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A novel inexpensive method for preparation of silk nanofibers from cocoons

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Abstract

This study introduces a novel method for the production of silk nano-fibers *via* employing a mechanical and straightforward alternative to other toxic and costly chemical approaches. For this purpose, silk nano-fibers were separated from the cocoon using mechanical homogenizer and probe ultrasound. After the preparation of silk nanofibers, the product was characterized by Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy. The results were then evaluated in comparison with previous techniques and the advantages of the proposed method were investigated.

Keywords: Silk; nano-fiber; physical technique; mechanical homogenizer.

Introduction

Extensive studies on materials have proven the potential therapeutic role of certain materials for different kinds of diseases such as cancer [1] as well as their application in procuring anti-microbial [2,3] or anti-inflammatory drugs [4]. Silk is one of the most common materials used in various industries due to its significant mechanical properties [5], biocompatibility [6] and luster [7] as well as its exceptional performance in textiles and also as a biodegradable material used in surgical sutures. The fibers in silk are produced by cultured silkworms, as well as over 30000 spider species and numerous worms of the Lepidoptera category which can metamorphic into butterflies, mites and moths. However, the silk that is usually used in medical industries throughout the world is produced by the Bombyx mori, a very particular a species of butterfly. In fact, fibrous protein is produced with its epithelial cells as line glands [5,8].

The row silk consists of two parallel columns of fibroin and sericin layers on the surface of silk fibroin fibers (SF). Fibroin fibers have recently attracted much interest due to their appealing properties such as biocompatibility, biodegradability

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(without immunological responses), strength and toughness which has made them quite applicable for tissue engineering of body parts, especially nerves [9], cartilage [10], bone [11,12], wound dressing [13] as well as for purposes of drug delivery [14,15]. Furthermore, these compounds are produced from fibroin, which possess good thermal stability in a wide range of temperatures up to 250°C, allowing it to function without loss of integrity in various applications [15,16]. Silk fibroin (SF) can be converted into powder, hydrogels, sponges, and fiber and film forms with various processes after dissolving in different solvents. Among these processes, one unique technique, known as electrospinning that produces nano-fibers as a controllable structure *via* preparing protein-based biological substances stands out the most [17].

Outstanding mechanical properties of silk are mainly attributed to its nano-fibrous structure as being hierarchical [18]. Nanofibers produced by this method are used to increase structural integrity and strength as well as promote cell adhesion and proliferation. These nano-fiber compounds are completely decomposed in traditional solvents such as lithium bromide aqueous solution or ethanol-water solution of calcium chloride to their constitutive molecules [19]. Often times upon dissolving, reformed nanofibers tend to show poor mechanical properties than raw silk. In addition, the process of nanofiber production with electrospinning technique is highly dependent on environmental conditions and is rather time consuming as well as complex and costly. Recently, direct extraction of nanofiber from natural materials by chemical and physical methods has been considered by researchers as an effective technique for the production of high resistance materials [17].

Accordingly, the aim of the present study is to procure silk nano-fiber from cocoon by mechanical process without the use of toxic chemical materials. This study seeks to introduce an inexpensive and safe way for the production of strong fibroin nanofibers while preserving its properties as an alternative to other costly and toxic method.

Constituent of fibroin

Silk fibers are mainly composed of two proteins, fibroin and sericin. Following degumming procedures, sericin, as the waste material is removed whereas fibroin strands remain. Fibroin protein, which contributes to 78% of the total weight of raw silk fibers has a highly oriented and crystallized structure. Each fibroin strands is named a fibroin filament. The filaments consist of fibers that contain 1000 micro-fibrils each. Inside these structures, hydrogen bonds hold strands of mico-fibrils together and are responsible for maintaining the structural integrity of fibroin fibers [5,9,20].

Micro-fibrils are the chief units which are packed together to form filaments and polymer compounds. These internal polymers consist of light (L) and heavy (H) chain polypeptides. The H-L complex forms *via* linkage with the disulfide bond at the C-terminal heavy chain, where in turn the heavy chains organize the β-sheet crystallite structure which is a key factor behind the mechanical properties of fibroin. Generally, fibroin polymers are mainly composed of alanine, glycine and serine and a small amount of cysteine [20].

Experiments

Instrumentation

The SERONTECHNOLOGIES (AIS2100) scanning electron microscope was used for imaging purposes. The Fourier-transform infrared spectroscopy of structures was observed using FT-IR in the range of 400–4000 cm-1 on a RAYLEIGH (WQF-510A) spectrophotometer using the KBr pellets. The Qsonica (Q700) probe device was used for homogenization of mixtures with ultrasonic irradiation.

