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Wet-spinning-based Molding Process of Gelatin for Tissue Regeneration

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TITLE:

Wet-spinning-based Molding Process of Gelatin for Tissue Regeneration

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KEYWORDS:

Gelatin, fiber, tube, wet spinning, molding, tissue engineering, regeneration, biomaterial

SUMMARY:

We developed and describe a protocol based on the wet spinning concept, for the construction of gelatin-based biomaterials used for the application of tissue engineering.

ABSTRACT:
This article presents an inexpensive method to fabricate gelatin, as a natural polymer, into monofilament fibers or other appropriate forms. Through the wet spinning method, gelatin fibers are produced by smooth extrusion in a suitable coagulation medium. To increase the functional surface of these gelatin fibers and their ability to mimic the features of tissues, gelatin can be molded into a tube form by referring to this concept. Examined by *in vitro* and *in vivo* tests, the gelatin tubes demonstrate a great potential for application in tissue engineering. Acting as a suitable filling gap material, gelatin tubes can be used to substitute the tissue in the damaged area (*e.g*., in the nervous or cardiovascular system), as well as to promote regeneration by providing a direct replacement of stem cells and neural circuitry. This protocol provides a detailed procedure for creating a biomaterial based on a natural polymer, and its implementation is expected to greatly benefit the development of correlative natural polymers, which help to realize tissue regeneration strategies.

INTRODUCTION:
The latest development in tissue regeneration involves the application of tissue engineering, which represents a challenge for the improvement of new therapeutic strategies in medical treatments. For example, the limited potential of nervous system regeneration, following injury or disease, poses a significant health problem worldwide. Due to the complexity of pathophysiological processes associated with the nervous system, the use of traditional autograft or the implementation of stabilization surgery has been shown to offer benefits in functional outcomes, but there is no strong evidence for the effects of spinal fixation surgery[1,2]. The tissue at the damaged area is lost and replaced with hypertrophically induced astrocytes[3], eventually forming a dense glial scar[4,5]. This matrix acts as a barrier that blocks the recovery of nerve function[6,7] and is, thus, greatly hinders regeneration. Therefore, a suitable filling gap material is expected to prevent the loss of tissue and reduce the formation of scar-associated connective tissue by maintaining the integrity of the damaged area, as well as by providing the direct replacement of neural cells and circuitry to promote axon regeneration.

Polymeric biomaterials have been preferred as scaffolds for tissue regeneration therapy, based on the regulation of cell or axon behavior and tissue progression through natural extracellular matrix (ECM) support. The fiber format is commonly considered as a building block for various materials, owing to its one-dimensional structure[8]. The fibers can generally be obtained by melt extrusion or wet spinning method; however, the large size and cost of the equipment and the difficulty to perform these methods are challenging. In addition, the majority of the work related to polymer fibers has been focused on synthetic or composite materials. Natural polymers as a source of biomaterial offer better biocompatibility properties for the human body. Nonetheless, to obtain the alignment of natural polymer fibers is relatively more difficult than of synthetic polymer sources[9]. Hence, the conversion of a natural polymer as a rich source of protein into biomaterial fibers is an important strategy—not only can the biomaterial fibers be directly isolated from the raw material, thus avoiding an unnecessary transformation to monomers, but the protein fibers also have a good appearance and favorable characteristics[10].

In this regard, we describe an inexpensive processing method for the manufacturing of natural polymer fibers through the basic concept of wet spinning, that can be implemented on the laboratory scale for tissue engineering. Wet spinning is performed by the extrusion and coagulation of a polymer solution into a suitable polymer nonsolvent. An appropriate, viscous solution doped into coagulation medium causes the polymer molecules to dissolve. Through the phase transition, the filaments then lose their solubility and are precipitated in the form of a solid polymer phase[11]. Referring to this concept, we then expanded the development of gelatin into the tube form by a molding process, which is considered proper for tissue regeneration application. In addition, intrinsically, we can also develop any shape of material from gelatin fibers (*e.g.*, gelatin conduit rolled up from several gelatin fibers), for other desired applications.

