



Preparation and characterization of ethanol-treated silk fibroin dense membranes for biomaterials application using waste silk fibers as raw material

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ABSTRACT

The possibility of producing valued devices from low cost natural resources is a subject of broad interest. The present study explores the preparation and characterization of silk fibroin dense membranes using waste silk fibers from textile processing. Morphology, crystallinity, thermal resistance and cytotoxicity of membranes as well as the changes on the secondary structure of silk fibroin were analyzed after undergoing treatment with ethanol. Membranes presented amorphous patterns as determined via X-ray diffraction. The secondary structure of silk fibroin on dense membranes was either random coil (silk I) or β -sheet (silk II), before and after ethanol treatment, respectively. The sterilized membranes presented no cytotoxicity to endothelial cells during *in vitro* assays. This fact stresses the material potential to be used in the fabrication of biomaterials, as coatings of cardiovascular devices and as membranes for wound dressing or drug delivery systems.

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1. Introduction

In 2007, the Brazilian production of raw silk fibers was around 1300 tons and employed approximately 8000 families (data from Bratac-Brazil). Brazilian silk production has been focused in the textile area which generates waste during the silk fibers processing that can be used as raw material for the study and development of silk-based materials. These pre-processed materials can present different properties when compared to the virgin fibers obtained from the silkworm cocoon, therefore, the materials made from this pre-processed silk could also present different characteristics and its chemical and physical properties were carefully characterized.

Silk is composed of two proteins: fibroin and sericin. Fibroin is the core filament of silk, while sericin is a glue-like protein surrounding the fibers to hold them together in the cocoon case. Silk fibroin (SF) from *Bombyx mori* silkworm is a fibrous protein which has a long history of use as textiles and surgical sutures. Recently, several researchers have investigated the use of SF in other areas, such as biotechnology and biomedical materials due to its properties including biocompatibility, mechanical strength, high thermal stability and microbial resistance (Um et al., 2001; Lovett et al., 2007; Wang et al., 2007; Yeo et al., 2008). Fibroin, as all fibrous

proteins, is not soluble in water due to its high concentration of hydrophobic amino acids residues, such as alanine, on its surface and in its bulk (Hossain et al., 2003). For the effectively dispersion of the SF, it is necessary to swell the compact fibrous structure and break down the hydrogen bond network leading to complete dispersion of individual fibroin molecules (Freddi et al., 1999). Gels, membranes or powders can be obtained from SF solution. SF membranes present a wide range of applications as biomaterials, for instance, wound dressing, drug delivery system or contact lenses.

The molecular conformation of SF membranes is an important parameter that needs to be controlled, since it will affect their physical and chemical properties. The process conditions, such as solution concentration, type of solvent and drying temperature can be used to control the molecular conformation in SF membrane (Putthanarat et al., 2002). Besides these parameters, the post-processing treatment can define the secondary structure of SF membranes. The addition of low dielectric constant organic solvents, such as methanol, ethanol or dioxane is the most common method to convert SF from random coil to β -sheet conformation (Freddi et al., 1999; Chen et al., 2001a,b; Um et al., 2001; Ha et al., 2003), which increases the crystallinity and diminishes the water solubility of the treated samples.

Since fibroin dense membranes are brittle in the dry state, they would be unsuitable for practical use (Li et al., 2002). However, in the wet form their malleability is increased allowing a larger range

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of applications. If the dry state is required and the brittleness is undesirable, silk fibroin properties can be improved by blending with other natural or synthetic polymers (Li et al., 2002; Jin et al., 2004a).

Most of the characterizations for SF dense membranes are made for methanol-treated samples in a dry state. For our purpose, the wet state is more adequate and ethanol instead methanol is applied to promote the crystallization treatment of SF.

The aim of this study was to use waste silk fibers to prepare dense SF membranes crystallized with ethanol and characterize its biocompatibility aiming applications as biomaterial (considered a specialty area). This is an example of a responsible and reasonable use of bioresources, which is a key strategy to support the development of technology by aiming value-added applications.

