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How ethanol treatment affects the physico-chemical and biological characteristics of silk fibroin nanofibrous scaffolds

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ABSTRACT

In this study, the effects of ethanol treatment on the mechanical and biological characteristics of the nanofibrous silk fibroin (NSF) scaffolds were evaluated. The results obtained from the mechanical tests confirmed that ethanol treatment significantly enhanced the physical properties of the scaffolds through the formation of a ß-sheet structure. It was shown that the ethanol treatment increased the mechanical property and cell viability, while decreased the porosity of the randomly arranged uniform nanofibers. The ultimate tensile strength for the NSF and ethanol-treated NSF (ET-NSF) scaffolds were 0.76 and 1.33 MPa, respectively. In addition, the ethanol treatment positively affected the proliferation rate of rat bone-marrow stromal cells (rBMSCs) without any detectable cytotoxicity. All the results obtained from this study strongly indicated the efficacy of ethanol treatment in enhancement of mechanical and biological characteristics of silk fibroin nanofibrous scaffolds. Copyright © 2015 VBRI press.

Keywords: Silk fibroin; tissue engineering; electrospinning; mechanical properties; ethanol treatment.

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Introduction

The *Bombyx mori* silk is composed of two types of proteins; fibroin protein as a water-insoluble core structural protein surrounded by a water-soluble glue-like protein named sericin. In contrast to sericin, fibroin protein has promising physic-chemical and biological properties making it an interesting biomaterial for tissue engineering applications **[1, 2]**. Silk fibroin protein has been widely used in tissue engineering **[3-6]**. This protein possesses a high concentration of hydrophobic amino acids that makes it insoluble in water. There are three crystalline structures (I, II and III) in silk fibroin protein; structure I) The silk before spinning from abdominal glands of *B. mori*, called α-helical structure which is a metastable structure. Structure II): The silk after spinning that is ß-sheet-rich nanocrystals, named also ß-sheet conformation which is water insoluble. Structure III): The water soluble fibroin lacking ß-sheet **[7-9]**. The silk fibroin requires to be dissolved for using as a biomaterial in fabrication of tissue engineering scaffolds **[10]**. High concentration chaotropic salts such as lithium bromide (LiBr) are required for dissolving the silk fibers, resulting ß-sheet-lacked and water soluble silk fibroin **[11,**

12]. On the other hand, molecular conformation of the silk fibroin is a vital parameter when is considered as a biomaterial. This parameter remarkably affects the physical and mechanical properties as well as biological behavior of the silk fibroin **[13]**. Some solutions such as methanol and ethanol are used to induce water insoluble β-sheet conformational transition **[1, 14]**. Several studies have used ethanol to induce the natural structure of the silk fibroin protein. However, the effects of ethanol treatment on mechanical and biological behaviors of the silk fibroin nanofibrous scaffolds, as two critical characteristics in tissue engineering scaffolds, have not been investigated before. In this study, the silk fibroin was electrospun and then treated with ethanol. Then, mechanical property was compared between ethanol-treated and -untreated nanofibrous scaffolds. In addition, the effect of ethanol treatment on response of rat bone marrow stromal cells (rBMSCs) was investigated.

Experimental

Materials

Silkworm cocoons were provided from Iranian silkworm research center (Guilan). Formic acid (98%), MTT solution and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich chemical company (USA). Cell culture medium and LDH kit were supplied from Gipco (Germany) and Zist Shimi companies (Iran), respectively. All other reagents and solvents were purchased from MERK chemical company (Germany).

The morphology of the nanofibrous scaffolds and chemical composition of the samples were investigated by scanning electron microscopy (SEM, AIS2100; Seron Technology, Uiwang-si, Gyeonggi-do, South Korea) and energy dispersive X-ray analyzer (EDX, Rontec, Germany). The average diameters of the fibers and the porosity of various layers were calculated by Image analysis program ImageJ (US National Institute of Health, Bethesda, MD).

Method

Bombyx mori (*B. mori*) silk fibroin solutions were prepared by a previously published protocol **[4]**. The samples were freeze dried and stored in a dry place at room temperature for the following analysis.