Gelatin, a biodegradable natural polymer, is formed from denatured and hydrolyzed collagen, including any semicrystalline, amorphous, or triple helical state of collagen[12]. It is well known that collagen is the essential structural protein in all connective tissues of vertebrates and invertebrates[13,14], which is similar to the protein structure of the main ECM that induces nerve growth and, simultaneously, replaces a large amount of glycosaminoglycan secreted during spinal cord injuries. Therefore, the use of gelatin as a source would be a great choice for any medical vehicle. Besides being an inexpensive source, gelatin is also biodegradable and cytocompatible and clinically proven to be a temporary defect filler[15]. Developed into a tube form, *in vitro* and *in vivo* tests described here demonstrate that gelatin has an excellent biocompatibility and suitability for future tissue engineering applications. Cultured with human adipose stem cells, gelatin tubes improve cell differentiation into neural progenitor cells by using positive nestin staining as a neural cell marker. Furthermore, gelatin as filling gap material, as produced by the method established in this study, is expected to be manageable and safe and to greatly benefit tissue engineers who are currently developing correlative natural polymers for the enhancement of tissue regeneration strategies.

PROTOCOL:

The fat tissues were obtained from orthopedic surgeries as certified by the Institutional Review Board of Tri-Service General Hospital, Taipei, Taiwan, R.O.C. Procedures involving animal subjects have been approved by the Animal Care Committee at National Defense Medical Center, Taiwan (R.O.C).

1. Wet Spinning Process

1.1. Solution preparation

1.1.1. Dissolve 5 g of gelatin powder in 100 mL of double-distilled water to obtain 5% (w/v) solution concentration.

1.1.2. Stir the mixture slowly at 60 - 70 °C overnight to achieve a completely homogeneous dispersion without any bubbles.

1.2. Wet spinning

1.2.1. Fiber formation

NOTE: A schematic of the method is shown in **Figure 1A**. The spinning setup is equipped with a peristaltic pump machine (see **Table of Materials**) that provides high-precision speed and controls the smooth delivery of the flow.

1.2.1.1. Plug a 26 G x 1/2 inch (0.45 mm x 12 mm) syringe as the solution injector into clear vinyl tubing.

1.2.1.2. Prepare 100 mL of 99.5% acetone solution in a beaker glass to be used as the coagulation bath.

1.2.1.3. Run the peristaltic pump machine at 21 rpm (3.25 mL/min) and let the gelatin solution stand for several seconds in acetone solution before rolling it up.

1.2.1.4. Take out the gelatin fibers from the acetone solution and immerse them into 2.5% (w/v) of polycaprolactone/dichloromethane (PCL/DCM) solution with 1:20 (w/w).

1.2.1.5. Let the gelatin fibers in the PCL/DCM solution dry overnight, under the hood at room temperature.

1.2.2. Tube formation

NOTE: A schematic of the method is shown in **Figure 1B**.

1.2.2.1. Use a peripheral venous catheter of 24 G x 3/4 inch (0.7 mm x 19 mm) as tube mold.

1.2.2.2. Load the catheter into the gelatin solution and hold it there for 3 s.

1.2.2.3. Load the catheter into the acetone solution and hold for 1 min.

1.2.2.4. Load the catheter again into the gelatin solution and hold it there for 3 s.

1.2.2.5. Repeat this alternate procedure 20x.

1.2.2.6. Take out the catheter from the acetone solution and let the molded tube dry at room temperature for 5 min.

1.2.2.7. Gently take out the gelatin tube from the catheter by using a hemostat and immerse the gelatin tube into 2.5% (w/v) of the PCL/DCM solution with 1:20 (w/w).

1.2.2.8. Let the gelatin tube in the PCL/DCM solution dry overnight, under the hood at room temperature.

2. Morphology of the Gelatin Tube

2.1. Mount the piece of dried gelatin tube on a carbon stub.

2.2. Put the sample into the ion sputter coater machine (see **Table of Materials**) and coat the sample with gold for 60 s; then, observe its morphology by scanning electron microscopy (see **Table of Materials**).