The structure and properties of dense SF membranes were determined by scanning electron microscopy (SEM), X-ray diffraction pattern (XRD), infrared spectroscopy with attenuated total reflection device (FTIR-ATR), thermogravimetric analysis (TGA), differential scanning calorimetric analysis (DSC) and mechanical tests. The changes on secondary structure by ethanol treatment were analyzed mainly by FTIR-ATR technique. The biocompatibility was evaluated by the ability of dense SF membranes to adherence and growth of endothelial cells. This characteristic is desired for materials to be implanted such as cardiovascular devices, since the growth of a cell layer on the surface of the device may improve its characteristics such as mechanical resistance (Feugier et al., 2005).

2. Methods

2.1. Preparation of silk fibroin solution and membranes

Waste silk fibers from silkworm (*B. mori*) were obtained from Bratac (São Paulo/Brazil), in the form of mixed pieces of fibers. These fibers were washed three times, during 30 min each time, in 0.5 wt% Na₂CO₃ solution at 85 °C to remove sericin. The fibers were then rinsed with water, and dried at room temperature. Purified fibroin (10 g) were dissolved in 100 mL of ternary solvent, CaCl₂–CH₃CH₂OH–H₂O (1:2:8 mol ratio), at 85 °C until total dissolution.

Dense membranes were prepared by dialysis of SF solution against distilled water for 4 days at room temperature. The final aqueous fibroin solution was cast onto polystyrene plate and dried at room temperature for 24 h. Some dense membranes were immersed in ethanol 70% v/v to induce structural change and reduce water solubility.

2.2. Characterization

Morphology of membranes was characterized by scanning electron microscopy (SEM). The analysis was performed on samples that were dried, coated with a gold layer and then examined in a LEO 440i scanning electron microscope (Oxford-Instruments, Oxford, UK).

Changes in the crystallinity of samples were followed with X-ray diffraction (XRD), performed on Rigaku-Ultima-RINT 2000 equipment (Rigaku, Texas, USA): (1) for silk fibroin salt original solution; (2) for the dialyzed solution; (3) for dense membranes before and (4) after ethanol treatment. In XRD analysis, the samples were used in wet form except in those samples that were not immersed in ethanol.

The changes induced by ethanol on the secondary structure of membranes were investigated by using a FTIR-ATR (Fourier transformed infrared spectroscopy with attenuated total reflection device) NICOLET-PROTEGÉ 460 equipment (Thermo Nicolet

Scientific, Massachusetts, USA). The optical element used for total reflection was a germanium crystal also provided by Nicolet. The membranes were dried and pressed against the crystal (by the sample holder) during measurements to ensure the highest contact surface between optical element and the sample.

Thermal properties of dense membranes treated with ethanol were obtained using the Shimadzu TGA-50 and the Shimadzu DSC-50 (Shimadzu, Kyoto, Japan). The range of temperature of 25–900 °C with a ramp rate of 10 °C/min and a N₂ flow of 25 mL/min were used in TGA analysis. Amounts of membrane weighing from 4 to 5.5 mg were used as samples, on platinum pans. For DSC analysis, the temperature range of 25–500 °C was used, with a ramp rate of 5 °C/min. The N₂ flow of 50 mL/min was applied and sample amounts ranging from 4 to 5.5 mg were used on aluminum pans.

Mechanical tests were performed using a SMS (Stable Micro Systems, Surrey UK) TA-xT2 texturometer, where tensile analyses were carried out. The samples were analyzed in a wet form and prepared following the procedures of ASTM D882-02 standard. All analyzed membranes had been treated in ethanol before running the mechanical tests and their average thickness was measured as ca. 100 µm. The equipment parameters used were: (a) pre-test speed: 3 mm/s, (b) speed test: 1 mm/s, (c) distance test: 40 mm and (d) initial distance: 50 mm. The strain was calculated as the ratio of the elongation to the gauge length of the test specimen, that is, the change in length per unit of original length (ASTM D638-02a).

2.3. Biocompatibility

2.3.1. Cytotoxicity test

Silk fibroin membranes were sterilized by humid heating. After sterilization, they were immersed in RPMI (Roswell Park Memorial Institute) culture medium of 6 cm²/mL concentration and left in the incubator at 37 °C for 72 h to fulfill the extraction condition.

