. However, this structure tends to convert to the β-sheet, which is thermodynamically more stable. The proportion of β-sheet in the molecular structure of the SF hydrogel is proportional to the degree of gelation, which is a function of time, temperature, concentration and pH of solution. The proportion of β-sheet in the molecular structure of SF will determine its stability, its solubility in water and its *in vivo* and *in vitro* degradability [5–7,10].

3.4. Thermal analysis (DSC)

The DSC thermogram (Fig. 4) depicts a peak at approximately 100 °C that is attributed to the loss of water during heating. The second endothermic peak at 290 °C is attributed to the thermal decomposition of the protein. This peak of degradation at 290 °C is characteristic of SF with β-sheet secondary conformation [16].

Thermal decomposition of SF is mainly influenced by molecular orientation of the protein [17]. Oriented fibers usually present a decomposition peak above 300 °C, while materials of SF with unoriented β-sheet conformation show a decomposition peak at between 290 and 295 °C. In contrast, amorphous materials of SF present peaks of decomposition below 290 °C [16]. Hence, the result obtained by DSC is in agreement with the result presented by XRD, which determined the secondary structure of fibroin hydrogel as a β-sheet. In both analyses, it was not possible to verify the presence of α-helix or random coil conformation. However, we cannot affirm that such conformations are not present, as they may occur in a low proportion when compared to the β-sheet. It should be emphasized that the hydrogels analyzed were freeze-dried, which may have caused the formation of a structure that is richer in β-sheets in the hydrogel, due to the dehydration of SF molecular chains.

3.5. Cytotoxicity

According to the cytotoxicity test results in Fig. 5, SF hydrogels are not toxic to cells. The cellular viability of SF hydrogels was kept between 90 and 100%, comparable to the negative control, in all concentrations of extract analyzed. Cell toxicity of SF hydrogels prepared by various methods has been reported in the literature through cytotoxicity tests and *in vitro* and *in vivo* cell growth [8,18–20].

The cytotoxicity test was conducted in hydrogels formed during dialysis of SF solution at 20 °C, without addition of crosslink agents. The biocompatibility of these materials combined with their tridimensional porous structure, their potential to calcify *in vitro* [1] and their high thermal and mechanical resistance, makes them promising biomaterials, especially for use in bone regeneration [21,22].

4. Discussion

Understanding the gelation of SF is important not only for producing gels, but also to provide information that allows better handling of the SF solution. The majority of solvents used to dissolve SF contain large quantities of salts [11]. These salts are responsible for keeping SF molecules stable in solution, but to prepare SF membrane, gel and powder, the salts have to be partially or totally removed by dialysis. Dialysis parameters, such as SF concentration, solvent type and pH, will influence the sol–gel transition of a SF solution [6,7,23], thus, these parameters should be carefully monitored both when SF hydrogel formation is desired or not.

Time and temperature of dialysis are very important parameters that will define the properties of SF derived materials. For example, whether the production of SF solution for membranes preparation is desired, dialysis should be interrupted before SF solution becomes

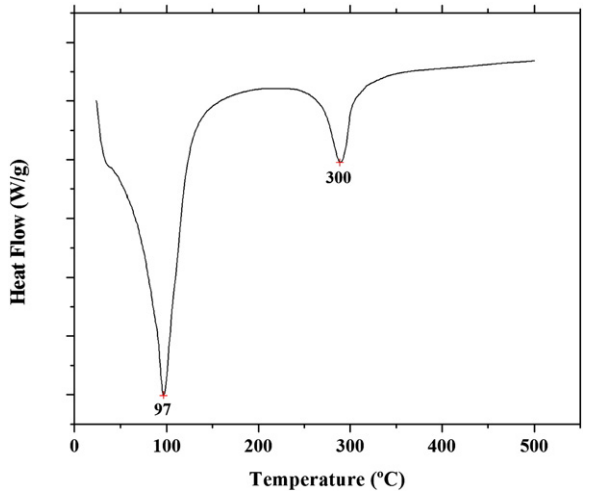

Fig. 4. DSC thermogram of silk fibroin hydrogel formed at 20 °C.

