

Production of Collagen Nanofibers and Unfolding of the Structure

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Received: February 25, 2018 / Accepted: March 24, 2018 / Published: May 25, 2018

Abstract: In this paper, we discuss two different collagen nanofiber production methods, namely, electrospinning and self-assembly of collagen fibers. The robustness and advantages of the methods are discussed. Scanning Electron Microscopy (SEM), Circular Dichroism (CD), Differential Scanning Calorimetry (DSC) methods are applied to compare the collagen fibrillary structures that are obtained by these techniques. The possible effects of applied methods and processes on the native structure are discussed and the future direction of the research is presented.

Key words: Collagen, Nanofibers, Electrospinning, Self-Assembly.

1. Introduction

Most of the scaffolding in mammals are composed of collagen, making 30% by weight of body protein tissues. Collagen has a unique triple helix configuration of three polypeptide subunits known as α -chains [1]. Type I collagen is predominant in higher order animals especially in the skin, tendon and bone where extreme forces are transmitted. Extracted Type I collagen is favored for biomedical applications, since in vitro under appropriate conditions it will spontaneously self-assemble to form biodegradable and biocompatible insoluble fibrils [2,3].

It is necessary to use different techniques to regenerate the collagen in required forms. One of these techniques is electrospinning. It is a highly versatile method to process solutions or melts, mainly of polymers, into continuous fibers with diameters ranging from a few micrometers to a few nanometers [4]. Another method is self-assembly of collagen fibers, obtained by co-extrusion of collagen solution and fiber formation buffer in a fiber formation bath. One syringe is filled by collagen solution and the other syringe is filled by the buffer solution. The collagen fibers are obtained in the solution bath by the effect of ionic interactions [5].

In this research, we presented two different collagen fiber production methods and discussed the possible effects of production techniques on the fiber structure: namely, electrospinning and self-assembly of collagen fibers. It is demonstrated that the electrospun collagen nanofibers are mostly denatured due to the electrospinning solvents and electromagnetic forces. Even though electrospinning is the only well-known

continues nanofiber production method. Self-assembly method is presented as an alternative technique to electrospinning. However, the fiber diameter in this method is limited by the inner diameter of the tubing that is employed in the production. We discussed the robustness and advantages of these methods and compare the collagen fiber structures.

2. Materials and Methods

Materials

Collagen type I was generously donated by the Kensey Nash Corporation, USA. Acetic acid (99%) was purchased from Merck KGaA. Tris, sodium phosphate dibasic and sodium chloride were purchased from Sigma-Aldrich and used as received.

Preparation of Collagen Solutions for Electrospinning Procedure

The collagen used in these experiments was a water-insoluble lyophilized foam powder consisting of tropocollagen extracted from bovine dermis and was used without further purification. Samples were prepared according to the following recipe: a 40% w/v collagen solution was prepared by dissolving collagen in HAc (40%)[6].

Electrospinning Procedure

The electrospinning procedure has been described in detail in Bürck, *J et al.* (2013) and Kazanci, M (2014) [4,7]. The prepared electrospinning solutions were loaded into a 2 ml syringe (Omnifix, B. Braun, Melsungen, Germany) with a blunt end nozzle, controlled by a syringe pump (Pump 33 Harvard Apparatus, Holliston, USA). The solution was pushed through a capillary blunt steel needle (21 gauge, 0.7 mm i.d. 50 mm length) at a constant speed (0.5 μ l/min). The steel needle was coupled to a high voltage source (Spellman Bertan Series 205B, NY, USA). The electric potential was needed to start the spinning process and thus form a jet. The applied DC voltage was held at 16 kV. A Cu collector was placed 15 cm from the needle tip to collect the electrospun collagen nanofibers. The nanofiber meshes were collected on cover-glasses placed onto the collector[7], as illustrated in Figure 1.

Production of Self-Assembled Collagen Fibers

Collagen fibers were self-assembled from a collagen solution, using a co-extrusion process. The system consists of two separate syringes. One 5-mL syringe contained a 1% w/v solution of collagen and the other syringe contained Fiber Formation Buffer (FFB) (135 mM NaCl, 30 mM TrizmaBase (Tris), and 5 mM sodium phosphate dibasic, pH 7.4). With the aid of a syringe pump (Harvard Apparatus Pump 33, Holliston, MA), the two solutions were simultaneously extruded into a bath of distilled water through a tubing system[8], as shown in Figure 2.