The cytotoxicity test was evaluated with Chinese hamster ovary cell line (CHO-k1). They were maintained in RPMI medium supplemented with antibiotic and antimicrobial (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% CO₂ 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, pH 7.4. The procedure for measurement was based in a colorimetric method which uses a tetrazolium compound (MTS or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) for determining the number of viable cells in proliferation (Cory et al., 1991). The microplates of 96 wells were prepared with 50 µL in quadruplicate of extracts diluted from 6.25% to 100% in RPMI medium. A suspension of CHO-k1 cells with 6 × 10⁴ cell/mL was prepared and 50 µL/well was pipetted in the microplates and incubated for 72 h at 37 °C in a humidified 5% CO₂ atmosphere. Blank and control medium of cells were also prepared. The cell viability was measured by adding 20 µL of MTS/PMS (phenazine methosulphate) (20:1) solution and incubated for 2 h at 37 °C in the humidified 5% CO₂ incubator. The microplates were read in a spectrophotometer at 495 nm. The test was compared with a negative control of HDPE (high-density polyethylene) and a positive control of phenol 0.3% in saline 0.9% solution. The Cytotoxicity Index for 50% of cell viability (CI₅₀) was graphically determined.

2.3.2. Biofunctionality

Human umbilical vein endothelial cells (HUVEC) from ATCC (CRL 1730) were maintained in F12 medium (Gibco®, Invitrogen Brasil Ltda., São Paulo, Brazil) supplemented with antibiotic and

antimicrobial solution at final concentration of 100 units/mL penicillin, 100 µg/mL streptomycin and 0.025 µg/mL amphotericin (Gibco®, Invitrogen Brasil Ltda., São Paulo, Brazil), 2 mM glutamine (Gibco®, Invitrogen Brasil Ltda., São Paulo, Brazil), 20 µg/mL endothelial cell growth supplement, 90 µg/mL heparin (Roche Brazil, São Paulo, Brazil) and 10% bovine fetal serum (Gibco®, Invitrogen Brasil Ltda., São Paulo, Brazil), the cells were maintained in an incubator at 37 °C in a humidified 5% CO₂ atmosphere until they reached confluence. For subculturing and experiments, cells were harvested using 0.05% trypsin and 0.02% EDTA (Sigma–Aldrich, St. Louis, Missouri, USA) in phosphate-buffered saline solution, pH 7.4.

Cell seeding onto SF membranes was performed after the silk fibroin membranes were sterilized by humid heating and placed on the button of a 12 multiwell plate. Three wells without silk fibroin membrane were used as control. Cell suspension were seeded at a concentration of 1.0×10^4 cells per well. The cells growing were accompanied by daily observation with a light inverted microscope with phase filter and the culture medium was changed every three days. Digital photographs were taken in 3rd and 14th days of culture.

2.4. Statistical analyses methodology

All results were performed on replicates: two membranes of each type for FTIR-ATR and XRD analyses; mechanical tests were performed on five replicates of each sample; the error bar of cell viability percentage was calculated for each extract concentration, from the standard deviation of values from four replicates (four wells for each concentration) and the cell counts were performed on five pictures taken from each well. The computer packages used for statistical analyses were Origin® and Minitab®.

3. Results and discussion

3.1. Morphology and XRD

Morphology of silk fibroin membranes is shown in Fig. 1. The SEM image exhibited regular surface without visible pores.

The XRD patterns of SF solution before and after dialysis and SF dense membranes before and after ethanol treatment are shown in Fig. 2. The profiles showed that the original salty and dialyzed SF solutions presents typical patterns of amorphous substances, as presented in Fig. 2(a) and (b), respectively. However, after dialysis, the solution revealed a halo around 2θ equals to about 27°, indicating their tendency to chain organization. This fact is in accordance to the loss of salt (calcium salt ions) in SF solution during dialysis, which diminishes the fibers dissociation and promotes hydrogen bond formation. The dialyzed solution is unstable, since there are fewer ions separating the protein molecules.