3. Culture of Human Adipose Stem Cells

3.1. Place the fat tissues (obtained from oral adipose tissue by clinical surgery) into a Petri dish containing 10 mL of transfer solution consisting of 0.1 M phosphate-buffered saline (PBS), 1% penicillin/streptomycin, and 0.1% glucose.

3.2. Cut the tissues into small pieces (less than 1 mm^2) with a scalpel blade.

3.3. Transfer the tissues into a 15 mL plastic tube and centrifuge at 500 x *g* for 5 min.

3.4. Remove the supernatant and incubate the sediment in a plastic tube containing 10 mL of Dulbecco's modified Eagle's medium (DMEM, consisting of 4 mM L-glutamine, 1 mM sodium pyruvate, and 15 mg/L phenol red) with 0.1% collagenase at 37 °C, in a humidified atmosphere containing 95% air and 5% CO_2, for 1 day.

3.5. Centrifuge the plastic tube at 500 x *g* for 5 min, discard the supernatant carefully (do not touch the sediment), and then, suspend the sediment gently by adding 10 mL of DMEM containing 10% fetal bovine serum (FBS) and let it stand for 1 day at 37 °C, in a humidified atmosphere containing 95% air and 5% CO_2.

3.6. Centrifuge the plastic tube at 500 x *g* for 5 min and discard the supernatant carefully; then, add 1 mL of stem cell medium (consisting of DMEM, 5% FBS, N-acetyl-L-cysteine, ascorbic acid-2-phosphate, 1% antibiotic/antimyotic, and insulin) to suspend the sediment, transfer it into a T25 culture flask containing 4 mL of stem cell medium, and let it stand for 3 days at 37 °C, in a humidified atmosphere containing 95% air and 5% CO_2.

3.7. Discard the supernatant, add 1 mL of 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), and incubate it at 37 °C, in a humidified atmosphere containing 95% air and 5% CO_2 for 3.5 min, to detach the cells from the bottom of the culture flask.

3.8. Add 1 mL of FBS, suspend the mixture, and transfer it to a microcentrifuge tube.

3.9. Centrifuge the suspension at 500 x *g* for 3.5 min, suspend the sediment with 1 mL of stem cell medium, and transfer it to the culture flask containing 5 mL of stem cell medium; then, keep it at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

3.10. Maintain the subculture by changing the stem cell medium every 3 days.

4. Cultivation of Cells on the Gelatin Tube

4.1. Sterilize the gelatin tube with UV light for 2 h; then, immerse it in 75% (v/v) ethanol and wash it 2x with stem cell medium to remove the residual ethanol.

4.2. Collect the human adipose stem cells (hASCs) from the culture flask.

4.2.1. Remove the medium from the culture flask.

4.2.2. Add 1 mL of 0.25% trypsin-EDTA and incubate at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2 for 3.5 min, to detach hASCs from the bottom of the culture flask.

4.2.3. Add 1 mL of FBS, suspend the mixture, and transfer it to a microcentrifuge tube.

4.2.4. Centrifuge the microcentrifuge tube at 500 x *g* for 3.5 min; then, discard the supernatant carefully, and suspend the sediment with 1 mL of stem cell medium.

4.3. Place the gelatin tube in a 6-well plate and seed 4 x 10^4 of hASCs in the tube with 3 mL of stem cell medium; incubate them for 2 weeks at 37 °C, in a humidified atmosphere containing 95% air and 5% CO_2.

4.4. Change the stem cell medium every 3 days to provide enough nutrition for cell growth.

5. Immunocytochemistry

5.1. Remove the stem cell medium and wash the well with PBS.

5.2. Fix the tube with cells with 0.1% acetic acid glacial for 30 min at room temperature.

5.3. Wash the well 2x with PBS.

5.4. Add a surfactant (NP-40, 0.05%) and incubate for 10 min at room temperature.

5.5. Wash the well 3x with PBS.

5.6. Add 2% normal goat serum and incubate at room temperature for 30 min.

5.7. Decant the solution, add primary antibody nestin (neural progenitor cell marker), and incubate overnight at 4 °C.

5.8. Wash the well 3x with PBS.

5.9. Add secondary antibody donkey anti-mouse-fluorescein isothiocyanate (FITC) and keep the sample in the dark for 2 h.

5.10. Wash the well 3x with PBS.

5.11. Add 1 mL of Hoechst 33342 to stain the nuclei and incubate for 10 min at room temperature.

5.12. Wash the well gently and observe it under a fluorescence microscope.

6. *In Vivo* Biocompatibility Test

NOTE: Rats with a weight between 201 - 225 g have been successfully tested using this protocol.

