Fabrication of Bovine Serum Albumin-Loaded Coaxial Electrospun Thread with an Aligned Core-Shell Fibrous Structure

Farzaneh Ghasemkhah, Masoud Latifi*, Afra Hadjizadeh, and Mohammad Ali Shokrgozard

Abstract- **Threads or yarns are key building blocks in constructing complicated fibrous structures for many biomedical applications such as scaffolds to direct cells in tissue engineering applications. In this study, protein-loaded aligned fibers with core-shell structures were successfully fabricated by combining coaxial electrospinning with a rotating disk collector. A model protein, bovine serum albumin (BSA) as a core was incorporated into shell material, including PCL/gelatin (50:50) hybrid and then electrospun aligned core-shell fibers on the rotating disk. After manually twisting the aligned fiber bundle, the prepared threads were crosslinked with genipin. The fibers and thread morphologies were characterized by scanning electron microscopy (SEM), fluorescent microscopy (FM), transmission electron microscopy (TEM), and Fourier transform infrared spectroscopy (FTIR), and the threads tensile properties were then evaluated. The well-aligned fibers (with a mean diameter of 1.472±0.444 µm) with uniform core-shell structures without any bead formation and homogeneous protein incorporation into them were obtained using a rotating wheel at 1300 rpm. BSA-loaded threads with a diameter of 150-250 µm were finally prepared from aligned fiber bundle by keeping their well-aligned structure. The tensile tests revealed that fibrous thread with the modulus of 34.2 cN/tex had proper tensile properties for tissue engineering approaches. FTIR thus proved that the crosslinking was successful between thread and genipin. After crosslinking, threads retained their fibrous structure and demonstrated the improvement in the tensile properties (modulus increased to 40.64 cN/tex). These findings are promising to promote biomedical agents-loaded fibrous threads for tissue suturing and tissue regeneration applications.**

Keywords: **coaxial electrospinning, core-shell fiber, aligned fibers, fibrous thread**

F. Ghasemkhah
Nanotechnology Institute, Amirkabir University of Technology, Tehran, Iran.

F. Ghasemkhah and M. Latifi
Department of Textile Engineering, Textile Excellence and Research Centers, Amirkabir University of Technology, Tehran, Iran.

A. Hadjizadeh
Department of Biomedical Engineering, Amirkabir University of Technology, Tehran, Iran.

M.A. Shokrgozar
National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran.

Correspondence should be addressed to M. Latifi
e-mail: latifi@aut.ac.ir

I. INTRODUCTION

Natural and synthetic fibrous materials in different forms [1-3] including bundle or yarn form have been widely applied in tissue engineering such as wound healing, artificial muscles and bone scaffolds [4,5], suture [6], and reinforcement element for brittle biocements [7,8]. Incorporating therapeutic agents into thread materials has significantly enhanced their regenerative capacity [9]. These structures have been generally made from both synthetic and natural micro-nanometer scale polymer fibers [10,11].

As a simple and low-cost method for producing continuous fibers with diameters ranging from submicron to several nanometers, the electrospinning technique has received tremendous attention in recent years [12]. Due to their outstanding properties such as large specific surface-to-volume ratio and highly porous microstructure with interconnected pores, the electrospun nanofibers offer numerous advantages in biomedical applications including tissue engineering scaffolds [13-15], drug release systems [16,17] and wound dressings [18], to name a few.

