A New Method to Prepare Porous Silk Fibroin Membranes Suitable for Tissue Scaffolding Applications

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ABSTRACT: A new method to prepare porous silk fibroin (SF) membranes without dialysis is proposed. Silk fibers were degummed to remove sericin and the resultant fibroin was dissolved in a $CaCl_2$-CH_3CH_2OH-H_2O ternary solvent. Rather than undergoing dialysis, a fibroin salty solution was diluted in water and then submitted to a mechanical agitation that led to a phase separation through foam formation on the solution surface. This foam was continually collected and then compacted between plates to remove the excess of water. The membranes presented large pores with diameters of greater than 100 μm (as shown by scanning electron microscopy - SEM), porosity of 68% and water content of 91% w/w. X-ray diffraction (XRD) and infrared spectroscopy (FTIR-ATR) indicated that the membranes present SF in a β-sheet structure even before the ethanol treatment. A typical elastic deformation profile and degradation under temperature were observed using calorimetric analysis (DSC), thermal gravimetric analysis (TGA) and mechanical tests. As indicated by the *in vitro* cytotoxicity tests, these membranes present potential for use as scaffolds. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 617–623, 2009

Key words: silk fibroin; porous membrane; biopolymer; biomaterial

INTRODUCTION

Silk, a natural polymer produced by the *Bombyx mori* silkworm, consists primarily of two proteins: fibroin and sericin. Fibroin is the protein that forms the silk filament and is coated by sericin, a glue-like protein that holds fibroin fibers together to form the cocoon case.[1] Silk fibroin (SF) had long been employed as textile fiber and surgical sutures, but recently its applicability in biomaterial applications has garnered much interest due to its biocompatibility with a variety of cell and tissue types.[1–3]

SF molecules are made of a heavy and light chain polypeptide of approximately 350 kDa and 25 kDa, respectively, and have 60–80% crystallinity depending on the frequency of its aminoacid sequence, Gly-Ala-Gly-Ala-Gly-Ser.[4,5] Two peptide conformations are commonly found in silk fibroin, labeled silk I and silk II. Silk I is the water-soluble structure existing in the silk glands before spinning and comprises a mixture of random coil and α-helix domains. Silk II, formed after spinning of silk fibers, is insoluble in water and presents an antiparallel β-sheet structure.[4,6,7] Regenerated silk materials can consist of both structural forms depending on the experimental conditions,[4,8] and the silk I structure can be easily converted to silk II by mechanical treatments, temperature changes or solvents.[3,4,6]

High mechanical strength, microbial resistance, thermal stability, ease of processing and biocompatibility are all attractive characteristics of fibroin. Based on these features, many applications have been proposed: substrates for cell culture, enzyme immobilization, drug-release agents, wound dressings, bone compatible materials and antithrombogenic materials.[3,9]

Motivated by the need for 3D biocompatible material structures in tissue engineering, several studies have reported the ability to form regenerated porous SF structures.[10] These 3D porous matrices are commonly used as scaffold materials on which cells can attach, multiply, migrate and function, as in the body's extracellular matrix.[11] These matrices must promote cell adhesion and growth, exhibit mechanical stability and degradation rates comparable with *in vivo* tissue growth.[11,12]

Regenerated SF materials, such as gels, films or porous structures, are commonly produced from a SF solution, which can be prepared by dispersing fibers in an appropriate solvent, generally containing bivalent salt ions. These salts are removed by dialysis, resulting in an aqueous fibroin solution. Nearly all fibroin solution preparation methods depend on this time-consuming dialysis step and are a complex

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process since the fibroin solution is unstable to temperature change and mechanical agitation.[13]

In this study, we sought to develop a new, easily scalable technique to produce regenerated SF membranes without the dialysis step. Membranes were prepared by compacting layers of fibroin foam into a porous structure that maintains its dimensional stability in neutral aqueous environments. These structures have good mechanical properties and, furthermore, can be easily processed, being suitable for large scale production.

This paper presents the conditions employed to form porous SF membranes and their physical and chemical characterization including morphological, thermal and crystallographic analyses, mechanical tests and infrared spectroscopy to analyze the peptide conformational structure. Cytotoxicity of SF membranes was assayed as a preliminary investigation of the membranes' cell compatibility.