The lyophilized silk fibroin was completely dissolved in formic acid for 3 hours to obtain 10% w/v fibroin/formic acid solutions. For electrospinning, the silk fibroin/formic acid solution was placed in a 3-ml syringe. A voltage of 18 kV/cm, 15 cm distance between the syringe and the collector (a rotating aluminum drum), and 0.3 ml/h constant flow rate were set-up according to a previously published work **[12]**. The NSF scaffolds were treated with ethanol 70% v/v for 1 hour at room temperature to induce water insoluble β-sheet conformational transition, dried under vacuum and then stored at room temperature.

Fourier transform infrared spectroscopy (FTIR) spectra were obtained in the spectral region of 400–4000 cm^{-1} with a resolution of 4 cm^{-1} to determine the changes induced by ethanol on the secondary structure of the electrospun silk fibroin **[3]**.

The morphology of the scaffolds was observed by SEM after coating with gold. The mechanical behavior of the scaffolds were investigated on a universal tensile testing machine at a crosshead speed of 1 $mm \cdot min^{-1}$ with a specified sample size (length = 20 mm and width = 10 mm) under ambient condition. The average diameters of the fibers and the porosity of various layers were measured as explained in our previous publication **[15]**.

The rBMSCs were isolated, expanded **[4]** and then cultured in a cell-culture flask incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC for 24, 48 and 72 hours. The MTT test was performed by a previously described method **[16]**. The data was normalized to positive control (as 100 % cell viability). In addition, the amount of lactate dehydrogenase (LDH) specific activity in cell culture medium was measured to assess the cytotoxicity effects of the samples. The data was normalized for total LDH released from 10^6 cells after freeze-thawing **[4]**. To observe the morphology of the rBMSCs cultured on the scaffolds, the samples were prepared for taking micrographs by SEM that operated at the acceleration voltage of 15 kV **[17]**. In addition, the chemical composition of the samples was determined semi-quantitatively by EDX.

Results and discussion

As can be seen in **Fig. 1**, the FTIR spectra of the ethanol-treated NSF (ET-NSF) scaffolds showed characteristic peaks of ß-sheet conformation at 1636 cm^{-1}, 1516 cm^{-1}, 1235 cm^{-1} and 965 cm^{-1}, while the characteristic peaks of α-helix at 621 cm^{-1} did not change **[18]**, which is in agreement with the obtained data by Li *et al.* **[18]**. **Fig. 1** also shows the SEM micrographs of the scaffolds before and after ethanol treatment. Randomly arranged nanofibers were formed uniformly without any bead formation, in which the average fiber diameters were 82 nm ± 12 and 121 nm ± 16 for the NSF and ET-NSF scaffolds, respectively. It was also shown that the pores' morphologies of the NSF and ET-NSF scaffolds were slightly different. The ethanol treatment yielded fibers with relatively larger diameters, which formed smaller pores.

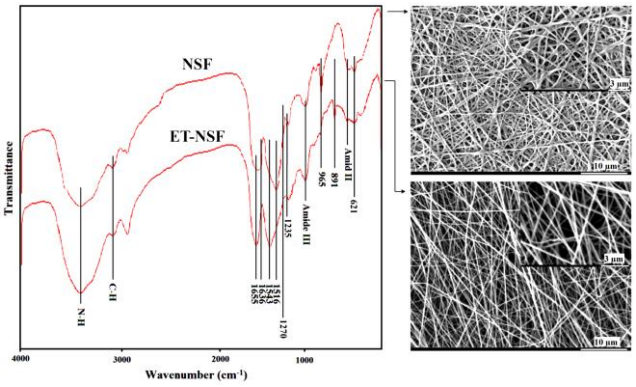

Fig. 1. FTIR spectra and SEM micrographs of the NSF scaffolds before and after ethanol treatment.

The mechanical tests also confirmed that ethanol treatment significantly enhanced the physical properties of the scaffolds through the formation of a ß-sheet structure. The ultimate tensile strength for the NSF and ET-NSF

scaffolds were 0.76 and 1.33 MPa, respectively. According to Chen et al. **[19]**, these results may be attributed to an increase in the flexibility of the ET-NSF scaffolds. Here, the elongation at break of 0.76% and 1.63% were recorded for the NSF and ET-NSF scaffolds, respectively.

The results obtained from MTT assay showed no significant difference between the proliferation rates of the samples up to 24 hours (independent sample *t*-test, $p>0.05$), as shown in **Fig. 1(a)**. However, the rate of the rBMSCs proliferation significantly increased after 48 hours for the ET-NSF in comparison with the NSF scaffolds.