Reagents and materials

LiBr (99 %) and Na_2CO_3 (98 %) were purchased from Merck (Germany). The silk cocoons were procured from Bombyx mori silkworm purchased from local traditional centers in the north of Iran.

Extraction of gum removed fibroin filaments

The crashed silk cocoons (as small sizes) were initially washed in hot distillate water several times. Then, 3 gr of the obtained materials were refluxed in Na_2CO_3 (0.5 % w/w) aqueous solution for half an hour and were rinsed with pure water. The entire process was then repeated twice. Finally, the gum removed fibroin filaments were dried at 40 ˚C under vacuum.

Preparation of silk nano-fiber

The silk nano-fibers were prepared using two methods: (i) Old chemical method which worked by dissolving silk in solvent and then using the electrospinning technique, (ii) Novel physical approach by means of using a mechanical homogenizer and ultrasonic irradiation.

Preparation of silk fibers (SF) with chemical method

Preparation of Fibroin solution

In this phase, the lithium bromide solution was used as a solvent for preparation of the fibroin fibers. 1 gr of the chopped fibroin (10 % w/v) was added to aqueous solution of LiBr (9.3 M) half an hour prior to soaking for easy dissolution. The mentioned two-phase materials were mixed at 60˚C overnight. Eventually, the yellow fibroin solution was obtained in the form of a viscous and sticky substance.

Fibroin solution dialysis

For purification of fibroin solution and LiBr removal, the prepared materials in the previous section were dialyzed by dialysis bags with extremely small pores (10 kDa molecular weight). The process was carried out as follows: fibroin solution was poured into dialysis bags with its two sides blocked and was floated in a large vessel containing deionized water. The water passed through the pores of the bag and the lithium and bromine small ions were washed and removed from the fibroin solution and transferred to the outside of the dialysis wall. It should be noted that the protein structures due to coarseness, were unable to pass through these fine pores. Fibroin soluble dialysis continued until complete isolation of lithium bromide (about 72 h). This is while the water of the container was changed every 8 hours. Afterward, the obtained pure fibroin was completely freeze-dried and eventually dissolved in the formic acid solution (10 % w/v) and prepared with a desirable concentration for electrospinning technique.

Electro-spining of SF solution

The process was initiated with proper concentration and viscosity. A high electric potential voltage (18 kV) with a flow rate of solution of 0.5 mL/h was applied to a droplet of the SF solution

on a target drum which was placed at a distance of 12 cm from the syringe tip.

Preparation of silk fibers (SF) with novel method

For this study, 1 gr of the gum removed fibroin filaments was split and crushed by surgical blade and was then poured into 200 mL of distillate water. Then, the mixture was chopped by a mechanical homogenizer set at 26000 RPM for 15 min (Figure 1). Fibroin particles were divided into very small pieces and were suspended in water. After that, the suspension was irradiated with an ultrasound probe (700 watt) until the nanofibers were completely broken apart. The process was repeated three times and the obtained fibers were separated by centrifuge in 5000 RPM and dispersed in 50 mL distillate water by ultrasound bath.

In the process of homogenization, the filaments were initially torn apart, so that after half an hour, fibrils appeared. The process was then continued so that microfibers, as smaller part of fibroin were dissented. Eventually a milky liquid was obtained. The suspension was eventually dried by freeze-drying. The resulting compound was a porous structure composed of nanofibers (Figure 2).

Results and discussion

Characterization silk fibers

FT-IR

In order to determine the silk nano-fiber structure the FT-IR technique was used at the scale of 400-4000 cm^{-1} or range infrared spectra. The results are depicted in Figure 3. The stretch vibrations band for the C-H aliphatic appeared at around 2900-3000 cm^{-1}.