EXPERIMENTAL

Preparation of silk fibroin solution

Silk fibroin of the *Bombyx mori* silkworm from Bratac (São Paulo-Brazil) was degummed three times with 0.5% (w/v) Na_2CO_3 solution at 85°C for 30 min and then rinsed with water and dried at room temperature. Degummed silk was dissolved in a ternary solvent of $CaCl_2$-CH_3CH_2OH-H_2O (1 : 2 : 8 mole ratio), at 85°C to a concentration of 0.1 g/mL.

Porous membrane preparation[14] (details of preparation are patented)

SF solution was diluted in distilled water (1 : 100) to diminish fibroin solubility and then foaming was induced by mechanical stirring using a Quimis 251D mechanical stirrer (Brazil) at approximately 620 rpm. This resulted in formation of a light foam on the surface of the solution, which was continually collected. The fibroin foam was compressed between polypropylene plates of 8 cm in diameter, applying a pressure from 1 to 3 kgf for 2 min, during 30 min, at about 25°C, in order to remove excess of water and promote foam agglomeration. At each interval of pressure loadings, more foam was aggregated to the plate and the excess of water was removed. Membranes were constructed by compressing several layers of compacted foam. Thickness and porosity of the membrane could be controlled by applying a compression force that was enough to reach the desired dimension (from 1 to 3 kgf). These kinds of membranes can be manufactured with different initial conditions, such as with different concentrations of silk fibroin solution, however, this factor does not affect the properties of membranes as long as the water added in the process is enough to provide the phase separation.

The stability of formed membranes was increased by their immersion in ethanol for 24 h. The membranes were then washed and stored in ultrapure water at 8°C. After one month, the membranes were still stable in water and did not present degradation. Freeze-dried samples were also shown to present dimensional stability. Mechanical tests were performed in order to investigate the effect of immersion time in ethanol on membrane mechanical properties.

Characterization

Morphology

Porous SF membranes were frozen in liquid nitrogen, fractured and then freeze-dried (Liobras, L101, Brazil) for 24 h. Surface and cross-section morphology of the porous membranes were observed with a LEO 440i (USA) scanning electron microscope.

Crystallinity

X-ray diffraction was performed on wet and lyophilized samples with a Rigaku-Ultima-RINT 2000 diffractometer (Japan) with Cu K radiation at 40 kV and 30 mA and scanning rate of 0.6°/min.

Molecular conformation

Fourier transform infrared spectroscopy with attenuated total reflection apparatus (FTIR-ATR) (Nicolet, Protégé 460, USA) was used to determine the secondary structure of silk fibroin porous membranes before and after ethanol treatment.

Thermal properties

Thermal gravimetric analysis (TGA) was performed by using a Shimadzu TGA-50 (Japan) analyzer at a heating rate of 10°C/min under nitrogen atmosphere (25 mL/min). Differential scanning calorimetry (DSC) measurements were performed with a Shimadzu DSC-50 (Japan) instrument under a nitrogen atmosphere (50 mL/min). Both analyses were performed on freeze-dried samples.

Mechanical properties

Tensile tests were performed using a Stable Micro Systems TA-xT2 texturometer (UK), according to the ASTM standard D882-02[15] at a cross-head speed of 1 mm/s. Samples were treated in ethanol for (a) one or (b) five days and then washed and stored in distilled water for one day before running the mechanical tests. The samples were taken from the storage

recipient and the excess of water was removed by standing the samples for 1 min in the environment.

Porosity and moisture

Porosity and moisture were calculated using the following equations:

$$\text{Porosity } (\%) = \frac{W_w - W_d}{V \cdot d} \times 100 \quad (1)$$

$$\text{Moisture } (\%) = \frac{W_w - W_d}{W_w} \times 100 \quad (2)$$

in which W_d and W_w are the mass of the membranes in the dry and wet states, respectively, d is the density of water and V is the volume of the wet membrane.

Residual calcium concentration

Porous SF membranes were dried and dissolved in chlorhydric acid (PA). The obtained solutions were then diluted in distilled water and analyzed using an atomic absorption spectrometer (Perkin Elmer, AAnalyst 100, USA) to determine the residual calcium concentration.

