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

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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.

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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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

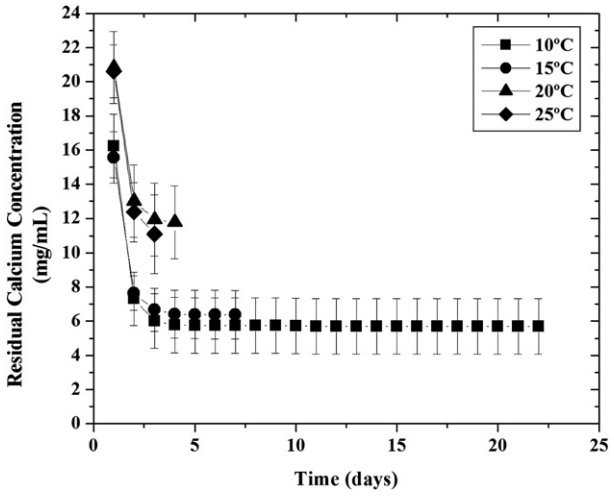

Fig. 1. Residual calcium concentration in silk fibroin solution during its dialysis until complete gelation is reached.