6.1. Anesthesia

6.1.1. Induce and maintain anesthesia; preferably, anesthetize a rat (8-week-old Sprague Dawley rat, female) *via* an intramuscular injection of tiletamine and zolazepam (25 mg/kg + 25 mg/kg, respectively) and xylazine (5 mg/kg). Perform a pain stimulation test by pressing the rat's fingers to confirm the anesthetization.

6.2. Sterilization of the surgical site

6.2.1. Shave and clean the skin of the rat's trapezius area (over the back of the neck) and sterilize it with povidone-iodine lotion (100 mg/mL).

6.2.2. Cover the rat with sterile surgical drapes to reduce bacterial transfer and subsequent contamination of the surgical site.

6.3. Implantation of the gelatin tube

6.3.1. Gently cut the skin of the rat's trapezius area with a scalpel blade.

6.3.2. Create a wound of 2 cm and place the gelatin tube directly on the layer between the fascia and the muscle.

6.4. Postimplantation surgery

6.4.1. Close the wound with a gut suture and sterilize it with povidone-iodine solution (100 mg/mL).

6.4.2. Induce the rat *via* an intravenous injection of ketoprofen (2.5 mg/kg) and cefazolin (15 mg/kg).

6.4.3. Keep the rat under aseptic conditions for 7 days.

6.5. Euthanasia

6.5.1. Place the rat into the euthanasia chamber with a flow of CO_2 inhalation (10% - 30% of the chamber volume per minute) for 10 min in 20 - 26 °C.

6.5.2. Cut the rat gently with a scalpel blade, remove the implanted tissue, and take photos for observation.

REPRESENTATIVE RESULTS:

In this study, we successfully developed the gelatin into fibers (**Figure 2A**) and tubes (**Figure 2B,C**) through the user-friendly wet spinning concept. These gelatin-based materials can be utilized as any medical tool, depending on their shapes. Considering that the functional surface and frame of such materials are more suitable for tissue regeneration, we examined the biocompatibility of gelatin tube by performing *in vitro* and *in vivo* tests.

To obtain an overview of the gelatin tube, first, we conducted morphological observations by using scanning electron microscopy (**Figure 3**). The results showed that the surface of the gelatin tube was not very smooth (**Figure 3A**), its inner diameter was more than 200 µm (**Figure 3B**), and its thickness was approximately 20 µm (**Figure 3C**).

Then, immunocytochemistry was performed to examine the biocompatibility of the gelatin tube *in vitro* (**Figure 4**). Initially, the gelatin tube was incubated with human adipose stem cells for 2 weeks and was stained with nestin as a neural cell marker (**Figure 4A**). Under the fluorescence microscope, the staining showed the positive nestin staining marker (green color) along with a bunch of detected nuclei (blue color), which suggested that the cells could penetrate and adhere well to the gelatin tube and differentiate into neural progenitor cells (**Figure 4B**). Further, the *in vivo* biocompatibility test of the gelatin tube showed analogous results. The gelatin tube was inserted into the fascia layer in a rat's trapezius area for 7 days. The result demonstrates that the implantation of the gelatin tube into the fascia layer indicated its safety and great biocompatibility, showed no indication of redness, swelling, or other inflammation symptoms of the local tissue, and was surrounded by well-developed connective tissues (**Figure 5A,B**).

FIGURE LEGENDS:

Figure 1: Scheme of the wet spinning setup. (**A**) Gelatin fibers. (**B**) Gelatin tube.

Figure 2: Bright view images of gelatin-based material obtained through the wet spinning method. (**A**) Gelatin fibers. (**B**) Gelatin tube. (**C**) Gelatin tube in PBS solution.