Cytotoxicity

In vitro cytotoxicity was performed with the Chinese hamster ovary cell line (CHO-k1). The cells were maintained in RPMI medium supplemented with antibiotics and antimicotic (100 units/mL penicillin, 100 μg/mL streptomycin and 0.025 μg/mL amphotericin), 2 m*M* 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 with 0.05% trypsin and 0.02% EDTA solutions in phosphate-buffered saline at pH 7.4. SF membranes were freeze-dried and sterilized by humid heating at 127°C for 16 min, and immersed in RPMI medium at 37°C for 48 h for extract preparation at a final concentration of 0.1 g/mL. The extract was used to carry out the cytotoxicity tests, as recommended by ISO 10,993.[16] Cytotoxicity test was performed in a 96-well microplate seeded with 3000 cells per well and extract dilutions of 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 (20 : 1) solution and incubated for 2 h at 37°C in a humidified 5% CO_2 incubator. Finally, the microplates were read in a spectrophotometer reader at 495 nm. The test was compared with a negative control of 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.

Figure 1 Morphology of silk fibroin porous membrane in a wet form.

RESULTS AND DISCUSSION

Morphology

Figure 1 presents a fibroin membrane prepared by the described process. Since the membrane is composed of several individual fibroin sheets, the strength of interaction between layers is critical—if this interaction is too weak, the film will delaminate into individual layers. The membranes, upon ethanol treatment, presented stability to such delamination but were flexible when wet. However, upon drying at room temperature, the membranes became extremely brittle. The lyophilized fibroin membrane surface [Fig. 2(a)] has an irregular structure with few visible pores; however, large pores of variable sizes were visible in the cross-section image of membranes [Fig. 2(b)]. The lack of pores that are orthogonal to the compression loading axis is probably due to the nature of the compression method, where it would be expected that the majority of void space would exist parallel to the loading axis. The majority of pores had a diameter of greater than 100 μm, which is thought to be one of the requirements for tissue scaffold materials.[17] The low porosity on the membrane surface is not a limitation, since a superficial layer can be easily removed, exposing a more porous one.

Porous SF structures may be prepared by different methods such as freezing thawing,[10] freezing-drying,[17,18] salt leaching,[19] or electrospinning.[20] However, these techniques include the time-consuming dialysis step. The present methodology does not require dialysis and can be used to prepare SF materials with similar pore shapes and sizes of some SF scaffolds reported in the literature.[17]

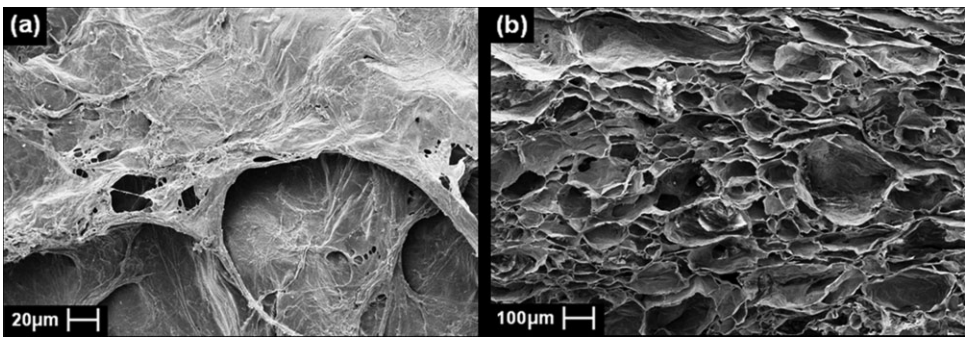

Figure 2 SEM image of (a) surface and (b) fracture of silk fibroin porous membrane.