Biomolecule-loaded scaffolding biomaterials offer numerous advantages in tissue engineering such as preserved bioactivity/functionality [19,20] and sustained release [21,22] of these molecules in different processing and application environments. For drug delivery applications, electrospinning provides two different methods for incorporating pharmaceuticals and other bioactive components into the fibers: blend electrospinning [23] and coaxial electrospinning [24]. Blend electro spinning involves simply mixing biomolecules with polymer solutions and provides medicated fibers with an initial burst release followed by a sustained release [23]. The initial intense burst release is related to the biomolecules

migration due to the rapid solvent evaporation and high ionic strength in the solution, which is localized on or near the fiber surface [25,26]. This initial burst release might cause a reduction of the drug's therapeutic efficiency and a shortening of the medicated fibers lifetime [27]. Besides, in blend electrospinning, biomolecules, especially proteins may lose their bioactivity due to conformational changes in the organic solvent environment [27]. In contrast, coaxial electrospinning provides a reservoir system for the biological agents that can protect them from an aggressive environment and modulate the diffusion rate of them due to the shell barrier [28-30]. Hence, coaxial electrospinning is regarded as one of the most remarkable breakthroughs in the field of susceptible biological agents release [29].

In coaxial electrospinning, at least two materials are independently delivered through a coaxial nozzle and drawn to form fibers with core-shell structures. In this case, the core solution which is injected through the inner needle, is most commonly hydrophilic to facilitate the preservation of the biomolecules bioactivity, whereas the shell solution which is injected through the outer needle consists of spinnable hydrophobic polymeric material to allow fiber formation after volatile organic solvent evaporation [31]. The process of coaxial electrospinning is conceptually similar to typical electrospinning. Under applied high voltage, the polymer solutions are drawn out from the nozzle and form a compound droplet with a core-shell structure at the end of the nozzle. As a high electric field is applied, the charge accumulation occurs on the surface of the shell solution, and then the charge-charge repulsion causes the elongation and stretch of shell solution to form a compound Taylor cone. Once the electrostatic force acting on the ejected polymeric solution overcomes the surface tension of the shell solution, a fine jet is formed from the cone. Via viscous dragging and contact friction, the stresses acting on the shell solution lead to the core solution shearing. The core solution deforms into the compound cone and a compound coaxial jet extends at the tip of the cone. As the solution jet moves away from the coaxial nozzle, the jet is rapidly thinned and dried as the two solvents evaporate. Finally, the core-shell nanofibers are formed and deposited on the surface of the grounded collector [32-34].

In a typical electrospinning process, ultrafine fibers are often collected in a randomly oriented and nonwoven form because of the instability of the charged jet and the chaos of the jet path [35,36]. However, obtaining aligned fibers with a highly ordered structure is desirable for some specific applications such as suture development. Among various attempts toward this goal [37-40], the collecting technique of rotating around a disc with high speed is mostly suitable for thread preparation due to its capability to obtain relatively long and aligned fiber bundles.

Threads or yarns are key building blocks to construct complicated fibrous structures for many applications in diverse areas including biomedical, such as sutures or scaffolds to direct cells in tissue engineering applications. In our previous study [41], the PCL/gelatin composition at different ratios was successfully fabricated as a shell layer of random core-shell fibers with the ability of controllable release, desirable mechanical properties, and sufficient cytocompatibility. In this study, a protein-loaded bundle from well-aligned core-shell fibers was prepared with the purpose of applying these threads to direct cells at angiogenesis approaches. In this regard, aligned core-shell structured fibers were firstly fabricated by combining coaxial electrospinning with a rotating disk collector. BSA (as a model protein) as a core material was incorporated into shell material, including PCL/gelatin (50:50) hybrid and aligned fibers were then fabricated on the rotating disk. After that, the aligned fiber bundle was manually pulled from the collector and manually twisted into an integrated thread, and further crosslinked with genipin. The fiber morphology was characterized by SEM and TEM. The loading of BSA inside the coaxially electrospun fibers was also visualized by FM. FTIR was conducted on the un-crosslinked thread and crosslinked one. Then, the tensile properties of the fibrous structures were evaluated for un-crosslinked and crosslinked threads.

II. EXPERIMENTAL

A. Materials

Poly(ε-caprolactone) (PCL) (M_w=80,000), gelatin type A (GT) from porcine skin, fluorescein isothiocyanate (FITC), and Rhodamine B isothiocyanate (RBITC) were all obtained from Sigma-Aldrich. 2,2,2-Trifluoroethanol (TFE) (purity≥99%) and BSA (purity≥98%) were purchased from Merck.