Figure 3: Morphology of the gelatin tube, observed by scanning electron microscopy. (**A**) A superficial view of the surface of the gelatin tube (the scale bar = 50 µm). (**B**) Inclined plane view (the scale bar = 200 µm). (**C**) Vertical view (the scale bar = 25 µm).

Figure 4: Observation of human adipose stem cell (hASC) growth on the gelatin tube. (**A**) Bright view image of gelatin tube-hASCs. (**B**) Under the fluorescence microscope, the staining showed the positive nestin staining marker along with a bunch of nuclei stained with Hoechst 33342. The gelatin tube provided an optimal environment for hASCs to adhere and grow inside the tube (the scale bar = 200 µm; blue color = nuclei; green color = nestin).

Figure 5: *In vivo* biocompatibility test of the gelatin tube. (**A**) The implantation of the gelatin tube into the fascia layer. (**B**) Observation of the local tissue environment implanted with the gelatin tube (red rectangle area) after 7 days. No redness, swelling, or other inflammation symptoms were observed, and the tube is surrounded by well-developed connective tissues 7 days after implantation. This is indicative of an excellent biocompatibility of the gelatin tube with the local tissue environment.

DISCUSSION:

We presented the development of gelatin-based biomaterials by using a simple wet spinning technique that can be applied in the study of natural polymers for tissue regeneration. This work demonstrated the possibility of gelatin fabrication as a great protein source without the addition of other sources, with the aim to optimize the properties of gelatin itself. The development of gelatin-based biomaterials was entirely carried out in room temperature (22 - 26 °C). A gentle solution preparation is a critical step within the protocol, as is maintaining an exact solution concentration and stirring the solution very smoothly to avoid any bubbles. The appearance of bubbles in the solution, especially when loaded into the syringe, will affect the form of gelatin in the coagulation medium.

Gelatin-based biomaterials were obtained through the phase transition of the gelatin solution in a suitable coagulation medium that causes the desolvation of the polymer molecules and precipitates the gelatin solution into a solid polymer phase[11]. By employing this basic concept of the wet spinning method, we utilized the shape of the gelatin into tube form to increase the functionality of its surface and to mimic the features of human tissues through the molding process. In this case, the adequate times to load, hold, and repeat the steps are necessary to build up an exact tube form. In this protocol, the homogeneity of the tube shape during the process cannot be controlled, considering the asynchronously of the transition phase from the bottom to the up part of the tube. Additionally, the preserved and sterilization methods for this material are limited because of its protein content.

Examined by *in vitro* and *in vivo* tests, the current results demonstrated that the gelatin tubes created with this protocol exhibit an excellent biocompatibility and have great potential applicability in tissue engineering. The inner diameter of the gelatin tubes obtained in this study was more than 200 µm (**Figure 3B**), which allows the cells to pass through and easily adhere to the tubes' inner surface[16]. In addition, the thickness and mildly asperous surface of the tube provides a proper template for cell attachment (**Figure 3A,C**). Consistently, the immunocytochemistry results showed that the cells penetrated and adhered to the gelatin tube—particularly the neural stem cells, as observed through the positive staining of the neural

cells with the marker nestin (**Figure 4B**). As the nestin marker was found in the area of the growth cones during the stages of axon extension, the positive staining of nestin is indicative of the cone growth[17]. Ultimately, this positive gelatin-hASCs combination can be implemented in the treatment of nervous system regeneration. The spinal cord is made up of groups of neuron axons and bundles of nerve fibers along the spinal vertebrae. Thus, the gelatin tube may not only provide appropriate filling gap material but will also guide nerve fiber growth and regeneration through the tube. Moreover, the great biocompatibility observed by the implantation of a gelatin tube into the fascia layer did not demonstrate any harmful and inflammation effect on the local tissues, and the tube was surrounded by well-developed connective tissues (**Figure 5A,B**).