XRD

X-ray diffraction patterns of wet and freeze-dried fibroin membranes are presented in Figure 3. Before lyophilization, fibroin molecules were plasticized by water and presented an amorphous structure with a broad peak, with maximum at $2\theta = 28°$, which could be related to silk I.[3] The same XRD profile was observed for wet samples before or after ethanol treatment. The loss of water promoted by membrane lyophilization induced their crystallization due to its molecular rearrangement and new hydrogen bond formation. Lyophilized samples showed peaks at $2\theta = 20.9°$ and $2\theta = 24.7°$, indicating the presence of silk I and silk II crystalline structures.[3,10]

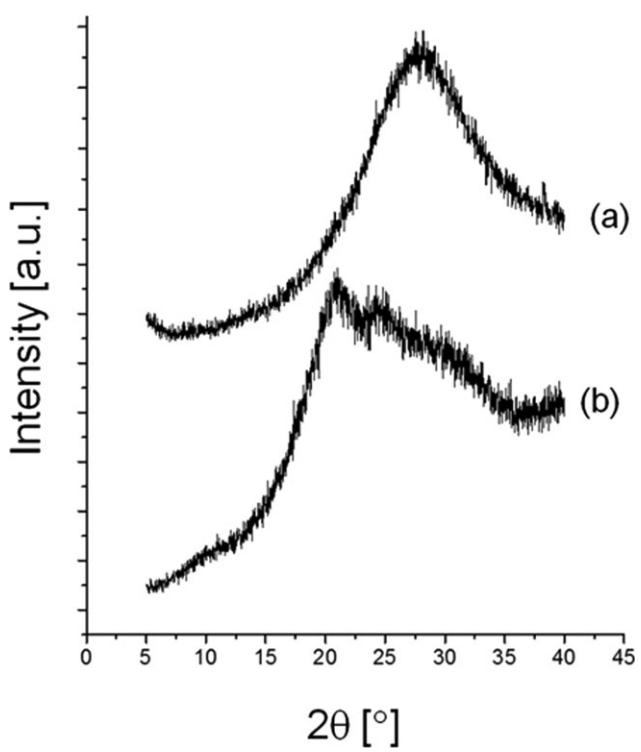

Figure 3 XRD spectra for (a) wet and (b) lyophilized silk fibroin porous membrane.

FTIR-ATR

FTIR spectra of both as prepared and ethanol-treated fibroin membranes are shown in Figure 4. Absorption bands at 1624 cm^{-1} (amide I) and 1527 cm^{-1} (amide II) were observed for nontreated membranes, and absorption bands at 1625 cm^{-1} (amide I) and 1533 cm^{-1} (amide II), for treated fibroin membranes. These results indicate that the porous SF membranes have a β-sheet structure even before the ethanol treatment.[2,8,21–25] It has already been reported[4] that fibroin foams favor the β-sheet conformation (silk II) at the dilution limit (<7%), while random coils or α-helices (silk I) dominate in more concentrated solutions. The alcohol treatment did not seem to change the fibroin molecule secondary structure, but it helped to stabilize the membranes, increasing the interaction of foam layers, probably due to the dehydration of the foam layers that can promote the formation of hydrogen bonds between them and, therefore, avoid their delamination.

Thermal analysis

Thermal analysis was performed on lyophilized fibroin membranes (Fig. 5). The initial weight loss at around 100°C is due to the loss of water, while the

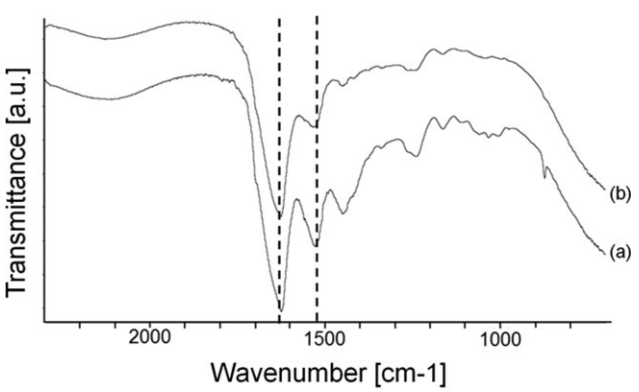

Figure 4 FTIR-ATR spectra for silk fibroin porous membrane (a) treated in ethanol and (b) not treated in ethanol.