As can be seen in **Fig. 2(b)**, there was no significant differences between the amounts of the LDH specific activity between the experimental groups after 72 hours. According to the results, the ethanol treatment positively affected the proliferation rate of the rBMSCs without any detectable cytotoxicity **[20]**.

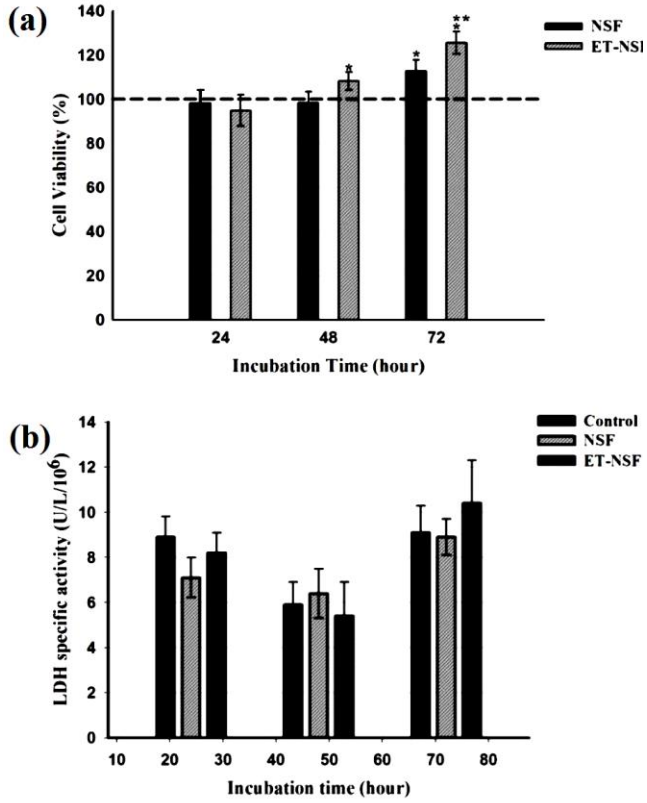

Fig. 2. MTT results **(a)** and LDH specific activity **(b)** of the NSF and ET-NSF scaffolds. *Significant difference with control ($p<0.05$). ** Significant difference with NSF scaffolds.

Fig. 3 illustrates the SEM micrographs of the cells in contact with the NSF and ET-NSF scaffolds after different time intervals. The cells were actively attached to both types of the scaffolds after 72 hours cell culture. The amount of the cells grown on the ET-NSF was clearly more than those of the cells on the NSF scaffolds after 48 and 72 hours, so that almost all the ET-NSF surfaces were occupied with the cells after 72 hours. In addition, EDX analysis was used to detect calcium deposit of the cells implanted on the scaffolds. It is worth mentioning that calcium and phosphorous were detected in the ECM of the cells on the samples.

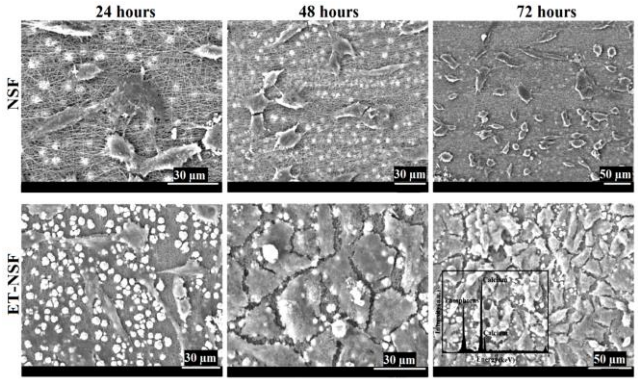

Fig. 3. SEM micrographs of the rBMSCs cultured on the NSF and ET-NSF scaffolds after 24, 48 and 72 hours incubation (In the corner: EDX pattern of calcium and phosphorus detected in the ECM of cells).

Conclusion

In the current study, ethanol treatment was found to successfully induce ß-sheet transition of the NSF scaffolds and decrease its water solubility, which must be minimized for using as biomedical applications. The data obtained from this study demonstrated that 70% (v/v) ethanol treatment positively affected the mechanical and biological properties, while decreased the porosity of the NSF scaffolds. Taken together, treating the nanofibrous silk fibroin with ethanol enhances its characteristics as a tissue engineering scaffold.

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