B. Electrospinning Solution Preparation

The shell solution with a concentration of 10 w/v% was prepared by mixing 10 w/v% gelatin/TFE and 10 w/v% PCL/TFE at weight ratios of 50:50. The mixed solutions were stirred for ~12 h prior to processing for adequate mixing. The core solution was prepared by adding BSA in distilled water (0.2 %).

C. Coaxial Electrospinning Setup for Aligned Fibers Formation

The schematic setup for preparing aligned core-shell fibers is shown in Fig. 1. In this setup, two fluids were independently fed through the inner and outer capillaries.

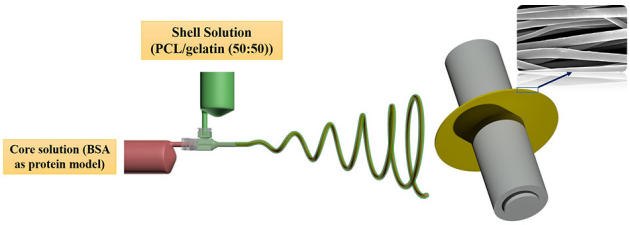

Fig. 1. Schematic of the setup used for preparing aligned core-shell fiber.

The inner and outer capillaries were respectively connected to two individual syringe pumps containing core and shell solutions without using Teflon tubes to reduce the dead space of connecting tubes and to minimize the solutions loss. The gauges of inner and outer needles of the coaxial nozzle were 20 (with inner diameter: 0.603 mm and outer diameter: 0.908 mm) and 14 (with inner diameter: 1.6 mm and outer diameter: 2.108 mm), respectively. A grounded aluminum rotating disk with a thickness of 200 µm and a diameter of 12 cm was used to collect fibers, and a copper electrode was directly connected to the coaxial nozzle with a high voltage. To obtain a stable Taylor cone with a core-shell structure, the injection rate of the inner and outer fluid should be controlled and balanced to the greatest extent. With an increase in the applied voltage to the threshold value for the determined solution flow rates, a stable compound Taylor and a steady coaxial fluid jet were formed and ejected out of the compound Taylor cone tip. In this regard, the flow rates of inner and outer solutions were appropriately adjusted so as to obtain a stabilized compound Taylor cone at a determined voltage. As the solution jet moved away from the coaxial nozzle toward the collector, the jet was rapidly thinned and dried associating with the two solvents evaporation to form composite fibers, which can be collected on a collecting screen in the form of either aligned bundles or non-woven mats, depending on the collecting device used. The aligned fiber bundles were manually pulled from the edge of the collector after accumulating sufficient fibers. In this study, well-aligned fibers could be collected when the collecting time was controlled within 15 min. One end of the fiber bundle was fixed with a blot, and the other end was then manually twisted into an integrated thread.

D. Crosslinking

The fibrous threads were crosslinked with genipin. Genipin is a small molecule that can easily penetrate into fibrous mats so that sufficient crosslinking can be achieved in a few days [42]. The electrospun threads were entirely soaked in a 0.5 w/v% genipin/ethanol solution for three days at room temperature to achieve enough crosslinking. Subsequently, threads were rinsed in ethanol three times to remove any residual crosslinker and then dried overnight at room temperature.

E. Morphological Characterization

E.1. Scanning Electron Microscopy (SEM)

The fiber and thread morphology was observed by SEM (Seron Technologies AIS2100, Korea) at a 10 kV accelerating voltage. The samples were coated with gold using a sputter coater (BALZERS SCD 004, Germany) before observation. The diameter of the electrospun fibers was analyzed using image visualization software (Image-J) by measuring about 120 counts for each sample.

E.2. Fluorescence Microscopy (FM)

The presence and distribution of the proteins inside the coaxial fibers were evaluated by FM. The samples were prepared using RBITC-conjugated BSA solution as the core and FITC was used to stain shell polymer. Then, the thin layer of core-shell fibers was collected on a glass slide for very short durations and was quickly analyzed by FM. The excitation wavelengths for RITC and FITC were 544 and 488 nm, respectively.

