Video Article

Synthesis of Keratin-based Nanofiber for Biomedical Engineering

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Abstract

Electrospinning, due to its versatility and potential for applications in various fields, is being frequently used to fabricate nanofibers. Production of these porous nanofibers is of great interest due to their unique physiochemical properties. Here we elaborate on the fabrication of keratin containing poly (ε-caprolactone) (PCL) nanofibers (*i.e.*, PCL/keratin composite fiber). Water soluble keratin was first extracted from human hair and mixed with PCL in different ratios. The blended solution of PCL/keratin was transformed into nanofibrous membranes using a laboratory designed electrospinning set up. Fiber morphology and mechanical properties of the obtained nanofiber were observed and measured using scanning electron microscopy and tensile tester. Furthermore, degradability and chemical properties of the nanofiber were studied by FTIR. SEM images showed uniform surface morphology for PCL/keratin fibers of different compositions. These PCL/keratin fibers also showed excellent mechanical properties such as Young's modulus and failure point. Fibroblast cells were able to attach and proliferate thus proving good cell viability. Based on the characteristics discussed above, we can strongly argue that the blended nanofibers of natural and synthetic polymers can represent an excellent development of composite materials that can be used for different biomedical applications.

Video Link

The video component of this article can be found at http://www.jove.com/video/53381/

Introduction

Electrospinning is recognized as a prevalent method of achieving polymer nanofibers. The fibers can be produced on a nanoscale and the fiber properties are customizable [1]. These developments and the characteristics of electrospun nanofibers have been especially interesting for their applications in biomedical engineering especially in tissue engineering. The electrospun nanofibers possess similarities to the extracellular matrix and thus promote cell adhesion, migration and proliferation[2]. Due to this similarity to the extracellular matrix (ECM), electrospun fibers can be used as materials to assist in wound dressing, drug delivery, and for engineering tissues such as liver, bone, heart, and muscle[3].

A variety of different polymers of synthetic and natural origin have been used to create electrospun fibers for different biomedical engineering applications[4]. Recently there has been growing interest in the development of composite nanofibers by blending synthetic and natural polymers[4]. In these compositions the final products typically inherit the mechanical strength associated with the synthetic polymer while also adopting biological cues and properties from the natural polymer.

In this experiment, PCL and keratin are presented as the synthetic and natural polymers to be used for the synthesis of a composite nanofiber. Keratin is a natural polymer that is found in hair, wool and nails. It contains many amino acid residues; of notable interest is cysteine[4,5]. Ideally a naturally occurring polymer would be biorenewable, biocompatible and biodegradable. Keratin possesses all three of these characteristics while also enhancing cell proliferation and attachment to the biomaterials it has been incorporated in[6].

Polycaprolactone (PCL) is a resorbable, synthetic polymer that is significant in tissue engineering[4]. This polymer has previously been praised for its structural and mechanical stability, however, it lacks cell affinity and exhibits a lengthy degradation rate. The hydrophobic nature of PCL is likely responsible for the lack of cell affinity[7]. However, PCL makes up for its limitations by being highly miscible with other polymers. A PCL/keratin composite should demonstrate the mechanical properties of PCL and incorporate the biological properties of keratin, making it an ideal choice for various biomedical applications.

Protocol

All protocol follows the guidelines of the North Carolina A&T State University Office of Research Compliance and Ethics.

1. Chemical Preparation for Keratin Extraction [4]

1. To prepare 1,000 ml of 2% wt/vol peracetic acid solution (PAS), under a fume hood add 20 ml of peracetic acid to 980 ml of Deionized (DI) water.
2. To prepare 1,000 ml of 100 mM Tris base solution (TBS), add 12.2 g of Tris Base to 1,000 ml of DI water and stir until completely dissolved.
3. Prepare diluted hydrochloric acid solution (DHAS) in a fume hood by pouring 4 ml of concentrated hydrochloric acid into 30 ml of DI water.
4. Procure approximately 20 g of human hair clippings that have not been chemically treated or altered. Hair can be any length.
5. Wash the hair thoroughly, by hand, with warm water and soap. Use deionized (DI) water for the final rinse.
6. Put the hair into two 600 ml beakers and place in an 80 ºC oven for 1 hr.
7. Drain the liquid from the bottom of the beaker and place back in the oven until the hair is completely dry.
8. Divide dried hair evenly between the 600 ml beakers, placing 10 g of hair into each beaker. Do not allow the hair to fill more than 500 ml.
9. Pour 500 ml of PAS into each beaker making sure to cover all of the hair. Cover the beakers with parafilm and store for 12 hr.