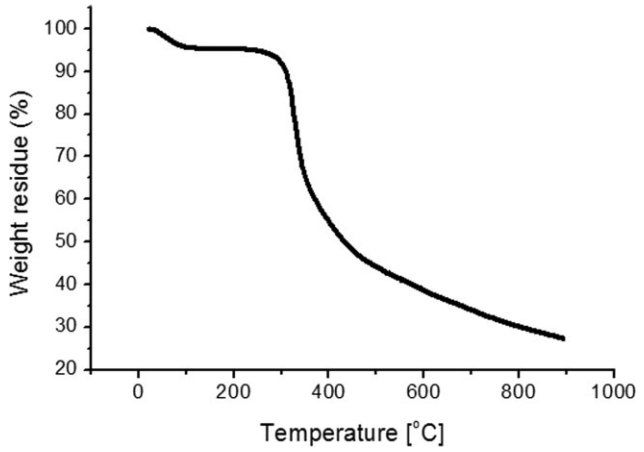

Figure 5 Thermogravimetric curves for silk fibroin porous membrane.

second weight loss, in a temperature range of 270–380°C, is associated with the breakdown of side chain groups of amino acid residues, as well the cleavage of peptide bonds.[2]

DSC thermograms (Fig. 6) present two endothermic peaks, one just below 100°C, due to the loss of water, and another at 296°C attributed to thermal degradation of silk fibroin. The decomposition behavior is similar to the one observed for nonoriented silk materials with a β-sheet structure.[2,21]

Mechanical tests

We investigated the effect of ethanol treatment on membrane mechanical properties. Figure 7 presents a typical tensile stress–strain profile of wet fibroin membranes treated in ethanol for one or five days. All analyzed membranes presented a typical elastic deformation profile. The average ultimate tensile strengths of the membranes treated in ethanol for

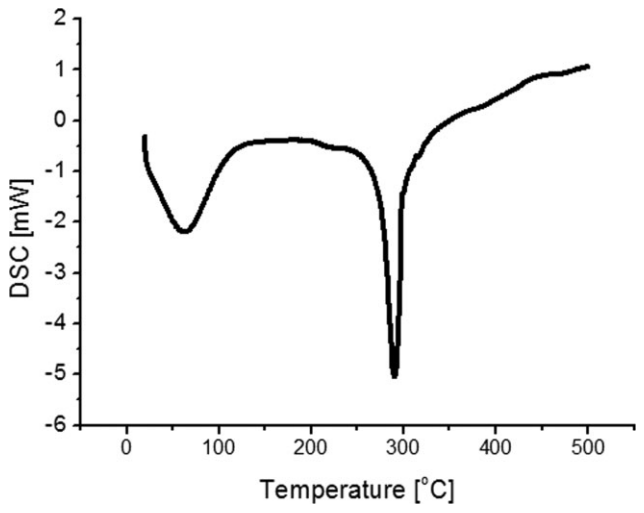

Figure 6 DSC thermograms for silk fibroin porous membrane.

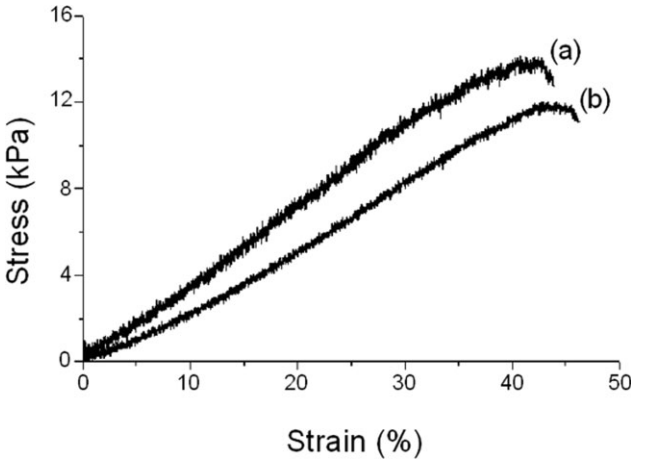

Figure 7 Typical tensile test profiles obtained for fibroin membranes treated in ethanol for (a) five days and (b) one day.

one and five days were calculated to be 11 ± 1 kPa and 14 ± 1 kPa, respectively. The immersion time in ethanol did not significantly change the tensile properties of the analyzed samples, suggesting that after 24 h in ethanol the membranes were already stabilized. More tests are being done in our laboratory to investigate the mechanical properties of lyophilized SF membranes. This data will be helpful to understand the effect of water plasticizing property on fibroin porous membranes and to compare with other SF scaffolds described in the literature.