E.3. Transmission Electron Microscopy (TEM)

To verify the core-shell structure of the fibers, they were examined using TEM (Zeiss-EM10C), operated at 100 kV. The samples for TEM observations were prepared by the direct deposition of the thin layer of electrospun fibers on carbon-coated copper grids.

F. Fourier Transform Infrared Spectroscopy (FTIR)

The un-crosslinked and crosslinked threads were characterized by FTIR (model: NEXUS 670, Nicolet). The infrared spectra of the samples were measured over a wavelength range of 350-4000 cm^{-1}.

G. Tensile Properties

Tensile properties of the electrospun threads were measured using a tensile tester (InstronElima EMT-3050). The fibrous threads were cut at random into pieces with a length of 20 cm and then weighed to obtain the linear mass density (tex) of the yarns. The tensile data were reported in cN per tex (cN/tex). The applied gauge length and crosshead speed were 20 mm and 10 mm/min, respectively. The tensile properties data are presented as mean ± standard deviation. Student's two-tailed t-test was performed using GraphPad Prism software to determine statistical differences. Differences were considered statistically significant at $P<0.05$ and $p<0.1$.

III. RESULTS AND DISCUSSION

A. Fiber Morphology and Structure

Core-shell fibers with hydrophilic BSA as the core material

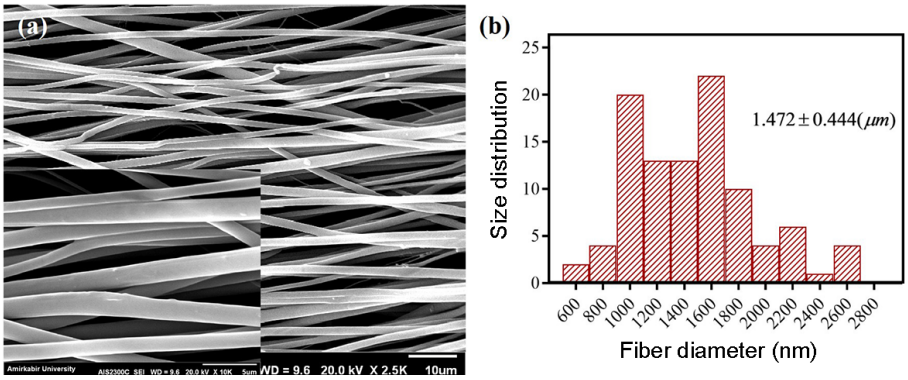

Fig. 2. (a) SEM images and (b) size distribution of BSA-loaded aligned core-shell fibers.

and the hybrid of hydrophilic gelatin/hydrophobic PCL as the shell layer were prepared by coaxial electrospinning. The bead free core-shell fibers were fabricated by optimizing the spinning parameters to form a stable and compound Taylor cone. The stable condition of the compound cone is required for uniform incorporation of core material into the shell solution and subsequently the formation of uniform core-shell fibers. To keep a compound Taylor cone in dynamic stabilization, it is necessary to control and balance the flow rates of core and shell solutions. Furthermore, at given solution flow rates, a stable compound Taylor cone was formed in a small range of applied voltage. In our previous study [41], the optimized conditions for steady electrospinning process were obtained to fabricate uniform core-shell fibers with PCL/gelatin as a shell and BSA as a core. At different weight ratios of PCL/gelatin hybrid shell, the inner/outer solutions flow rates, as well as the voltage, were appropriately adjusted so as to obtain a stable compound Taylor cone [41]. In this study, with regard to the pervious study, the voltage was adjusted at the previous optimized condition to obtain a stabilized compound Taylor cone. Finally, the stabilized compound Taylor cone and bead free core-shell fibers were obtained at a flow rate of 0.1 and 0.7 mL/h of the inner and outer fluids, 10 kV voltage, and 12 cm distance. The results demonstrated that the ultrafine fibers exhibiting a beadless and smooth surface were obtained from PCL/gelatin blend solution as shell material, as shown in Fig. 2. It shows the SEM images and size distribution of the aligned fibers with core-shell structures, taken from the rotating wheel collector. A rotating wheel collector can create a strongly concentrated electrostatic field to attract the charged jet traveling