2. Preparation of Keratin Extract Solution

1. Separate the hair from the PAS using a 500 µm sieve. Collect the waste PAS in a separate container. Rinse the hair thoroughly with DI water to remove any leftover PAS.
2. Place the rinsed hair in a 500 ml flask. Pour 400 ml of TBS into the flask, making sure that the hair is covered. Place flask in a shaking bath set to 38 ºC, 65 rpm for 1 hr.
3. After 1 hr, remove from shaking bath and pour the liquid, approximately 400 ml of keratin extraction solution (KES), into a 1,000 ml beaker.
4. Pour 400 ml of DI water into the flask containing the hair ensuring that the hair is covered and place back in the shaking bath for 1 hr. Remove the flask from the shaking bath and pour the remaining 400 ml of the KES into the 1,000 ml beaker with the previously collected KES. The hair is no longer needed and can be dumped out of the flask and discarded in the nearest trash receptacle.

3. Concentration of Keratin Extract Solution

1. Fill rotodistiller bath to the top line with distilled water and set to 90 ºC. Set rotation speed to 200 rpm. Turn on the coolant chiller-pump and set to -10 ºC.
2. Using a pH meter to monitor the pH of the KES, use a pipette to slowly add 1 mM DHAS to the KES until reaching a pH 7.0. Stir slowly as the solution is added.
3. Pour the neutralized KES into the 500 ml round-bottom flask until about quarter full. Run the distiller according to manufacturer's protocol for 1.25 hr.
    1. Repeat step 3.3 until all of the KES has been distilled. Ensure that the flask is connected tightly.

4. Dialysis of Keratin Extract Solution

1. Pour KES solution equally into 12 separate 14 ml conical tubes. Centrifuge the tubes at 1,050 x g for 10 min.
2. Pour the centrifuged KES into a clean beaker, making sure to pour away from the collected debris. Repeat until all of the KES has been centrifuged.
3. Fill a 2,000 ml graduated cylinder with DI water.
4. Cut dialysis tubing cellulose membrane to 24 inches, and clip one end of the tubing to hold it shut.
5. Dip the length of the tube in the cylinder of DI water to make it more flexible and easier to open.
6. Open the non-clipped end of the dialysis tubing cellulose membrane and slowly pour 60 ml of centrifuged KES solution into the tubing. Use another clip to close this end of the tubing.
7. Put the dialysis tube in the 2,000 ml cylinder of DI water and allow to sit for 24 h. Change the DI water in the cylinder every 3 to 4 hr.
8. After the 24 hr period, empty the dialyzed KES solution into capped jars, being sure to leave space at the top and place in the freezer at -20° C for 24 hr.

5. Lyophilization of Keratin Extract Solution

1. Set the lyophilizer to -86 ºC.
2. Remove the jars containing frozen KES from the freezer, take the caps off of the jars, and place them into freeze dryer ampoules. Place the seals on the ampoules and turn the knob to create a vacuum pressure of 0.133 mBar. Lyophilize the samples for approximately 48 hr, until all of the moisture is gone.

6. Preparation of Electrospinning Solutions (10 wt % Keratin Solution)

1. Remove the Keratin Powder from the lyophilizer and weigh it.
2. Add DI water to the Keratin powder to create a 10% weight/weight Keratin solution.

7. Preparation of 10% wt PCL Solution

1. Obtain PCL (ε-caprolactone polymer,Mn 70 - 90 kDa), and trifluoroethanol (TFE). Prepare a 10% by weight PCL in TFE by stirring O/N and obtain a homogeneous mixture.