Characterization

Circular Dichroism (CD) spectroscopy, Scanning Electron Microscopy (SEM) and Differential scanning calorimetry (DSC) were employed to characterize the structure of collagen nanofiber meshes.

Circular Dichroism Spectroscopy (CD):

CD spectra were recorded using a J-815 spectropolarimeter (JascoCo., Tokyo, Japan). The instrument was

routinely calibrated with a 0.06% (w/v) aqueous solution of ammonium D-10-(+)-camphorsulfonate at 290.5 nm. The spectra were scanned between 260 and 190 at 0.1 nm intervals. Three repeat scans at a scan rate of 10 nm min⁻¹, 8 s response time, and 1 nm bandwidth were averaged for each sample and its respective blank. The experimental details were given in Aras *et al.* (2015) [8].

Scanning Electron Microscopy (SEM):

Scanning electron micrographs were obtained with a Jeol JSM7500F. Images were acquired at an acceleration voltage of 1.5 kV and a working distance between 4 and 5 mm, using a through-the-lens secondary electron detector. Prior to the investigations, samples were coated with a few nm of Pt.

Differential Scanning Calorimetry (DSC):

Calorimetric measurements of samples (native collagen powder, electrospun collagen nanofibers that were obtained from TFE and HAc) were carried out on DSC-60 Shimadzu (Japan). Measurements consisted of a single upward scan from 20 to 150 C at a heating rate of 5C/min. Data analysis was performed using the Ta60 software (version 2.21) supplied with the instrument.

3. Results and Discussion

Circular Dichroism (CD):

CD utilizes the differential absorption of left- and right- handed circular polarized light in an asymmetric environment to assess secondary structure. The CD spectra of self-assembled collagen fibers and electrospun collagen nanofibers in 0.05M HAc are presented in Figure 3. We employed Ackerman *et al.* method [9] to extract the fraction of triple helix, mainly relying on the equilibrium state of collagen spectra. The PP-II peak at 221.5 nm exhibited positive values at 15C and transformed to a negative ellipticity at 90 C, which indicated that collagen completely unfolded at that temperature. Figure 3 shows the spectra of (a) the electrospun collagen nanofibers, (b) self-assembled collagen fibers, where the electrospun collagen nanofiber sample displayed the lowest amount of PP-II. The self-assembled collagen solution scored a higher PP-II fraction.

Scanning Electron Microscopy (SEM):

Fig.4 (a) and Fig.4 (b) demonstrate the SEM images of the electrospun nanofibers and self-assembled collagen fibers. Electrospinning yielded randomly oriented and interconnected fibrous meshes with the fiber diameter in the nanometer range. The electrospun nanofiber diameters were mainly determined by the collagen concentrations and type of electrospinning solvents. For example, 40% collagen solution in HAc produced electrospun nanofiber meshes with the fiber diameter ranging between 150 and 200 nm. Whereas self-assembled collagen nanofibers diameters are determined by the inner tube dimensions. The collagen fibers in Figure 4 (b) are consisting of multi collagen fibrils wrapped together. The fiber diameters are in the range of a couple of tens micrometers.

Differential Scanning Calorimetry (DSC):

Fig. 5 shows the DSC results of self-assembled collagen fibers and electrospun collagen nanofibers. The

characteristic endothermic peak is shifted to lower temperatures for electrospun nanofibers. The peak shift is more pronounced for the electrospun collagen nanofibers. The enthalpy effects are caused by collagen-collagen and collagen water interactions [10]. It can be concluded that less hydrogen bonds exist in the electrospun collagen nanofibers compared with the self-assembled structure where the right-handed triple helix structure of collagen is stabilized by interchain hydrogen bonds. When subjected to heating, the original triple helical structure of collagen molecules is unraveled the most in the nanofibers and the least energy is needed to break up the structure.