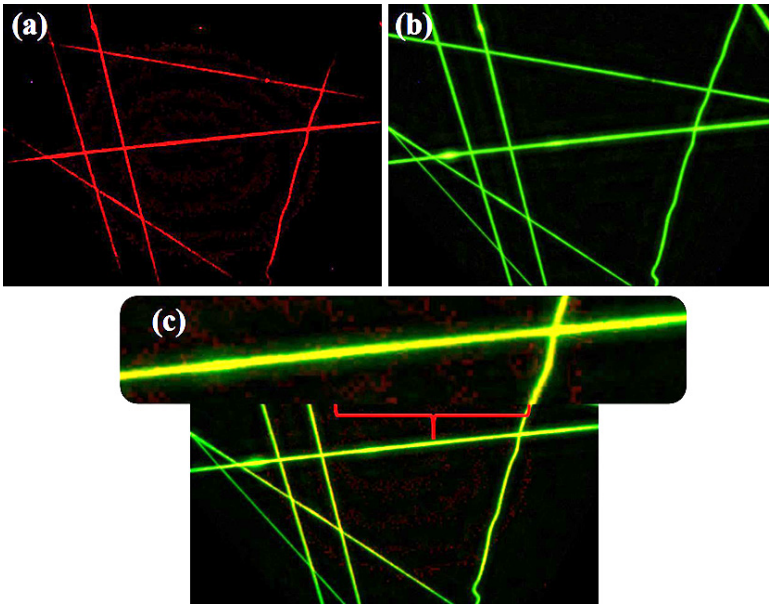

Fig. 3. FM images of core-shell fibers which: (a) RBITC (red), (b) FITC (green) were used to stain BSA core and PCL shell, respectively, and (c) then merge of red and green fluorescent images.

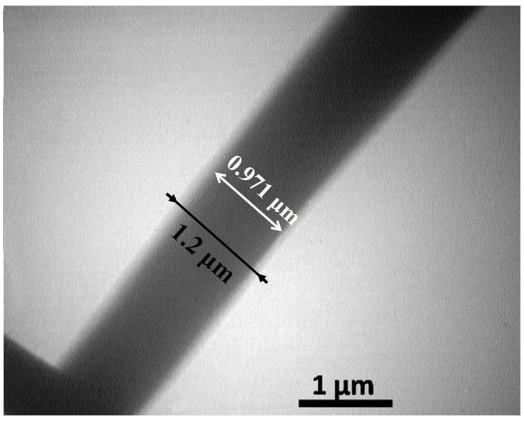

Fig. 4. TEM image of coaxial electrospun fibers. Core and shell parts were indicated by arrows.

toward it and wind fibers along its edge. Finally, the well-aligned fibrous mats were obtained by adjusting the rotating speed and electrospinning parameters. In this study, the well-aligned fibers with a mean diameter of 1.472±0.444 µm were obtained using a rotating wheel at 1300 rpm.

B. Protein Distribution in Fibers

As the SEM images could not provide evidence that the protein (BSA) was successfully incorporated into fibers, the fluorescent staining of core-shell fibers was performed. The RITC-conjugated BSA was loaded as the core solution, while the shell polymer contained FITC (green stain) to study protein distribution inside fibers by fluorescent microscopy. Fig. 3a demonstrates the successful loading and uniform distribution of protein (indicated by red stain) inside PCL/GT (50:50) fibers. To visualize the core-shell structure, fluorescent images were taken from core-shell fibers, where shell polymer and core solution were respectively stained with FITC (green stain) and RITC (red stain). Figs. 3a and 3b shows the fluorescence images of the fibers, featuring red and green light emissions, respectively. To observe the core-shell structure, green, and red fluorescent images were merged (Fig. 3c). However, using two different fluorescent stains for core and shell materials, the boundaries between the core and shell materials became indistinguishable. This could probably be due to the ultrafine core and shell diameter of fibers, which could not be recognized by the low power magnification of the fluorescent microscope.

