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Preparation of Porous Silk Fibroin Materials Cross-linked by Carbodiimide (EDC)

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Abstract: A cross-linked porous silk fibroin (SF) material was prepared by freeze-drying.1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N- hydroxysuccinimide (NHS) and 2-morpholinoethanesulfonic acid (MES) were added in different concentrations of SF solutions. The mixed solution was poured into aluminum vessels to make a layer of about 2 mm thickness. Then they were frozen for 6 hrs at -80, -50, -20 and -10 ℃, respectively, and lyophilized. After 36 hrs porous silk fibroin materials were obtained. Compared to the uncross-linked porous SF materials, cross-linked materials show an obvious decrease in water solubility, indicating that effective cross-linking reaction took place in silk fibroin. The results of FT-IR and Raman spectra show that EDC reacted with the carboxyl of SF, mediating acyl isourea or acid anhydride formation. The resultant acid anhydride may readily react with amino groups of SF to yield amide bonds. The influence of freezing temperature and concentration of the silk fibroin solution on the structure and properties of materials were also investigated. The porous SF materials, with average pore size 32.0-332.5 μm and porosity 59.7%- 90.9%, could be prepared by freeze-drying. Thicker solution and lower freezing temperature resulted in smaller pore size and porosity.

Keywords: Silk fibroin, porous materials, cross-linking, EDC.

1. Introduction

In recent years, silk fibroin is widely used as a starting material for advanced biomedical applications such as controlled drug release carriers [1], artificial cruciate ligament [2], wound cover materials, bone tissue engineering scaffolds etc. Dal et al. [3] implanted 3D mesh works made up of silk fibroin-based nonwovens into the subcutaneous tissue of mice for six months. The mesh works not only are biologically well tolerated but even guide the de novo production of a vascularized RCT. Unger et al. [4] examined primary human endothelial cells, the human endothelial cell lines, HPMEC-ST1.6R and ISO-HAS-1 for adherence and growth patterns on the fibroin nets. They indicated that silk fibroin nets are highly endothelial cell-compatible scaffolding materials that support the growth, normal and inducible cell functions and angiogenesis potential of human endothelial cells in vitro. Kaplan et al. [5] found that the electrospun silk fibroin-based scaffolds supported hMSC growth and differentiation towards osteogenic outcomes. The three-dimensional scaffold will hopefully be applied to tissue engineering matrix, tissue inducing materials and cell cultural substrates.

In this study, freeze-drying method was applied in preparation of porous *Bombyx mori (B. mori)* silk fibroin materials, and the cross-linked SF material were obtained by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as cross-linking agent and N-hydroxysuccinimide (NHS), 2-morpholinoethanesulfonic acid (MES) as assisting agent. The influence of freezing temperature and concentration of the silk fibroin solution on the structure and properties of the porous materials were investigated.

2. Experiment

2.1 Preparation of cross-linked porous SF material

Degummed *B. mori* silk fibroin fibers were dissolved in triad solvent $CaCl_2$-CH_3CH_2OH-H_2O (mole ratio=1:2:8) at 72±2 ℃ for 1h. After dialyzing against water for 4d, the filtrated SF solution was stirred slowly at 37±2 ℃ to let it evaporate and get concentrated up to 5 ~ 10 wt %, and the solution of 7.5%, 5.0% and 2.5 wt % were obtained by diluting it with deionized water. 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and 2-morpholinoethanesulfonic acid (MES), of weight ratios of 20%, 10% and 20% against the total weight of silk

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fibroin, were added into different concentrations of SF solutions, respectively. The mixed solution was poured into aluminum dish to make a layer of about 2 mm thickness. The dish was placed for 3hrs in a freezing chamber at desired temperature (-80, -50, -20 and -10 ℃), respectively, and lyophilized. After 36 hrs porous silk fibroin materials were obtained.

2.2 Measurement

2.2.1 X-ray diffraction (XRD)

X-ray diffraction was performed by a X'PERT PRO MPD diffractometer with CuK_α radiation at a scanning rate of 2 °min^{-1}.

2.2.2 Fourier transform infrared spectra (FT-IR)

The spongy porous SF materials were cut into micro-particles with radius less than 40μm, and the samples were prepared in KBr pellets. FT-IR spectra were obtained by a Nicolet Avatar-IR360.

2.2.3 Raman spectra

The spectra were recorded using a Dilor LabRam-1B spectrometer, operating at a resolution of 1cm^{-1}. The Spectra Physics Model 164 argon ion laser was operated at 632.8nm with about 6mW power.

2.2.4 Pore characteristics

The cross-sectional morphology of porous SF materials was observed by Hitachi S-570 Scanning Electron Microscope (SEM). The border of each pore in top layer was defined according to gradient method. Thus the pictures of the top layer of its cross sections were obtained. Each pore area x_1, x_2, …… x_n and the area (S) of porous SF material in statistic range, were calculated according to the limits of each pore in bmp and the number of picture points in the whole picture. And the pore radius (r_i) of each pore, and average pore radius $\bar{R}$ were given by the Eq. (1) and (2).

$$r_i = \sqrt{\frac{4x_i}{\pi}} \tag{1}$$

$$\bar{R} = \frac{\sum_{i=1}^{n} r_i}{n}\pi \tag{2}$$

The density and porosity of the cross-linked porous SF materials were measured by liquid displacement. A sample of weight W was left in ethanol covered for 1 h and vacuumized to force the ethanol through the pores. Then the ethanol-impregnated sample was immersed in a known volume V_1 of ethanol in a graduated cylinder. The total volume was V_2 and the weight of the composite was W_1. The density ρ (g/cm^3) and the porosity M (%) of the sample were calculated using Eq. (3) and (4) (the density of ethanol is 0.78g/ cm^3).

$$\rho = \frac{W}{V_2 - V_1} \tag{3}$$

$$M = \frac{(W_1 - W)}{\rho_1 \times (V_2 - V_1)} \tag{4}$$

2.2.5 Water solubility

Water solubility of porous SF materials was determined by shaking 250 mg of sample in 25 ml of water at 37 ℃ for 24h. The weight of the dissolved fraction was determined by eq. (5), using ultraviolet absorbance of the supernatant at 278nm.

$$S = \frac{KAV}{W} \times 100\% \tag{5}$$

Where S is water solubility (%), K is 0.1832 mg/ml, (experimental constant determined from weight loss), A is absorbance at 278 nm, V is the volume of the solution (ml), and W is the initial weight of sample (mg).

3. Results and Discussion

3.1 X-ray diffraction (XRD)

The main diffraction peaks of silk I crystal are at 12.2° (d=7.25 Å, medium strong), 19.7° (d=4.5 Å, strong), 24.7° (d=3.60 Å, medium), 28.2° (d=3.16 Å, medium); and those of silk II are at 9.1° (d=9.7 Å, medium strong), 18.9° (d=4.69 Å, medium strong) and 20.7° (d=4.30 Å, very strong) [6].

As shown in Figure 1, the XRD curves of all kinds of uncross-linked porous SF materials are comparatively smooth. No matter on what technical conditions the porous SF materials are prepared, no peaks, which attribute to either the Silk Ior Silk Ⅱ, appear on XRD curves, indicating that the condensed structure of porous SF materials is mainly an