XRD diffractograms of dense membranes before and after ethanol treatment, exhibited in Fig. 2(c) and (d), showed amorphous patterns but with a strong tendency for organization. Before ethanol treatment, the pattern of dense membrane showed halos around 18.7–26.2°. Modifications in crystal structure can be noticed during the film formation. These transitions are indicated by the displacement from the first halo at 25–30° (SF dialyzed solution) to 18.7–26.2° (SF dense membrane). After ethanol treatment, dense membranes show a more intense halo indicating that the protein chains are more organized. Um et al. (2001) prepared SF dense membranes following the same methodology used in this study, except from the crystallization step, where the authors used methanol instead of ethanol. As results from XRD analysis, the authors showed peaks associated to β -sheet structure. These peaks are normally at 9.1°, 18.9° and 20.7° corresponding to β -sheet crys-

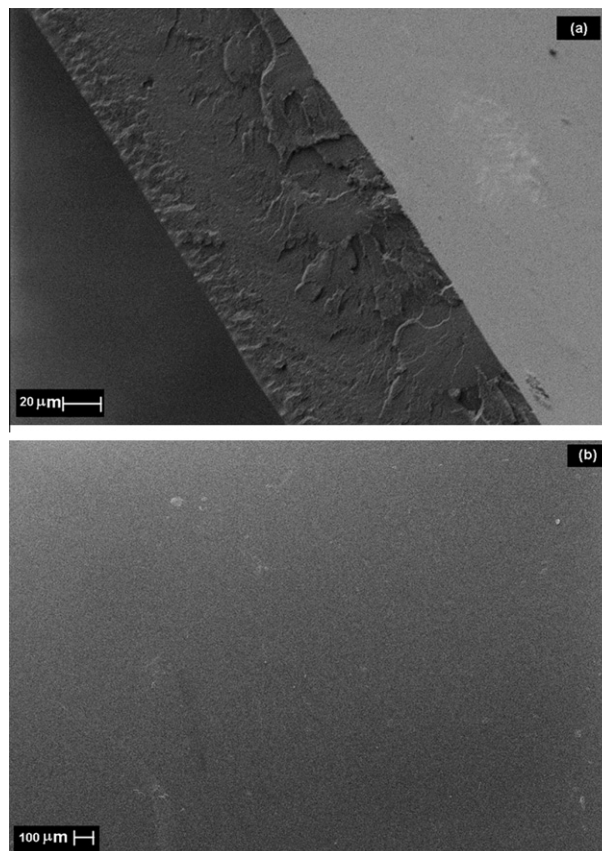


Fig. 1. SEM image of (a) fracture and (b) surface of SF dense membranes.

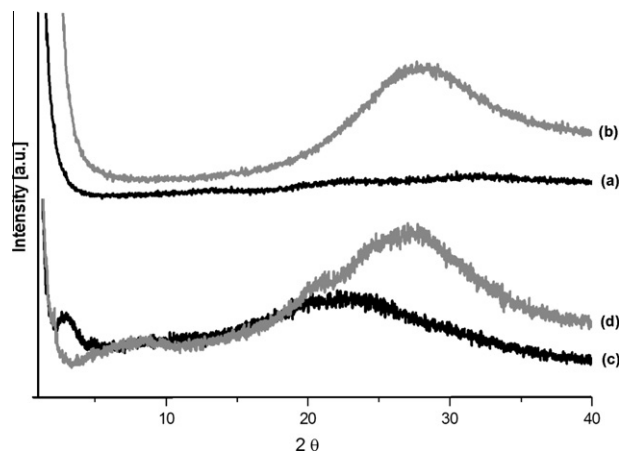


Fig. 2. XRD diffractograms of SF solution (a) before and (b) after dialysis; SF dense membrane (c) not treated and (d) treated in ethanol.

talline spacing of 9.70, 4.69 and 4.30 Å, respectively. The peaks associated to random coil structure are at 12.2°, 19.7°, 24.7° and 28.2° corresponding to crystalline planar space of 7.28, 5.5, 3.6 and 3.16 Å, respectively (Li et al., 2002).