C. TEM Characterization

TEM was also applied to obtain further evidence that BSA was indeed encapsulated within the shell material. Fig. 4 represents TEM images of the core-shell fibers, which shows a relatively uniform core-shell structure in which BSA was encapsulated by the PCL/gelatin shell with clear boundaries. As shown, the core-shell structure of the obtained coaxially electrospun fibers was successfully demonstrated by TEM images, indicated by the difference in electron density between the inner core and outer shell of the fibers. The sharp boundaries between core and shell layers in TEM images are attributed to the immiscibility of core-shell solutions and the high-speed processing characteristic of electrospinning, which would significantly prevent the mixing of core-shell fluids.

D. Fibrous Threads

BSA-loaded PCL/gelatin hybrid threads with a diameter of 150-250 µm were prepared from aligned fiber bundles with core-shell structures using coaxial electrospinning. SEM observations were conducted to explore the morphology and orientation of the fibers in the prepared threads, as given in Fig. 5. We can see that the fibers kept a well-aligned structure and coaxial electrospinning afforded uniform BSA-loaded threads composed of fibers with smooth surfaces without any bead formation (as shown in Fig. 5b). Fig. 5c shows SEM images of fibers in structures of thread after crosslinking with genipin. Conventional natural polymers, in particular, those derived from the extracellular matrix, will hydrolyze when in contact with an aqueous solution [43]. In this regard, the gelatin part of

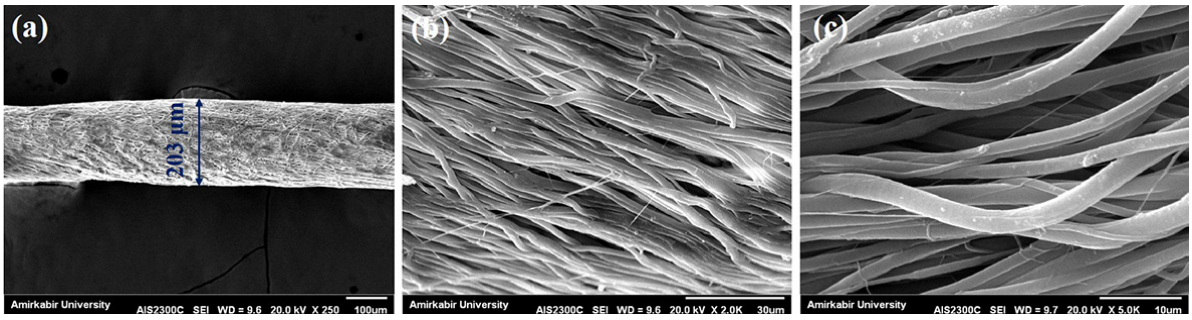

Fig. 5. SEM images of: (a) the core-shell fibrous threads, (b) core-shell fibers of thread without crosslinking, and (c) core-shell fibers of thread crosslinked with genipin.

fibrous threads is dissolved and loses its three-dimensional structure in aqueous conditions at *in-vivo* and *in-vitro* conditions. Crosslinking is an effective method that can ameliorate the mechanical performance and temporal stability. Normal crosslinkers often show cytotoxicity if they are not removed completely before cell culture. Herein, genipin was chosen to treat the scaffolds. Genipin is a biocompatible, natural crosslinking agent and has been widely used to chemically crosslink natural polymers and biological tissues between primary amine groups [44]. Genipin has also been reported to show much less cytotoxicity than other agents [45]. SEM images revealed that fibers with crosslinking treatment could retain their stable morphology. Threads with genipin treatment retained their fibrous structure and integrity (Fig. 5c). Due to fiber swelling, the mean diameter showed a slight increase after crosslinking as compared to untreated samples.