8. Preparation of Keratin /PCL Solution

1. Obtain the 10 wt % PCL solution previously prepared and 10 wt % keratin solution. Add keratin into the PCL solution drop wise in order to create 10 ml PCL/keratin solutions of 90:10, 80:20, 70:30, and 60:40 ratios.
2. Use a vortexer to obtain a homogeneous mixture of PCL/Keratin solution before electrospinning.

9. Production of Electrospun PCL/keratin Fiber

1. Place approximately 8 ml of the PCL/keratin solution in a 10 ml disposable syringe fitted with a 0.5-mm diameter plastic tube. Place the syringe in a syringe pump where the flow rate is set to be 2.5 ml/hr.
2. Apply voltage to the tip of the needle connected to the tube (positioned ~23 cm from the fiber collecting drum, and ~30 degree from the horizontal). Apply a 19 - 22 kV voltage to the tube and rotate the collector drum at around 200 rpm.
    1. Wrap the collector drum with aluminum foil before running the sample. Ensure that the aluminum foil is stable by applying the labeling tape on either end.
    Note: The fibers will form on the aluminum foil (See **Figure 2**), store the fiber covered foil at RT.

10. Mechanical Analysis of PCL/Keratin Nanofibers

1. Cut the sample into 60 mm by 8 mm. Make sure to record the thickness by using digital micrometer. For each composition prepared, use five samples.
2. Attach the 60 x 8 mm sample in the tensile tester. Use a custom designed specimen holder to attach the sample. Sandwich the sample between specimen holders which is made from an index card using double-sided tape. Apply the 10 N load cell with a displacement rate of 10 mm/min.
    Note: The specimen holder was designed to be 62 mm by 40 mm with an inner window of 50 mm by 38 mm.
3. Record extension and load values through software according to software protocol.
4. Calculate stress by dividing the force by area of the sample tested. Next, calculate strain by dividing the change in length by initial length.
5. Generate the stress-strain curve by plotting stress in y-axis for corresponding strain value in x-axis.
6. Calculate the Young's modulus from the linear region where slope equals to modulus of elasticity. Determine tensile strength from the stress strain plot by taking 0.2% offset in strain and drawing a parallel line to the linear region curve.
7. Perform the statistical analysis of the mechanical data obtained in triplicate previously using one-way analysis of variance using analysis software[8]. *p* values <0.05 were considered statistically significant.

11. Surface Morphology and Structural Characterization

1. Use scanning electron microscope (SEM) with parameters of accelerating voltage of 15 kV and current of 5 µA to observe the morphology of the electrospun fibers.
    1. Prior to imaging with SEM, cut a 2 cm^2 section of the fiber and attach it to a SEM stage using copper tape. Place the stage inside the sputter coater and coat with gold at 15 mA for 1 min and 30 sec to create a gold layer approximately 11 nm thick. Load the sample into the chamber of the SEM. Observe the fiber samples.
2. Use Fourier transform infrared spectroscopy (FTIR) to characterize the chemical bonding between the PCL and keratin. Place the 2 cm^2 section of the electrospun nanofiber membrane in a magnetic holder and purge the system with dry air before testing. Obtain spectra for 200 scans and analyze the spectra using standard software according to software protocol.
3. Perform spectrum analysis using standard software and use triplicates of each ratio of nanofiber according to the manufacturer's protocol.

12. Study of Cell-fiber Interaction

1. Cut fibers into 16 mm^2 samples. Use a biocompatible, silicone-based glue to fix each sample to coverslips with a 12 mm diameter.
2. Glue one end of the fiber sample to the back of the cover slip, wrap the sample around the coverslip, then glue the free end of the sample to the back of the cover slip as well, leaving the top of the slip covered with only the fiber sample.
3. Place the fiber samples in a 24-well plate. Sterilize the samples by immersing in 80% ethanol for 1 hr.
4. Rinse the ethanol from the samples using DI water. Then, pipette 2 ml of basal medium onto the samples, making sure to cover the entire sample. Remove the medium after 5 min.
5. Use fibroblast 3T3 cells to seed the fiber samples. Suspend the cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% fetal bovine serum (FBS) so that there are approximately 62,000 cells/cm^2 per 1 ml of DMEM.
6. Drop 1 ml of the 3T3 cell-containing DMEM solution into each well plate ensuring that each fiber sample is covered with approximately 62,000 cells/cm^2. Incubate the well-plates for 24 hr at 37 °C and 5% CO_2. Note: Use 5% CO_2 because that level can usually maintain the pH of the cell media in physiological range for days.