Although SF is amorphous in the analyzed samples, the XRD diffractogram present some order. From Bragg law ($\lambda = 2d \sin \theta$), the film presents average distances (d) between polymeric chains of: 3.7 Å for dialyzed solution, 4.1 Å for dense membrane not treated with ethanol, 3.4 and 4.2 Å for dense membrane treated with ethanol. Comparing these results with those reported by Um et al. (2001), Chen et al. (2001b), and Jin et al. (2004a), it is possible to observe differences in the distances of SF polymeric chains. These

differences can probably be attributed to the fact that ethanol (and not methanol) was used for treatment and that wet membranes were analyzed (instead of the dried form). The distances indicate that probably SF presents random coil conformation in dialyzed solution and dense membranes not treated in ethanol, while dense membranes treated in ethanol exhibit halos that are associated to both β -sheet and random coil conformations.

3.2. FTIR-ATR

Although the dried membranes are compressed against the crystal (by the sample holder) during measurements, the resolution of spectra will depend on the contact area between optical element and the sample. FTIR-ATR can provide some poorly resolved peaks depending on the roughness of the surface that is in contact with the crystal, however it is the most valuable technique to measure the changes on surfaces and interfaces. FTIR-ATR is a powerful technique to investigate structures, as the knowledge of the vibration origins of amide bonds and others can be often applied to study the molecular conformation of silk fibroin fibers or films (Min et al., 2004; Kong et al., 2004). The more informative infrared bands to analyze proteins are the amide bands: amide I, amide II and amide III. Amide I vibrations represent CO stretching, while amide II vibrations represent the bending of NH bond associated with CN stretching (Franks, 1993). Amide III vibration is associated to the combination of NH deformation and the CN stretching vibration (Magnani et al., 1991).

FTIR-ATR spectra of dense membranes before and after ethanol treatment are shown in Fig. 3(a) and (b), respectively. Dense membranes not treated in ethanol showed absorption bands at 1530 cm^{-1} to amide II and at 1656 to amide I. After ethanol treatment, the absorption bands were shown at 1623 , 1700 , attributed to amide I, at 1527 cm^{-1} to amide II.

These results indicated that the secondary structure of dense membranes changed after ethanol treatment from random coil to β -sheet conformation (Franks, 1993; Freddi et al., 1999; Um et al., 2001; Chen et al., 2001a; Cardenas et al., 2002; Li et al., 2002). The band at 1700 cm^{-1} can be indicative of the antiparallel arrangement of the fibroin chains in the β -sheet domains (Freddi et al., 1999).

3.3. Thermal analysis

Thermogravimetric curves of SF dense membranes are shown in Fig. 4. Both membranes showed similar curves with two regions of mass loss: the first one was presented near $100\text{ }^\circ\text{C}$ and is attributed to the loss of unbound water and the second one was presented in

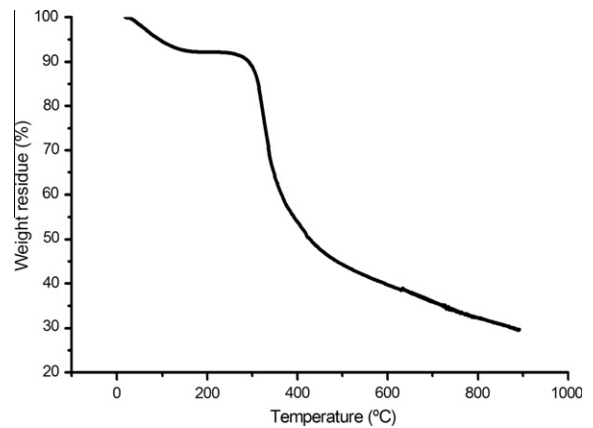


Fig. 4. Thermogravimetric curves of SF dense membrane treated with ethanol.

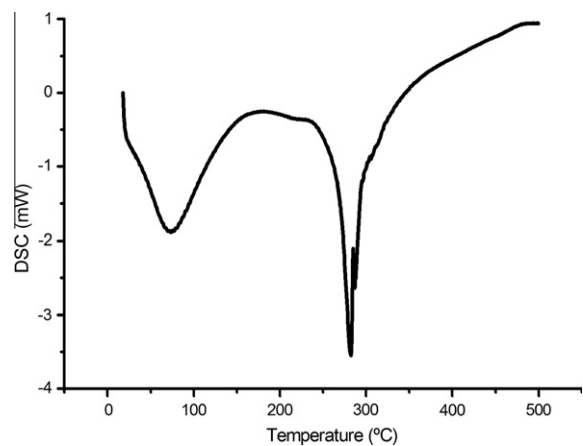


Fig. 5. DSC thermograms of SF dense membrane treated with ethanol.

a range of $270\text{--}380\text{ }^\circ\text{C}$ and is associated with the breakdown of side chain groups of amino acid residues as well the cleavage of peptide bonds (Um et al., 2001).