Figure 1. Mechanically homogenization with 26000 RPM

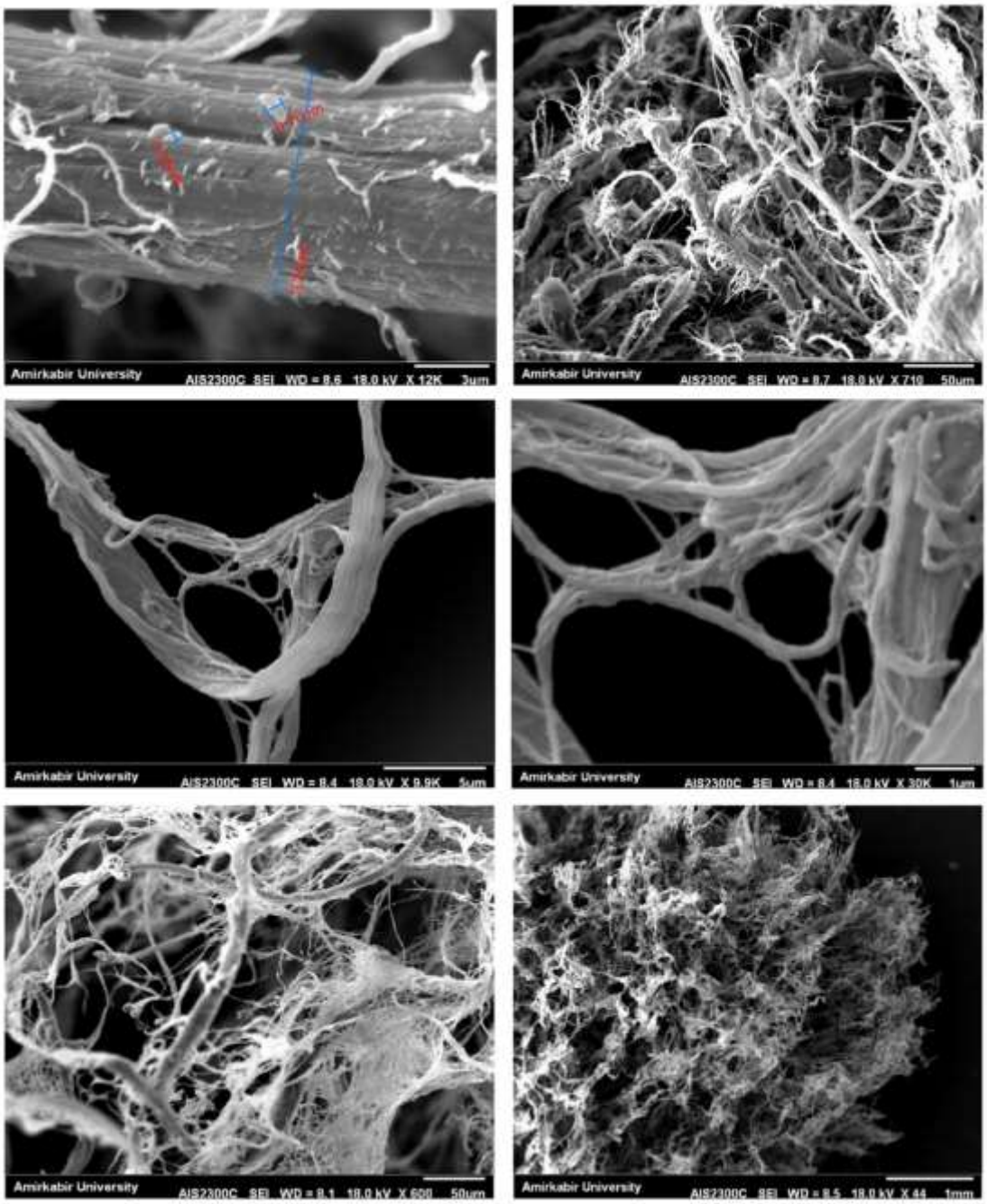

Figure 2. SEM images of the filaments in the various stages

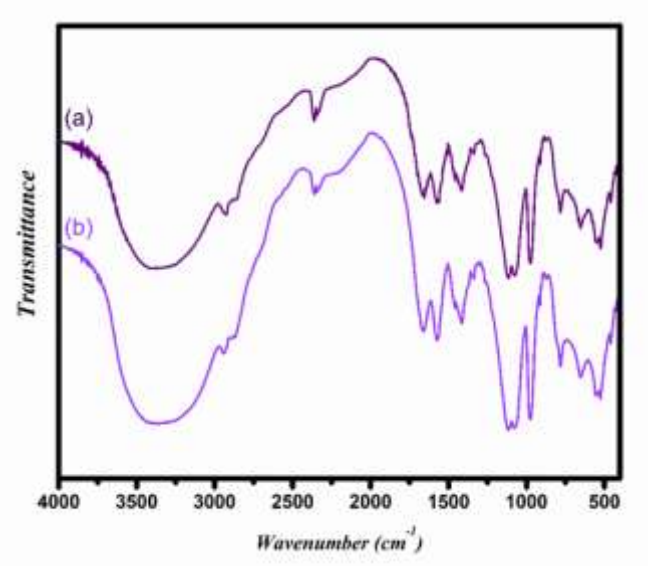

Figure 3. FT-IR spectra of (a) old chemical method (b) novel physical method

The absorption peak for the C=O groups was observed in the 1650 cm^{-1} area. The absorption peak at 1513 cm^{-1} is attributed to the C-C alkane bending vibration. The peak of 3286 cm^{-1} area corresponds to the N-H vibration in the overlapping with the O-H broad band at the 3000-3300 cm^{-1} range. Also, the vibration and stretch band of C-N amine was observed at 1224 cm^{-1}. Considering chemical compounds of silk, the FT-IR result can be related to fibroin fibers.