13. Degradation of Nanofiber Matrix

1. Cut dried PCL/keratin nanofiber membranes into square approximately 30 mm x 30 mm.
2. Sterilize the 900 mm^2 samples with 80% alcohol for a 10 min incubation period and wash thoroughly with DI water. Incubate the samples in 15 ml of PBS, pH 7.5, at 37 ºC. Replace the buffer every 3 days.
3. Take the membranes out of the solution at specified intervals, 1 week and 7 weeks, and rinse with DI water.

4. Place the membrane samples into freeze dryer ampoules. Place the seals on the ampoules and turn the knob to create a vacuum. Lyophilize for 24 hr, until completely dry.
5. Attach the dried sample it to a SEM stage using copper tape. Place the stage inside the sputter coater and coat with gold at 15 mA for 1 min and 30 sec.
6. Load the sample into the chamber of the SEM. Observe the fiber samples at an accelerating voltage of 1.5 kV and 5 µA current.

Representative Results

Fiber Morphology
SEM images of the fibers were obtained for all the fiber compositions. See **Figure 3**. Fiber image confirms that the fibers are randomly oriented.

Mechanical Testing
Mechanically strong fibers are generally required for various tissue engineering applications. These fibers should retain sufficient strength and flexibility under certain stress and environmental conditions[9]. Generally, scaffolds are desired to have moduli close to that of the target tissue to avoid any stress shielding effects and to maintain sufficient strength during *in vivo* and/or *in vitro* cell growth[10]. Tensile Tester was used to measure the Young's modulus and breaking strength of the fiber. Young modulus (MPa) of PCL/keratin at ratios of 100:00, 90:10, 80:20, and 70:30 was found to be 10 ± 2, 8 ± 1, 5 ± 1.5, and 4.5 ± 1.6, respectively[4]. Similarly, breaking strength (MPa) of PCL/keratin at ratios of 100:00, 90:10, 80:20, and 70:30 was found to be 3 ± 1.2, 2 ± 0.5, 1 ± 0.2, and 1 ± 0.3 respectively[4] as seen in **Table 1**. **Figure 4** displays the graphical trend of variation of Young's modulus versus PCL.keratin ratios. The trendline in the graph aids in further understanding the breaking elongation rate.

Structural and Morphological Characterization
In **Figure 5**, FTIR transmittance spectra for PCL/keratin composite nanofibers show bands at 2,950 cm^{-1}, 1,050 cm^{-1}, and 1,240 cm^{-1} due to asymmetrical stretching vibrations of CH_2, C-O and C-O-C groups, respectively. The carbonyl absorption peak at 1720 cm^{-1} that is characteristic of PCL is also visible[4,11]. Absorption bands at (3,286 cm^{-1}), (3,056 - 3,075 cm^{-1}), (1,600 - 1,700 cm^{-1}), (1,480 - 1,580 cm^{-1}), and (1,220 - 1,300 cm^{-1}) are indicative of keratin proteins. The bands have been denoted as amide A, B, I, II, and III, respectively.

The bands and peaks present in the FTIR spectra change with the increased keratin concentrations in the composite. Their appearance indicates the presence and structural conformation of keratin chains, however, the interactions between the peaks and bands did cause some difficulty. For example, the amide I band present at 1,600 - 1,700 cm^{-1} is typically used to study keratin, however, the band is slightly disfigured by the PCL peak. Fortunately, the amide II band will suffice to prove keratin presence, keratin chain conformation, and bonding interactions between the PCL and keratin functional groups.

Cell-Fiber Interaction
Using Scanning Electron Microscopy the cell-fiber interaction was studied. **Figure 6** shows the SEM images of the 3T3 fibroblast cells that were cultured on the nanofiber samples for 24 hr. The adhesion of the cells to the nanofiber samples is visible and shows that the cell filopodia tend to follow the alignment of the nanofibers when they are similar in diameter. Almar Blue (AB) assay was performed to quantify the viability of 3T3 cells in the fibers. AB is the chemical resazurin. This non-fluorescent dye enters the living cells, mitochondrial reductases reduces resazurin to resorrufin which is pink and fluorescent. There was not significant difference in the toxicity levels of different ratios of PCL/keratin.