E. Fourier Transform Infrared (FT-IR) Spectroscopy

FTIR measurements were conducted on the un-crosslinked fibrous thread and crosslinked fibrous thread to determine whether crosslinking of the PCL/gelatin fibrous thread affected the primary gelatin-containing structure (Fig. 6). Qualitative analysis by FTIR detected a characteristic peak at 1730 cm^{-1} that is implicated in the stretching vibration of carbonyl groups associated with the ester bonds in PCL. Another characteristic bands of PCL were also observed at 2949 cm^{-1} (asymmetric CH_2 stretching), 2865 cm^{-1} (symmetric CH_2 stretching), 1293 cm^{-1} (C–O and C–C stretching), 1240 cm^{-1} (asymmetric C–O–C stretching) and 1170 cm^{-1} (symmetric C–O–C stretching) [46-48]. For un-crosslinked thread, common bands of protein appeared at approximately 1640 cm^{-1} (amide I) and 1540 cm^{-1} (amide II), corresponding to the stretching vibrations of C=O bond, and coupling of bending of N–H bond and stretching of C–N bonds, respectively [46-48]. These two peaks are sensitive markers of polypeptide secondary structure, which were evident in gelatin-containing threads [48]. In addition, the un-crosslinked thread had amide A peak (N-H stretching vibration) at 3330 cm^{-1} and amide III peak (C-N stretch plus N-H in phase bending) at 1240 cm^{-1}. As shown in Fig. 6, a little shift was detected for amide A peak from 3330 cm^{-1} for the as-spun thread to 3412 cm^{-1} for the crosslinked ones. The mechanism for gelatin crosslinking with genipin generally involves spontaneous reactions of the amino group NH_2 of gelatin with genipin. As a result of the chemical reactions with genipin, the peak intensity of the primary amine in crosslinked thread decreased, this may be attributed to the consumption of the amine ($-NH_2$) groups during crosslinking reactions [49]. These features suggest that the carboxymethyl group of genipin has reacted with the amino group of gelatin to form a secondary amide [49,50].

F. Tensile Behavior

The tensile properties of core-shell fibrous threads are important for their successful applications in tissue engineering. The tensile performance of core-shell structured threads was analyzed by tensile stress-strain measurements. Fig. 7 illustrates representative stress-strain curves of BSA-loaded core-shell fibrous threads with and without crosslinking. These curves exhibited similar bilinear behavior with an initial elastic region followed by a plastic part. Based on the mechanical evaluations as shown in Table I, core-shell fibrous threads had, respectively, Young's modulus and ultimate tensile strength of 34.2 and 1.65 cN/tex and the strain-at-break of 20.78%. The mechanical performance of the hybrid fibrous thread is affected by the properties of its components. The PCL fibers showed a very soft and flexible characteristic with low Young's modulus and high elongation-at-break, but gelatin fibers were rigid and

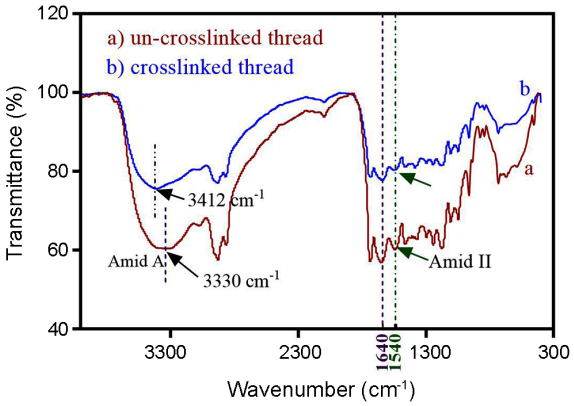

Fig. 6. FTIR analysis of: (a) un-crosslinked thread and (b) crosslinked thread.