SEM

Figure 4 illustrates the results of scanning electron microscopy for size and surface morphology study of prepared nano-fibers using the two methods. The SEM images of the structures revealed that the fibers obtained from both chemical and novel physical method were separated and woven together. The fibers were also connected at short distances, similar to a bridge and the diameter of the fibers was less than 100 nm. It is clear that the diameter size distribution of the prepared old chemical method (Figure 2-a) fibers are narrower than the novel physical method (Figure 2-b). As is evident in SEM images, the general structure of both products are similar and the extracted fibers obtained using the novel method can morphologically replace those obtained using the previous method.

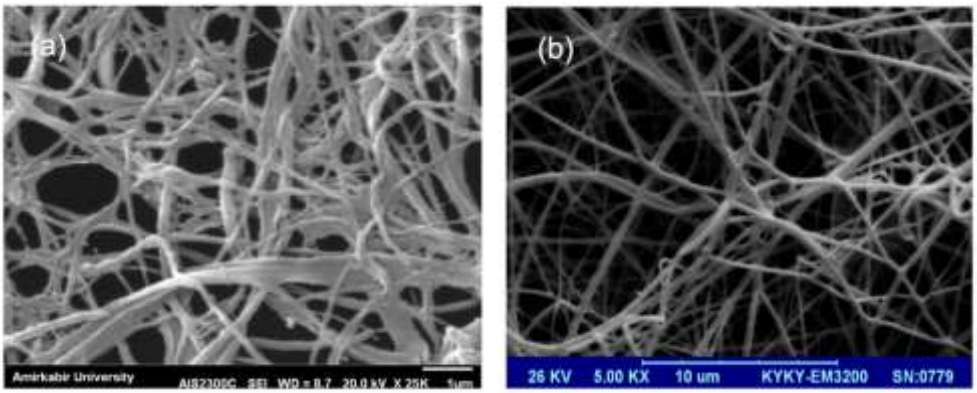

Figure 4. SEM images of silk nano-fibres prepared by (a) old chemical method (b) novel physical method

Cell adhesion and cell spreading assay

For this assay SF nano-wovens were cut out with punch (10 mm in diameter) and put onto the three well culture plates of 48 wells. Then these fibers were washed twice with PBS and sterilized in a solution of 70% alcohol for 10 min. Eventually, the process of sterilization was completed with a 20-30 min exposure to UV light which is sufficient to ensure complete disinfection. For cell culture, 300 µl of a cell suspension containing 1×106 HBMSCs (Human Bone Marrow Mesenchymal Stem Cells) used in each well. Culture expanded in α-MEM medium, 10% fetal bovine serum, 1% penicillin and streptomycin and 1% NEAA (nonessential amino acid). The cells were allowed to adhere completely in 3 days after which cell-containing nanofibers were prepared for SEM imaging.

The first loosely adherent cells were removed by washing once PBS, and the remaining bound cells were fixed with 2.5% glutaraldehyde overnight. The second day nanofibers were washed with PBS for 30 min and placed in PBS for 18h. The third day samples were fixed in 1% Osmium Tetroxide and finally dehydrated in 30% (two times for every 30 min), 50% (40 min), 70% (40 min), 80% (40 min), 90% (60 min), 100% (3 times for every 60 min) ethanol. After fixation, scanning electron microscopy was used to investigate the macroscopic morphology and state of cell adhesion to SF nanofibers. Figure 5 illustrates happy cells with good cell-cell adhesion and strong junction with nanofibers (Figure 5).