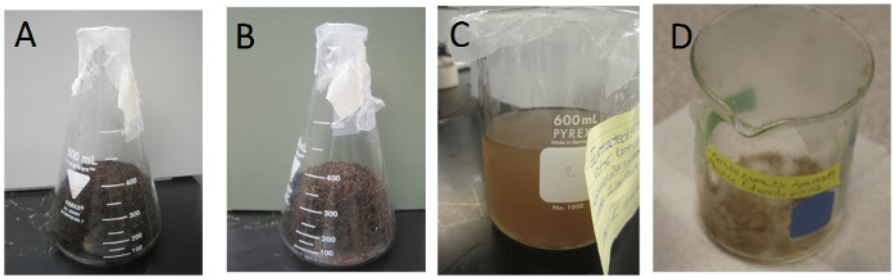

Figure 1. Pictures of the Keratin Extraction Process. (**A**) Cleaned human hair before extraction; (**B**) Hair after keratin extraction; (**C**) Keratin extract solution; (**D**) Lyophilized keratin powder. Please click here to view a larger version of this figure.

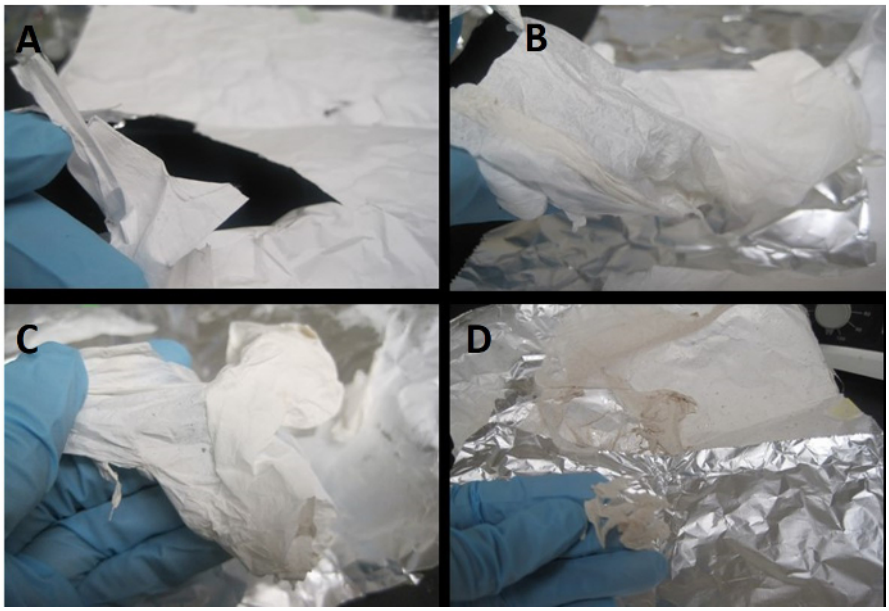

Figure 2. Digital Camera Pictures of Electrospun Fibers. Images of as synthesized fibers of CL/keratin with different ratios collected on aluminum foil. Please click here to view a larger version of this figure.