4. Conclusion

In the previous work [7], it was demonstrated that not just the solvent, but also the electrospinning process itself denatures collagen. We also noticed that HAc derived collagen nanofibers have relatively higher PP-II fraction [7]. We were also able to illustrate the local evidence of partially preserved triple helix structure in HAc derived collagen nanofibers by using SEM methods[6]. However, the motif is detected only at certain regions. Therefore to be able to keep the native structure of collagen fully preserved, some other alternative production methods might be a solution. The self-assembly of collagen fibers is one of them, nevertheless, it is extremely difficult to achieve continuous fibers at the nanoscale by employing alternative production methods other than electrospinning.

Collagen fibers production is a complex process, and many parameters could affect the final structure of the fibers. In this paper, we investigated the structure of collagen that are produced by different techniques by using CD, SEM and DSC. We demonstrated that collagen was mostly unfolded in the electrospinning process, while the native structure was preserved in the self-assembly method. After the spinning process we observed refolding to a certain degree, but the PP-II fraction did not exceed 42%. We also evaluated the structure of self-assembled collagen fibers. The results were compared with the electrospun nanofibers. CD spectroscopy illustrated that self-assembled collagen fibers scored relatively high PP-II values(%86), comparing with the electrospun collagen nanofibers. However, the dimensions of the self-assembled collagen fibers are limited by the inner tubing diameter and the final fiber product is extremely brittle. Therefore, there is no alternative for electrospinning process to obtain continuous nanofibers, but the application of the electrospun collagen nanofibers in the biomedical field is restricted by their being vulnerable to the physiological conditions. Afterward, the crosslinking agents should be applied to the nanofibers in order to preserve the morphology of the nanofibers.

Acknowledgments

This study is supported by TUBITAK's Co-Funded Brain Circulation Program-2236, (Project number 112C025). We would like to thank Bianca Posselt (Institute of Biological Interfaces, IBG-2, KIT) for technical assistance during the CD measurements.