Porosity and moisture degree

The porosity and moisture of SF membranes were calculated to be 68% and 91%, respectively. Porosity of SF scaffolds prepared by other techniques such as freeze-drying, freeze-thawing or electrospinning can achieve values of higher than 80%.[17,18] Porosity of SF membranes, prepared by the method proposed, may probably be tuned to high values by an accurate control of the compression stress applied to agglomerate the foam layers. However, further studies should be done to evaluate how stable the membranes would be when lower compression stress is applied.

Residual calcium concentration

The residual calcium concentration of fibroin membranes after immersing them in water for five days was calculated to be 5.5% (w/w). This result shows the high affinity of the membranes for calcium, making them suitable for use as a bone regeneration matrix. Kino et al.[26] reported that SF films prepared by casting are prone to mineralize during *in vitro* calcification experiments when the calcium concentration in the films is higher than 5%. These authors added

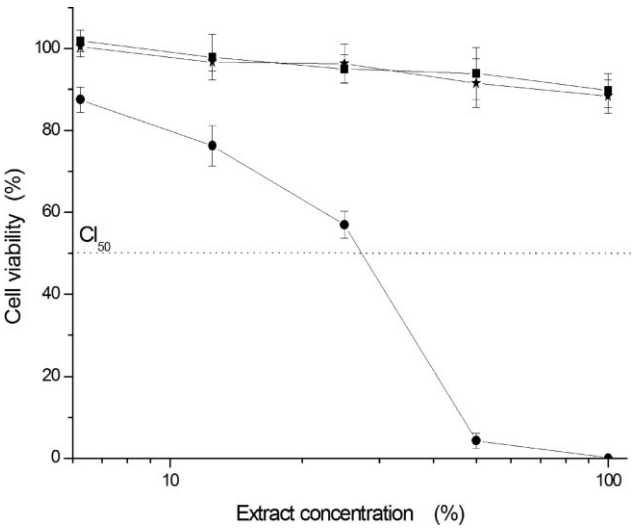

Figure 8 Cell viability curve for silk fibroin membrane (★); negative control – HDPE (■); positive control – 0.5% v/v phenol solution (●).

calcium chloride to the SF dialyzed solution in order to achieve the required calcium concentration. The porous membranes prepared by our method do not require calcium chloride addition or the dialysis step. *In vitro* calcification experiments on silk fibroin porous membranes produced by the proposed method have been carried out in our laboratory and deposition of calcium phosphates, such as hydroxyapatite, was observed.[27] The quantity of residual calcium may be adjusted by altering the processing conditions, such as the dilution rate of silk fibroin solution in water, immersion of membranes in water, competitive salt binding, and other methods.

Cytotoxicity test

The cytotoxicity assay can be used as a screening test to predict the cell compatibility of new biomaterials.[28] According to Figure 8, porous SF membranes do not present IC_{50} value for any extract concentration analyzed. As such, SF porous membranes can be initially considered as a non-cytotoxic material, being suitable for exploration in the biomaterials field. This characteristic is ensured by the natural conditions used in the production method, which do not employ any hazardous solvent or material. Many researchers have investigated the biocompatibility of SF-derived materials and have shown that SF is compatible with several types of cells and, in most cases, they promote cell adherence and growth.[20,29,30] Further studies will be carried out on porous SF membranes obtained by the proposed method to verify the potential of those materials for cell adherence and growth and, therefore, assess the feasibility of using them as scaffolds in the tissue-engineering field.

CONCLUSIONS

We have proposed a new method to produce SF membranes using a simple, industrially scalable approach that eliminates the need for time-intensive dialysis. Membranes produced using this method can be prepared easily in large scale, with properties that make them attractive candidates for use as biomaterials, scaffolds in tissue engineering. These properties include the interconnection of pores that are large enough to allow cell growth, high porosity (above 60%), mechanical and thermal stability (with SF β-sheet), suitable calcium content, and noncytotoxicity.

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