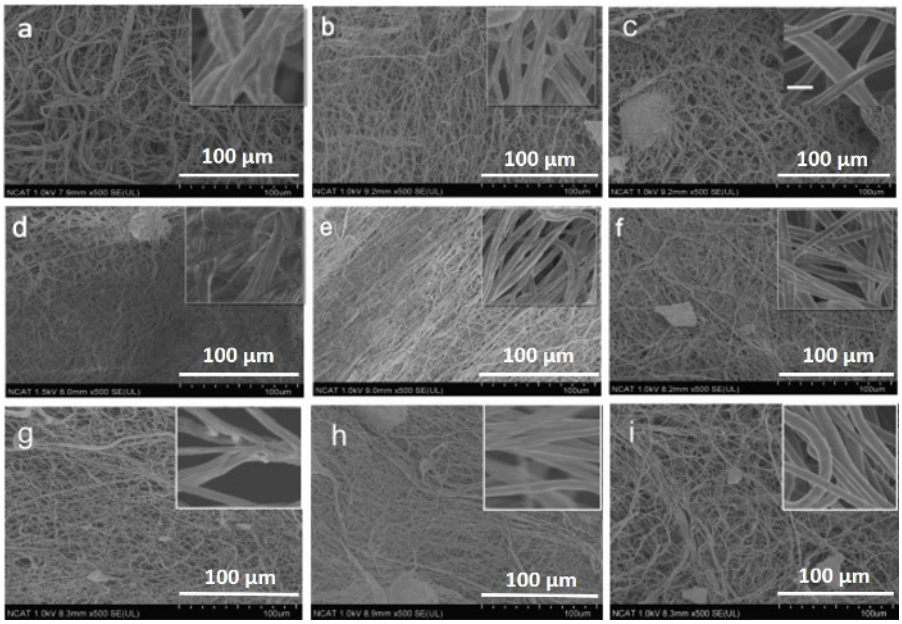

Figure 3. SEM Images of PCL/keratin Nanofibers. (**A-C**) images of the nanofibers spun from solutions with PCL/keratin ratios of 70:30, 80:20, and 90:10, respectively. The insets show higher magnification images of each corresponding SEM image. SEM images (**D-F**) and (**G-I**) represent the fibers shown in images (**A-C**) after 1- and 7-week degradation tests, respectively. The scale bar in the insets represents 500 nm (see scale bar in image **C**). Please click here to view a larger version of this figure.

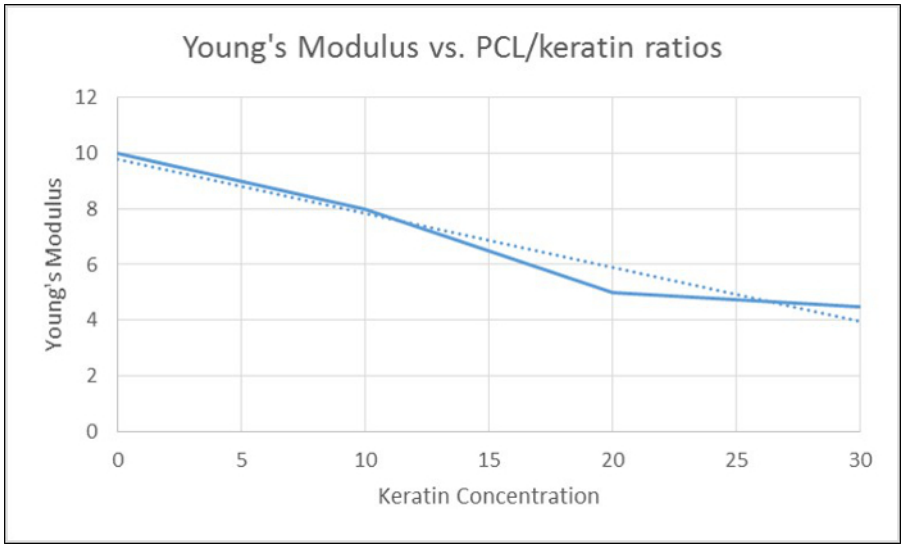

Figure 4. Graph of Modulus versus PCL/Keratin Ratios. A graph of keratin concentration versus Young's Modulus shows the graphical trend of variation of the Young's Modulus. The trendline aids in understanding breaking elongation rate. Please click here to view a larger version of this figure.