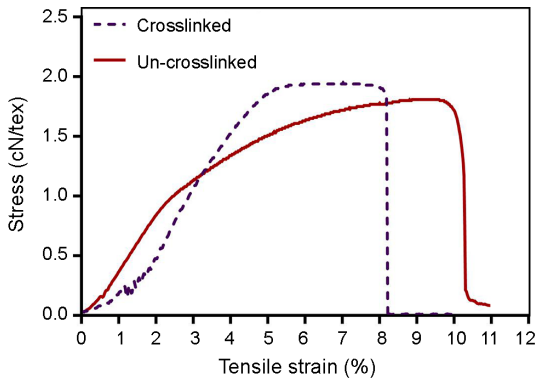

Fig. 7. Tensile properties of the core-shell structured scaffolds: representative stress-strain curves of without crosslinking and with crosslinking treatment.

TABLE I
MECHANICAL PROPERTIES OF FIBROUS THREADS WITH CORE-SHELL STRUCTURE (SAMPLE NUMBER=5).

Mechanical properties	Un-crosslinked fibrous thread	Crosslinked fibrous thread	n
Young's modulus (cN/tex)	34.205±2.872	40.64±11.155	5
Ultimate strength (cN/tex)	1.645±0.163	1.662±0.395	5
Strain-at-break (%)	20.789±3.04	14.265±4.154	5

brittle and exhibited a high modulus and low elongation-at-break. In our previous study [41], an increase of Young's modulus was achieved with increasing the gelatin content in the shell material for different blending ratios. The crystalline morphology of electrospun polymeric fibers strongly influenced the mechanical properties [51,52], and gelatin mainly contributed to the crystallinity of the electrospun mats [53,54]. In this regard, it was assumed that by combining the gelatin into PCL fibers, in addition to the biological performance, the tensile properties could be enhanced and became more proper for tissue engineering approaches. Moreover, fiber orientation in the thread will affect the interaction between the fibers and consequently influence their mechanical behavior [55]. Compared with un-crosslinked threads, fibrous threads crosslinked with genipin showed improvement in tensile properties with increment in Young's modulus from 34.20 cN/tex to 40.64 cN/tex ($p<0.1$) and significant decrement in the strain-at-break from 20.78% to 14.26% ($p<0.05$). However, a significant improvement ($p>0.5$) was not observed in terms of the ultimate tensile strength (1.66 cN/tex).

IV. CONCLUSION

In this study, BSA-loaded aligned fibers with core-shell structures were successfully fabricated by combining coaxial electrospinning with a rotating disk collector. A model protein, BSA (as a protein model) as a core was incorporated into the shell material, including PCL/gelatin (50:50) hybrid and aligned fibers were then fabricated on the rotating disk. After collecting the aligned fiber bundle and twisting manually to prepare BSA-loaded fibrous thread, the threads were crosslinked with genipin. The results revealed that coaxial electrospinning is a promising method for protein incorporation into fibers with a uniform fibrous structure. FM and TEM imaging confirmed the successful production of uniform core-shell fibers in which protein was homogeneously incorporated into the shell. Finally, BSA-loaded PCL/gelatin hybrid threads with a diameter of 150-250 μm were prepared from aligned fiber bundles with core-shell structure and SEM imaging demonstrated that the fibers kept a well-aligned structure and coaxial electrospinning afforded uniform BSA-loaded threads composed of fibers with smooth surfaces without any bead formation. SEM observation and FTIR analysis thus proved that the crosslinking was successful between gelatin parts of thread with genipin (as a crosslinker). The tensile tests revealed that fibrous thread with a modulus of 34.2 cN/tex had proper tensile properties. Also, it was found that crosslinked threads with genipin retained their fibrous structure and demonstrated an improvement in the tensile properties of the fibrous thread with increment in Young's modulus from 34.20 cN/tex to 40.64 cN/tex, which are the two critical characteristics for tissue engineering.

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