Characterization of Silk Fibroin/Hyaluronic Acid Blend Films Cross-linked with EDC

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Abstract: Silk fibroin (SF) / hyaluronic acid (HA) blend films were prepared using 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as crosslinking agent and N-hydroxysuccinimide (NHS), 2-morpholinoethanesulfonic acid (MES) as assistant agents. The physico-chemical properties of the blend films were examined and analyzed. The FTIR shows that HA interferes with SF to form crystal structures, whereas EDC induces SF to form Silk I. Compared to the uncross-linked blend films, the EDC cross-linked blend films show an obvious increase in elongation at break, and a decrease in tensile strength and Young's modulus. The flexibility of the films improved significantly. L929 cells grew well on SF/HA blend films, demonstrating that the blend films cross-linked with EDC have no significant toxicity.

Keywords: Silk fibroin, hyaluronic acid, films, blends, crosslinking, structure, cyotocompatibility.

1. Introduction

In biological systems, besides protein there are large amounts of hyaluronic acid and other mucopolysaccharide in the extracellular matrix of the cartilage tissue and dermal connective tissue of vertebrates. Protein and polysaccharide, the two main components of extracellular matrix, have a few favorable effects on several cellular functions such as adhesion, migration, and proliferation. These components maintain normal structure and function of the organism. Ideal characteristics for the substrate to support tissue ingrowth include special and compositional properties that attract and guide the activity of the cell. Silk synthesized by Bombyx mori, consists of two kinds of proteins, sericin and fibroin (SF). SF is the structural fibrous protein and constitutes about 75% of the intact silk; sericin which surrounds and binds the fibroin fibers constitutes about 25% of the intact silk. The SF molecule consists of heavy chain, light chain and P25 glycoprotein of ~350 kDa, ~26 kDa and ~30 kDa, respectively, and the ratio is H:L:P25=6:6:1 [1]. SF has a good biocompatibility and silk has been commercially used as surgical sutures for decades. It is also an attractive natural fibrous protein for biomedical applications due to its permeability to oxygen and water, relatively low inflammatory response and protease susceptibility. Recently, there are many reports about the SF materials which have been widely applied in controlled drug delivery system [2], blood vessel engineering [3], peripheral nerve regeneration materials [4], femur defects [5], bone tissue engineering [6], artificial ligament [7], artificial tendon [8] etc.

Hyaluronic acid (HA) is a mucopolysaccharide found in various types of tissues. Its immunoneutrality makes it an excellent building block among biomaterials to be employed in tissue engineering [9]. HA has been widely applied in ophthalmology [10], osteoarthritis [11], wound healing [12], drug delivery [13] and tissue engineering [14] which demonstrates that HA has a good biocompatibility [15-16].

In this study, SF/HA blend films were prepared using 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as cross-linking agent and (NHS) N-hydroxysuccinimide, 2-morpholinoethansulfonic acid (MES) as assistant agents. The physico-chemical properties of SF/HA blend films as well as attachment and growth of fibroblast cells on films were investigated.

2. Experimental

2.1 Materials

Bombyx mori raw silk was purchased from Zhejiang province of China. Hyaluronic acid (sodium salt, Mw = 2.5×10^6) was purchased from Shandong Freda Co., Ltd. (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma Chemical Co., 2-morpholinoethanesulfonic acid (MES) was purchased from Fluka Chemical Co., L929 cells were provided by

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School of Medicine of Soochow University, DMEM (Dulbeccu's Modified Eagle Medium, Gibico) was purchased from Shanghai Xi Basi Biological Technology Co., Ltd.

2.2 Preparation of SF/HA blend films

Raw silk fibers were degummed three times with 0.05% (w/w) Na_2CO_3 solution at 100 ºC for 30 min and rinsed thoroughly and then dried in an oven. The extracted silk fibroin was dissolved in a ternary solvent system of $CaCl_2/CH_3CH_2OH/H_2O$ (1:2:8 in molar ration) at 70±2 ºC for 1 h. SF solution was obtained after dialysis and filtration. The HA aqueous solution was obtained by dissolving HA powder in deionized water.

The SF and HA solutions were mixed together at weight ratios of 100/0, 90/10, 80/20, 60/40, 40/60, 20/80, and 0/100, respectively. The EDC, NHS and MES were added to give the weight ratios of 20%, 10% and 20% against the total weight of SF and HA in solution, respectively. The blend films were prepared by casting the mixed solutions on the polyethylene dish and dried at 60ºC. The thickness of the blend films were about 50 μm.

2.3 Scanning Electron Microscopy (SEM)

Surface morphology of blend films was observed by HITACHI.S-570 scanning electron microscopy.

2.4 Fourier Transform Infrared (FT-IR)

The blend films were cut into micro-particles with radius less than 40 μm, and then samples were prepared in KBr pellets. FT-IR spectra were obtained with a Nicolet Avatar-IR360.

2.5 Tensile strength and elongation at break

Tensile strength and elongation at break were measured by an Instron 3365 Tester at 20 ºC, 65% RH with a head speed of 20 mm/min. The specimen was 30 mm wide and 30 mm long between clamps.

2.6 Young's Modulus

Initial tensile modulus was performed with an Y391 Yarn Elasticity Tester. The samples were cut into 120 mm×5 mm strips. Elongation value was obtained after a drawing load of 0.735 N was exerted on the samples for 5 secs. The experiment was performed under the following conditions: pretension of 0.2 N and gauge length of 50 mm. The initial tensile modulus was calculated according to the Equation (1):

$$E = \frac{P \times L_0}{S \times \Delta L} \tag{1}$$

Where E is initial tensile modulus (MPa), P is drawing load (N), L_0 is specimen gripping length at pretension (mm), S is specimen cross-section area (mm^2) and ΔL is elongation value in 5 s (mm).

2.7 Cell culture

L929 fibroblasts were cultivated in 1% penicillin/streptomycin-10% FBS-Dulbecco's modified Eagles medium at 37 ºC in air with 5 (v/v)% CO_2. The cells were trypsinized in the logarithmic growth state and evenly seeded (1×10^4cells/well) into 24 well plates to continue cultivation. The pictures of the L929 were obtained using inverted microscope (Olympus) after culturing for 5 days. The blend films were immersed in deionized water at 37ºC for 3 days and PBS for another 3 days, then were cut and placed carefully at the bottom of 24 well plates. Collagen film which was used as a negative control was obtained by casting collagen solution on 24 well plates directly, dried at 60 ºC, then treated with 75% alcohol for 2 hrs and immersed in deionized water for 7 days.

3. Results and discussion

3.1 Morphology

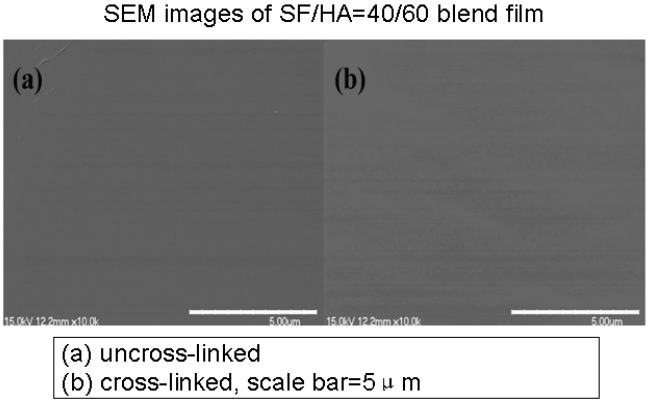

Figure 1 SEM images of SF/HA=40/60 blend film.