DSC thermograms of SF dense membranes are shown in Fig. 5. Endothermic peaks below $100\text{ }^\circ\text{C}$ are associated to water loss during heating. Thermal degradation peaks are present at 283 and $287\text{ }^\circ\text{C}$. The presence of double degradation peak indicates that endothermic reactions of different structures occurred. The decomposition behavior of silk is influenced by the intrinsic morphological and physical properties of the sample, with the degree of

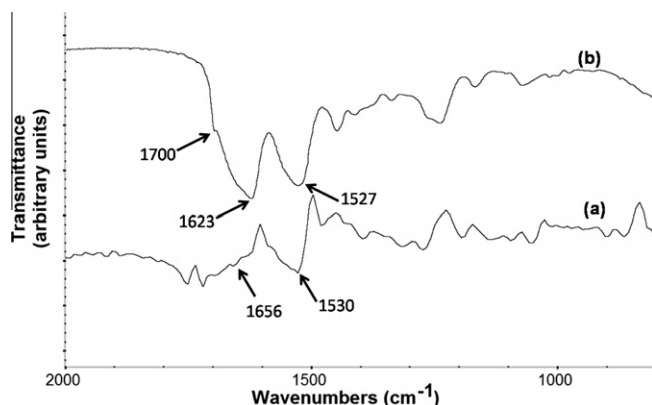


Fig. 3. FTIR-ATR spectra of SF dense membrane (a) not treated with ethanol and (b) treated with ethanol.

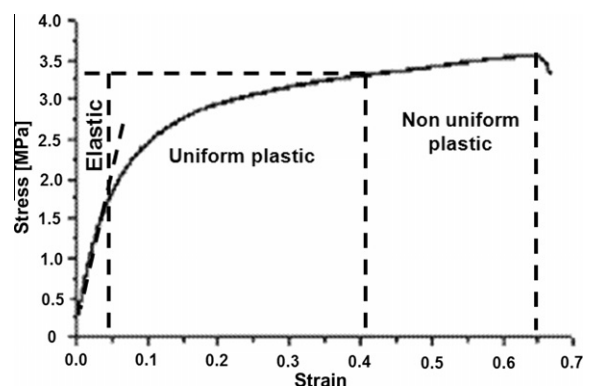


Fig. 6. Typical profile of tensile test of silk fibroin dense membranes treated with ethanol.

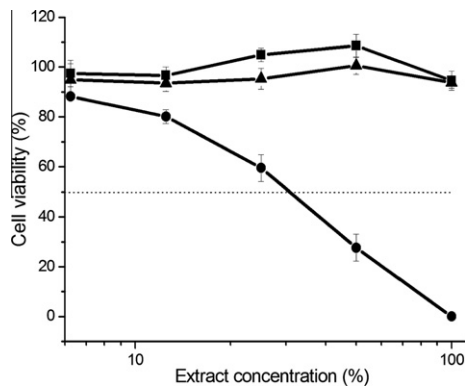


Fig. 7. Viability of the CHO-k1 cells under the cytotoxicity test. ■ – SF dense membrane; ● – positive control (phenol solution 0.5% v/v); ▲ – negative control (HDPE – high-density polyethylene).

molecular orientation being one of the most important parameters (Tsukada et al., 1996). Well-oriented silk fibers normally exhibit a decomposition peak located at above 300 °C, no oriented silk materials with β -sheet structure, usually decompose in the 290–295 °C and amorphous silk fibroin occurs at a lower temperature, normally less than 290 °C (Freddi et al., 1999). The membranes analyzed, showed decomposition peaks on the temperature range associated to amorphous SF, confirming the XRD results presented previously.