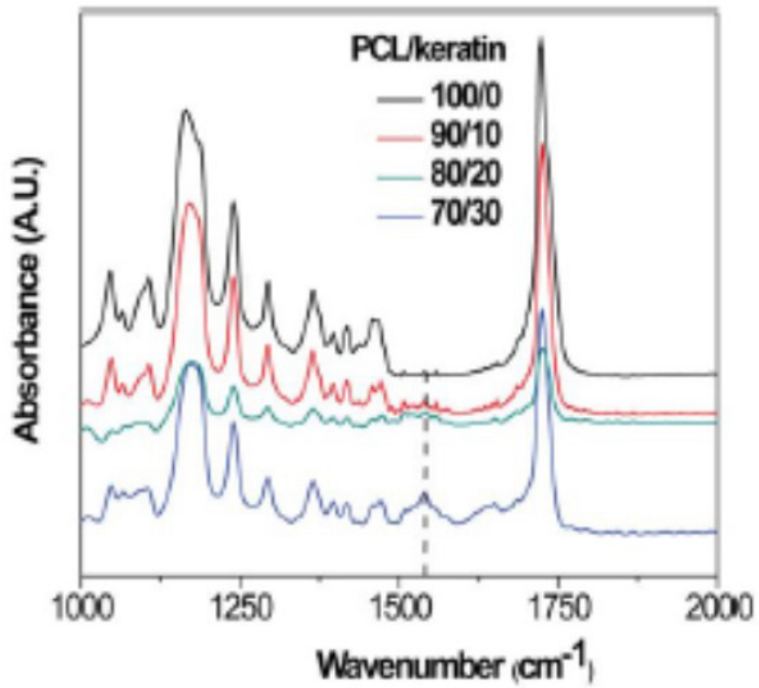

Figure 5. FTIR Spectra of PCL/keratin Nanofibers with Different Ratios. The spectrum confirms the bonding of PCL to keratin. The major peak measured at 1,722 cm^{-1} agrees with standard basic measurements of PCL absorption band and is visible in all spectra. Please click here to view a larger version of this figure.