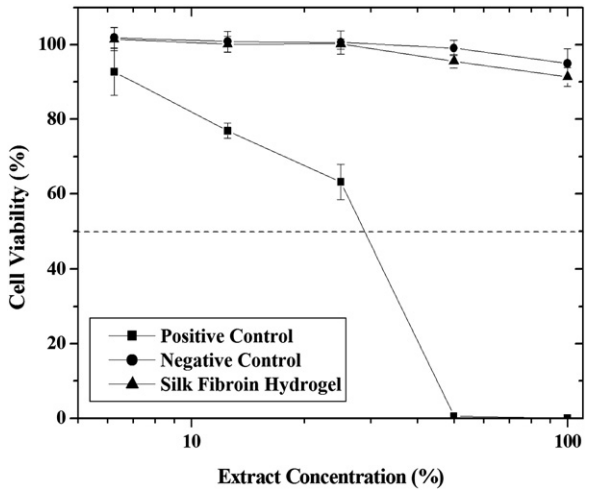

Fig. 5. Cell viability of silk fibroin hydrogel, determined by *in vitro* cytotoxicity test.

unstable. Visualization of this point is relatively difficult, but seems to be very close to the point of hydrogel formation during dialysis.

From the fourth day of dialysis, the residual calcium concentration in the hydrogel remained constant for dialysis at 10 and 15 °C. If the goal is to obtain SF membranes, the dialysis may be interrupted on the third or fourth day without gelation of the solution during the membrane formation. For the concentration of the SF analyzed, dialysis at 20 and 25 °C should be interrupted before the fourth day, to avoid hydrogel formation.

At higher temperatures, quicker gelation kinetics was found, not necessarily in association with the same salt concentration decrease (which was expected, as mass transfer may be favored). In other words, temperature is the major control factor in SF gelation kinetics, rather than calcium salt concentration. Understanding the process kinetics will allow us to control the SF gel or sol state by regulating the gelation kinetics and interrupting the process at the desired point rather than modifying the thermodynamic (gel or sol) equilibrium state.

The hydrogels produced during the dialysis process have a cylindrical form, molded by the dialysis membrane shape. These hydrogels can be molded *in situ* if the SF solution is poured in its unstable state or if a reagent capable of accelerating or inducing the hydrogel formation is added to the dialyzed solution [6,8,9]. Moldable hydrogels or solutions that gelify *in situ* may be of great interest in the medical area, in bone reconstitution or wound treatments [1,13,18,22,24]. The absence of cytotoxicity and deposition of calcium phosphates in the hydrogels during *in vitro* calcification tests [1] are very promising results, indicating the possibility of their use for biomaterials in bone regeneration. Thus, the possibility to mold a hydrogel incorporated with growth factors or calcification inducers may have a great potential for the treatment of bone defects caused by fractures or diseases such as osteoporosis.

5. Conclusion

The sol–gel transition of SF solution during dialysis was studied, focusing on the correlation of the gelation time, temperature and residual calcium concentration. Increasing the temperature of dialysis leads to a shorter gelation time and to a higher residual calcium concentration in the hydrogels formed. The sol–gel transition of SF takes place throughout dialysis due to the drastic decrease of salt ions in the SF solution, which promotes the aggregation of fibroin molecules, and leads to the formation of β-sheets that stabilize the hydrogel. However, one can regulate the sol–gel transition of a SF solution by regulating the process kinetics, temperature, and calcium concentration.

Inducing the sol–gel transition during dialysis of SF solution allows the formation of stable porous hydrogels that have been previously characterized as non-cytotoxic and prone to *in vitro* calcification [1]. These characteristics suggest that SF hydrogels, prepared by simply dialyzing a SF solution, without any post-treatment, are potential candidates for use as biomaterials in the bone regeneration field.

Acknowledgements

The authors thank *CAPES* (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and *CNPq* (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for financial support.

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