3.4. Mechanical tests

The average values of stress and strain at rupture for dense membranes were found to be 3.5 MPa and 0.68, respectively. Typical profile of tensile tests (Fig. 6) to SF dense membranes presented three distinct regions: (1) linear, corresponding to elastic phase; (2) curve region, following the linear region, corresponding to uniform plastic deformation; (3) non-uniform plastic deformation region.

The stress values at rupture observed are inferior to those reported for polymeric films, such as those made from PLLA (Ouchi

et al., 2006). However, these values are close to those presented for biopolymeric films such as those composed by chitosan (Lauto et al., 2001; Jin et al., 2004b).

The wet form of tested membranes probably influenced greatly on the magnitude of stress–strain values. This effect was studied by Jin et al. (2004b) for chitosan films cross-linked with genipin. They concluded that the increase in films moisture led to increased strain and decreased stress values.

3.5. Biocompatibility

The sterilization procedure did not change the SF membranes stability. The response of the cytotoxicity test, which evaluated the CHO-k1 viability of SF membranes extract dilutions is shown in Fig. 7. In this figure, it is possible to observe that the SF membrane did not present cytotoxic effect.

Micrographs where HUVECs are adhered and grown onto SF membranes surface in a period of two weeks are shown in Fig. 8. The cells density increased by the same growth rate when membranes were compared to the positive control wells of the cell culture plates.

It is possible to say that qualitatively, SF membranes present high potential to be used in applications where cytocompatibility is required. The rate of cell number growth (observed by comparing the increase in Fig. 8 from (a) to (b) versus (c) to (d)) is more important for analysis than the absolute number of cells (Fig. 8 from (a) to (c) or (b) to (d)), as the latter is highly influenced by the cell seeding step of the test.

4. Conclusion

The use of waste silk fibers is an alternative to the most common method of using the silkworm cocoons in the preparation of silk derived materials. Silk fibroin from waste silk fibers can be processed into dense membranes with similar characteristics to those reported for SF from the virgin cocoon. After ethanol treatment, dense membranes are crystallized to the β -sheet conformation and are suitable for adherence and growth of endothelial cells. These results indicate that SF membranes, prepared from waste

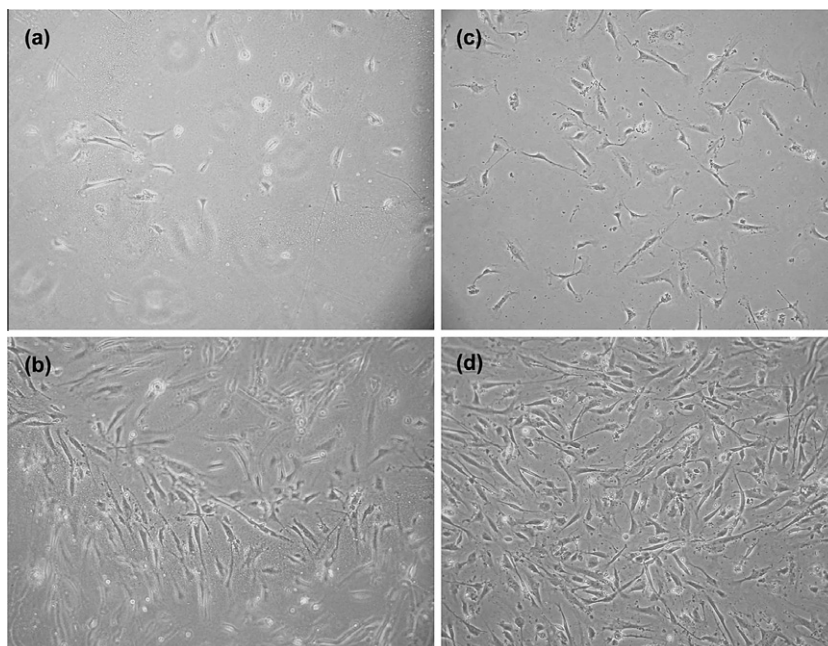


Fig. 8. HUVEC growing onto SF membrane (a, b) and control well (c, d).

silk fibers, are promising candidates for biomaterials application, such as coating or manufacturing of medical devices, membranes for wound dressing and drug delivery systems.

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