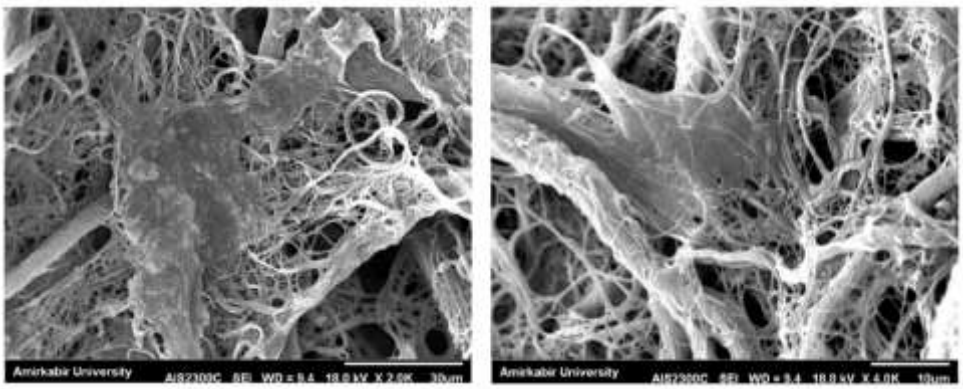

Figure 5. SEM images of cell adhesion on the silk nano-fibres

Cell viability and cell proliferation

Cell viability and proliferation were measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma, USA) staining. For this method the HBMSCs were initially seeded on scaffolds, and were then considered in 3-time points (24 h, 72 h and 7 days), and were finally incubated in MTT solution (5 mg/mL MTT in PBS) in a 5% CO_2 and 37°C for 3h. The absorbance was measured at 570 nm (Figure 6). Comparing the cell viability and proliferation of HBMSCs cultured on electrospun SF and mechanical method SF nanofibers, with MTT assay showed no statistical difference after these time points ($p>0.05$).

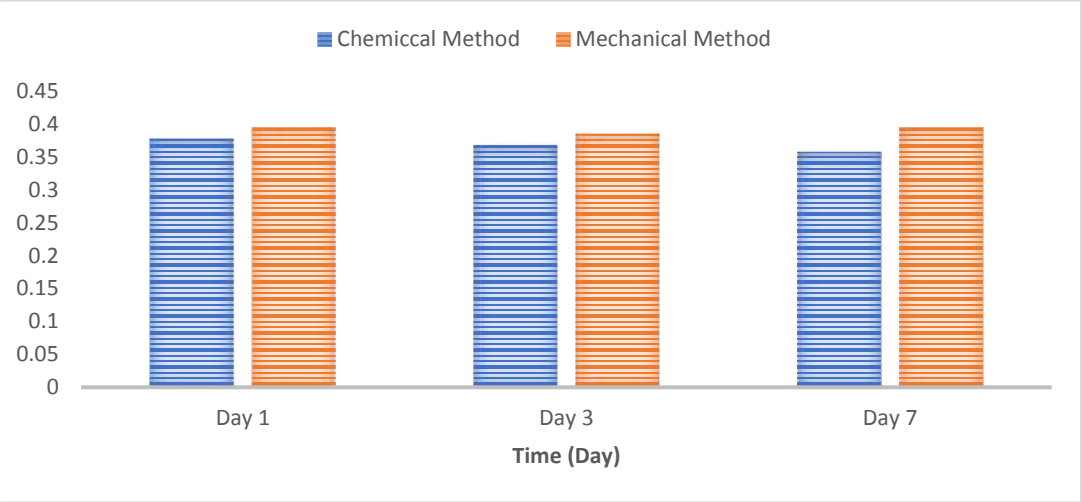

Figure 6. MTT test of prepared silk nanofibers by two methods at three-time points

Comparing the new mechanical technique with previous methods

The proposed method avoids the use of toxic solvents to prepare the desired product, whereas, previous chemical methods, after dissolving silk and regeneration of nano-fibers, used chemical toxic compounds at significant quantities to obtain the final structure. Also, traditional chemical methods were not economical with a large body of materials and energy being consumed for dissolving and later application of electrospinning. In addition, a substantial amount of time was required in the traditional methods for the preparation of the product compared with the new mechanical method on top of which the old methods were very sensitive to environmental conditions. Therefore, using mechanical homogenizer and ultrasonic irradiation is a desirable and cost-effective way for the production of silk nano-fibers compared with other methods.

Conclusion

A new physical technique was used in this study for the preparation of silk nano-fibers which avoided the use of toxic chemical solvents. The accuracy and exactitude of the produced structure was confirmed by Fourier-transform infrared spectroscopy (FTIR) and scanning electron microscope. The nanofibers obtained by this method, due to their bulky structure, can be used as filler in defects as well as suitable scaffolds for bone, cartilage, or even soft tissue after mixing with hydrogels or ceramic materials.

In addition to the value and desirability of the obtained products

using this method compared to the traditional products, it has been shown that this method can be used as an affordable, fast and environmentally friendly technique. The conclusion should also include negative results and recommendations based on the results. In such cases where the study has led to clear cut finding, it is preferable to give the conclusions in the form of a series of numbered points.

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