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Figures

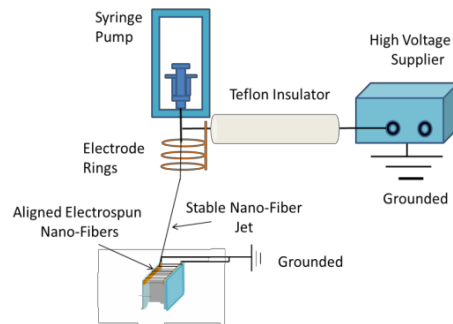


Figure 1. Schematic Diagram of Electrospinning

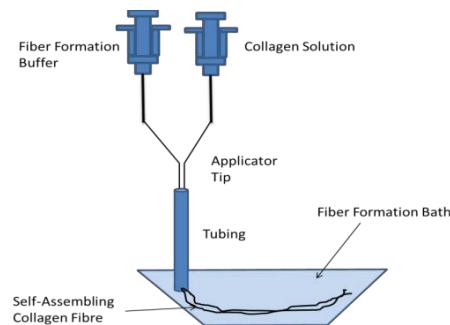


Figure2. Schematic Diagram of Production of Self-Assembled Nanofibers

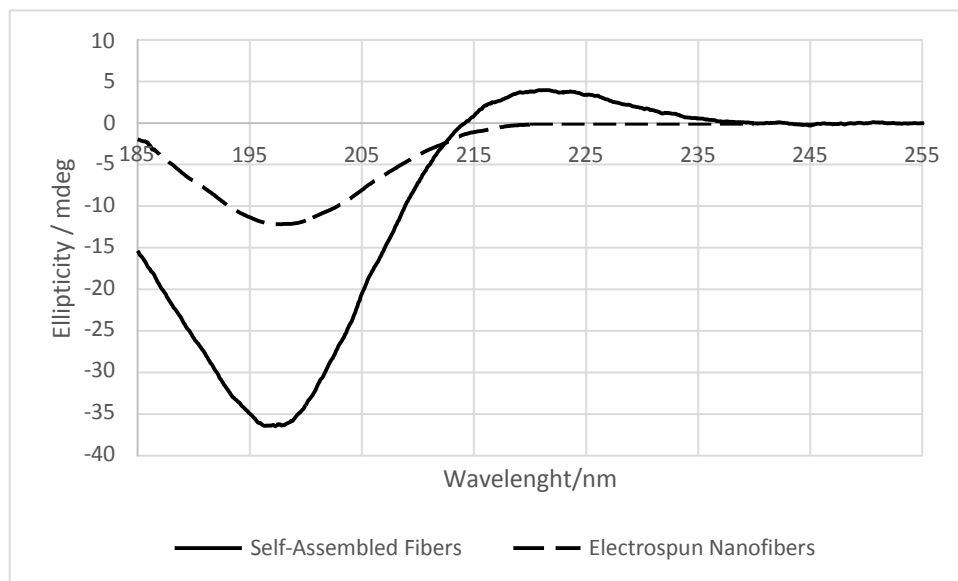


Figure3. CD Spectra of Electrospun Collagen Nanofibers and Self-Assembled Collagen Fibers

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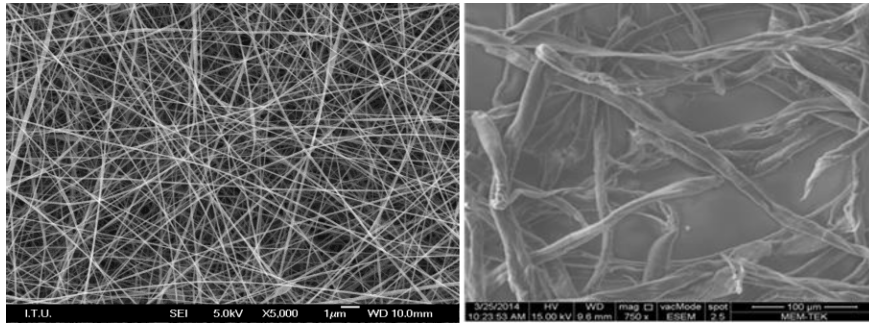


Figure 4. SEM Images of Collagen Nanofibers (a) Electrospun Collagen Nanofibers (b) Self-Assembled CollagenFibers

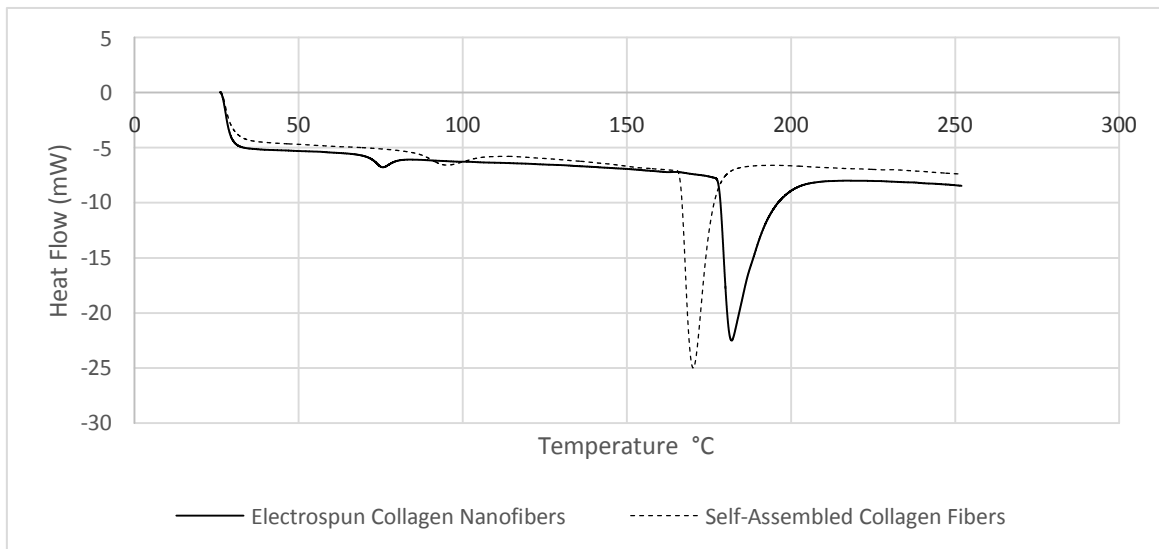


Figure 5. DSC Results of Electrospun Nanofibers and Self-Assembled Fiber

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