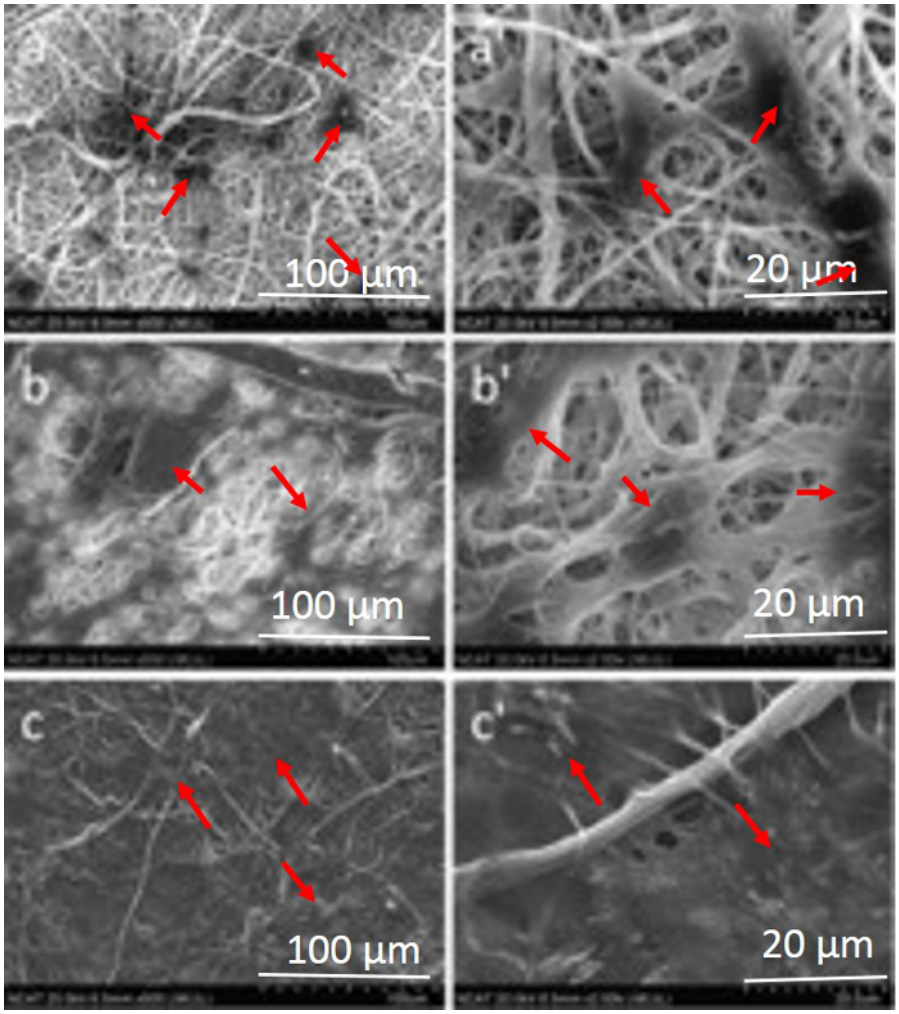

Figure 6. SEM Images Showing the Morphology of 3T3 Fibroblast Cells Seeded on PCL/keratin Nanofiber Membrane. Images **A**, **B** and **C** represent the PCL/keratin with ratios of 90/10, 80/20, and 70/30, respectively. Images (**A'**), (**B'**), and (**C'**) are higher magnification images of (**A**), (**B**), and (**C**), respectively. The darker areas on the images indicate the location of each fibroblast cells attached on top of and throughout the nanofiber topography. Please click here to view a larger version of this figure.

Ratios	Young's Modulus (Mpa)	Breaking Strength (Mpa)
100/0	10 ± 2	3 ± 1.2
90/10	8 ± 1	2 ± 0.5
80/20	5 ± 1.5	1 ± 0.2
70/30	4.5 ± 1.6	1 ± 0.3

Table 1. Mechanical Properties of PCL/Keratin Fibers

Discussion

Extraction of keratin from human hair was successfully achieved. The peracetic acid acted as an oxidizing agent on the human hair, allowing the keratin to be extracted by the Tris Base. The production of keratin powder was small scale due to the fact that it was only done for research purposes. This procedure has already been established in industry for large-scale production. The purpose of extracting the small-scale keratin was to control contamination, batch variability, and cost-effectiveness.

The keratin extraction is the limiting step of this procedure. The yield of keratin powder is very low, 0.7 - 2%. 20g of human hair yielded 0.14 - 0.4 g keratin. Another critical step in producing the electrospun fibers is formulating a solution that is suitable for electrospinning. Keratin was easily dispersed in DI water, however, upon electrospinning, the keratin/water solution did not result in fiber formation. To provide the necessary molecular interactions to create nanofibers a copolymer was introduced to the solution. PCL dissolved in TFE, was able to interact more strongly with keratin through hydrogen bonding. The TFE was largely responsible for stability of the copolymer complexes because of its electronegativity and acidic behavior.

Mixing the keratin solution with the PCL solution presented new challenges due to that fact that PCL is known to be hydrophobic while the keratin is known to be hydrophilic. As we increased the ratio of keratin it was difficult to obtain the homogeneous mixture. This issue was resolved by adding keratin solution drop wise to the PCL solution, and vortexing it manually for 30 min.

SEM images show excellent surface morphology that is ideal for cell growth and proliferation. The comparative FTIR results demonstrate good miscibility between PCL and keratin in the electrospun fibers. This may be because of intermolecular hydrogen bonding between PCL and keratin. Another factor is the speed with which electrospun fibers solidify preventing PCL aggregation in the mixture. The structural and mechanical integrity is sustained by the molecular interactions between the PCL and keratin, making the material suitable for regenerative medicine applications. These electrospun fibers were found to have young moduli close that of the native tissue. Mechanically strong fiber is able to support cell adhesion and proliferation. The PCL/keratin nanofibers exhibited good uniformity, structural integrity, suitable mechanical properties, and cellular compatibility. Young's modulus (MPa) for PCL/keratin at ratios of 100:00, 90:10, 80:20, and 70:30 was found to be 10 ± 2, 8 ± 1, 5 ± 1.5, and 4.5 ± 1.6, respectively. The moduli decreased as the ratio of keratin added increased. Cell adhesion and proliferation on the PCL/keratin fibers confirms that the fibers are not toxic and provide support for cells growth. The filopodia growth along the nanofibers indicates favorable interaction between the fibroblasts and the PCL/keratin fibers.

Electrospinning technique was successfully used to synthesize the PCL/Keratin based nanofibers. This technique, unlike other existing methods, has proven to be reliable and cost-effective and can potentially be used in large-scale nanofiber production. From this study, we conclude that PCL/Keratin based composite nanofibrous scaffolds have the potential to be used for biomedical applications and mimic natural ECM for tissue engineering applications.

Disclosures

The authors declare that they have no competing financial interests.

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