In conclusion, we successfully developed a simple and useful protocol for the construction of gelatin-based biomaterials with excellent biocompatibility and cell environment that can be used in prospect tissue engineering applications, such as in nervous system regeneration, blood vessel replacement, urinary tract reconstruction, and in the rebuilding of organ parts. This protocol can be implemented by tissue engineers who are currently developing correlative natural polymers without any synthetic polymer addition. The protocol can be easily expanded upon and can be used to customize the design of specific biomaterials. In the future, this protocol can be adapted to produce protein-based biomaterials on a mass scale, thus helping to realize tissue regeneration.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

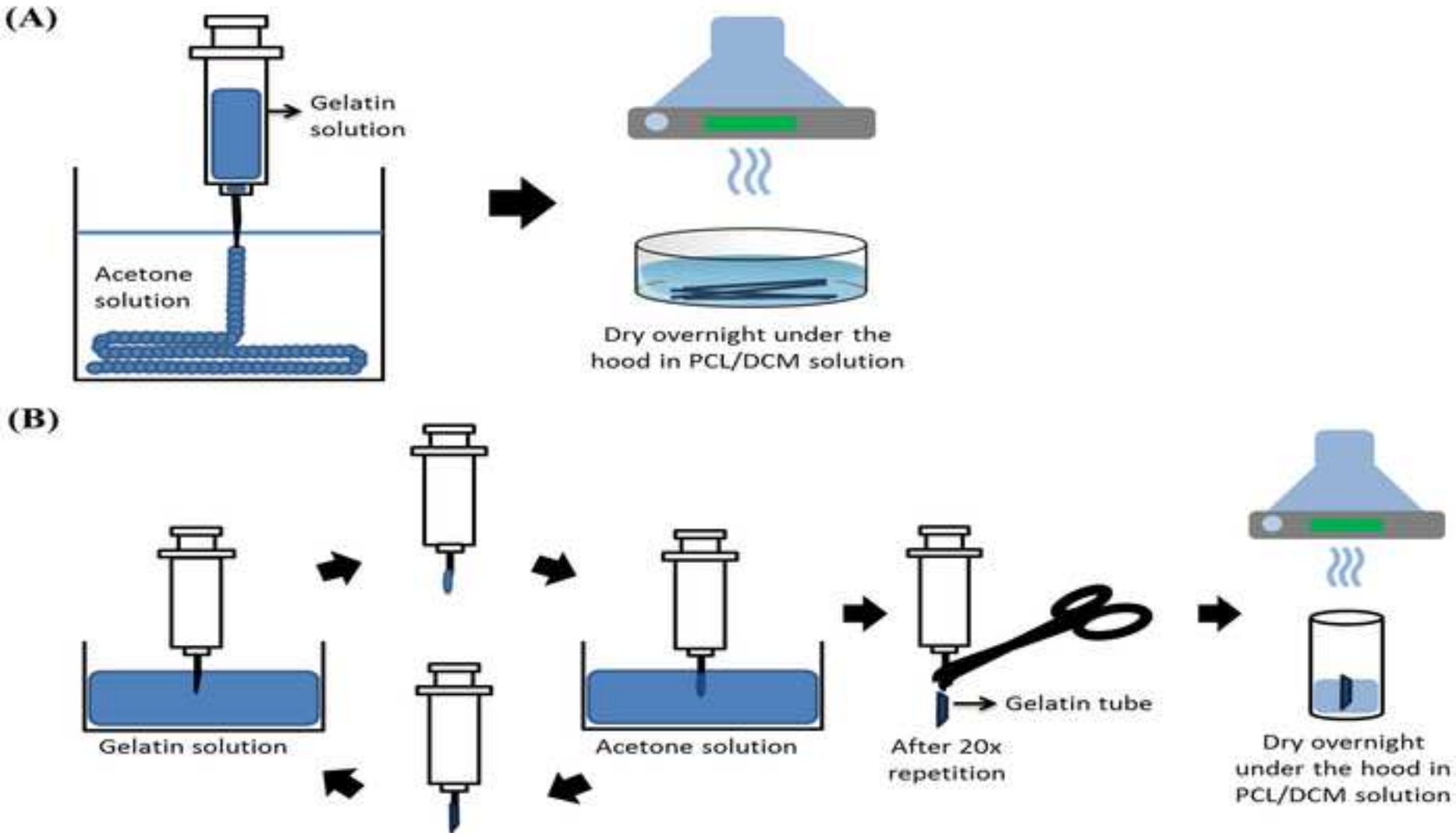

Figure 2

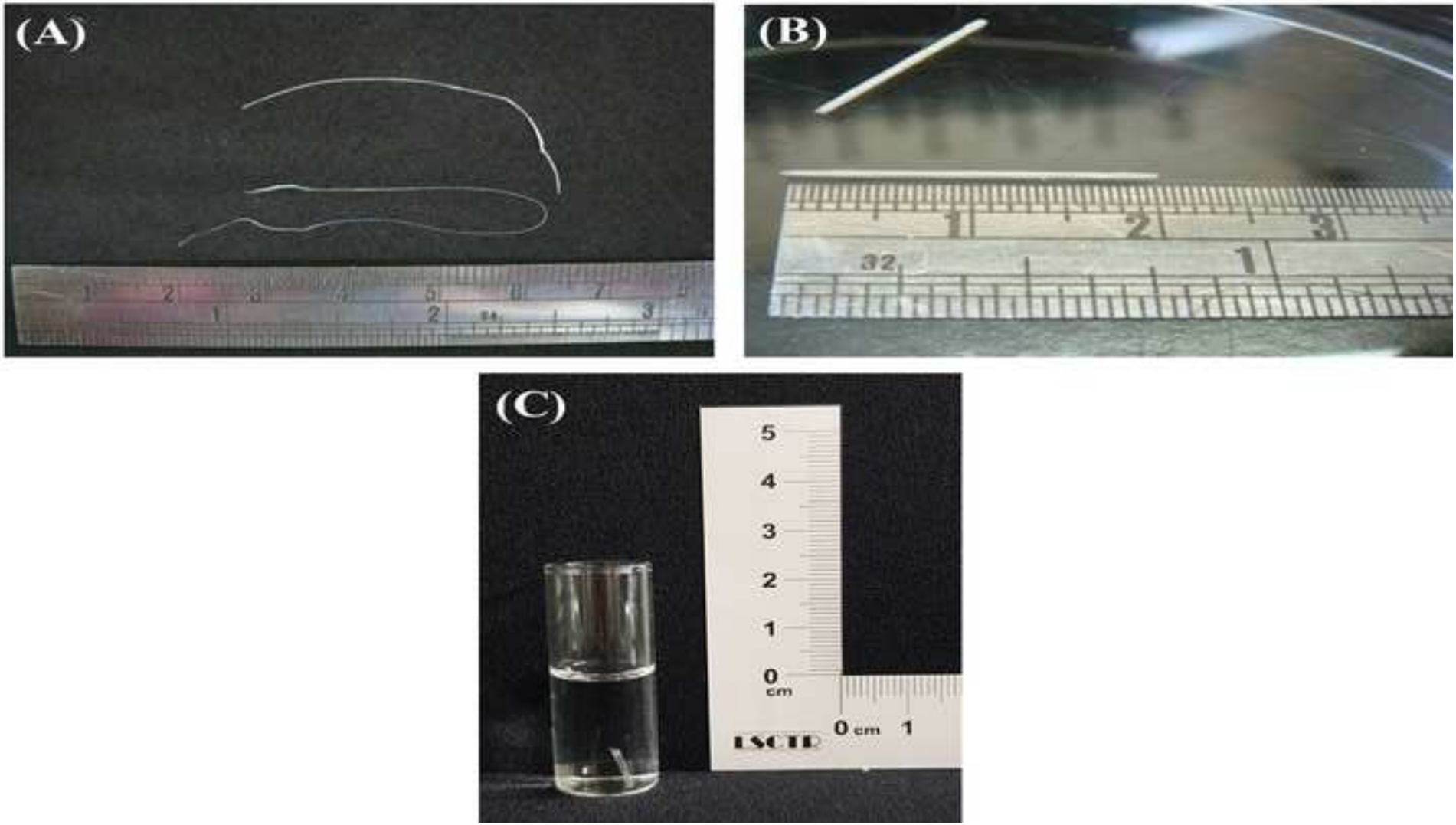

Figure 3

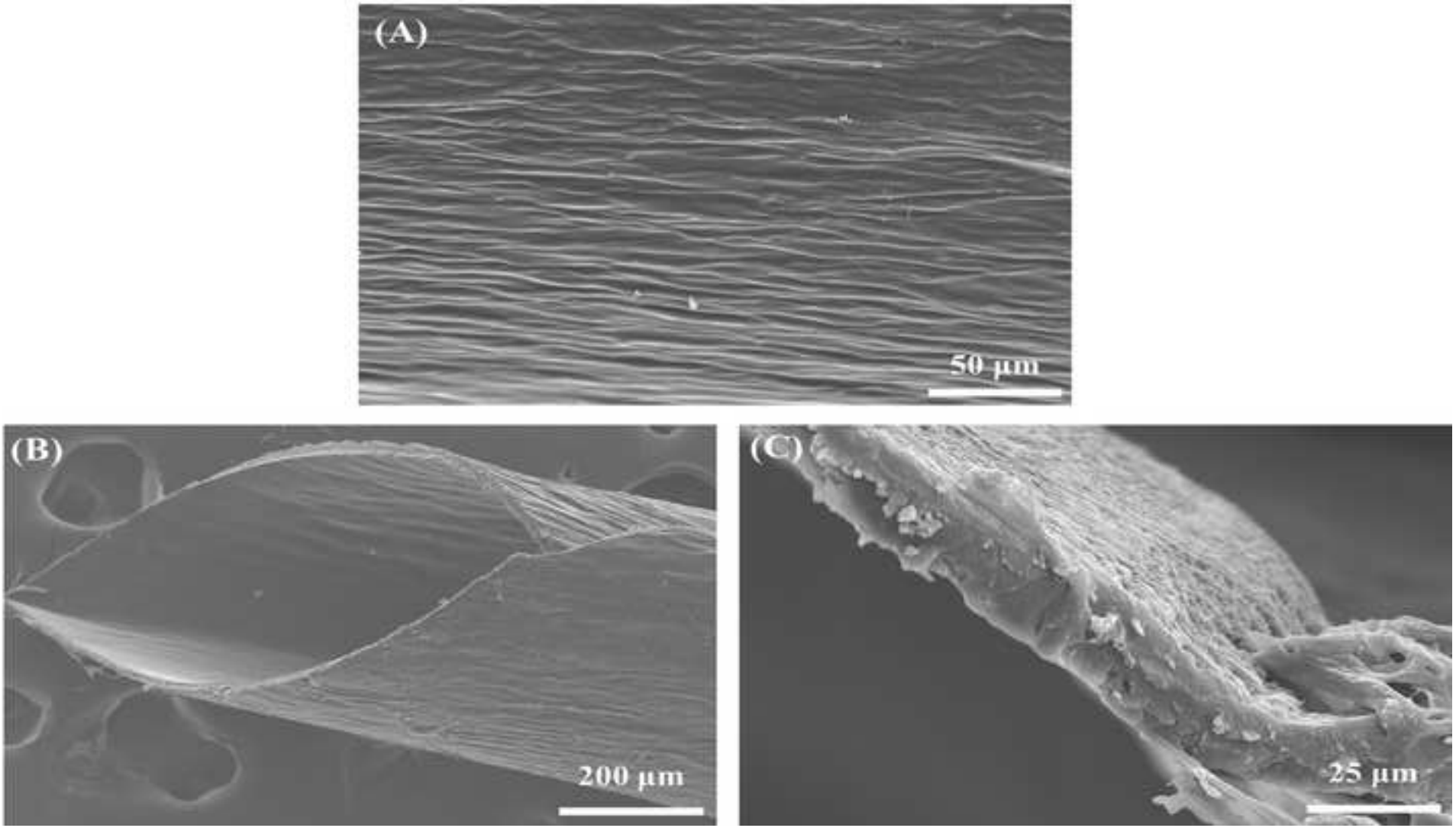

Figure 4

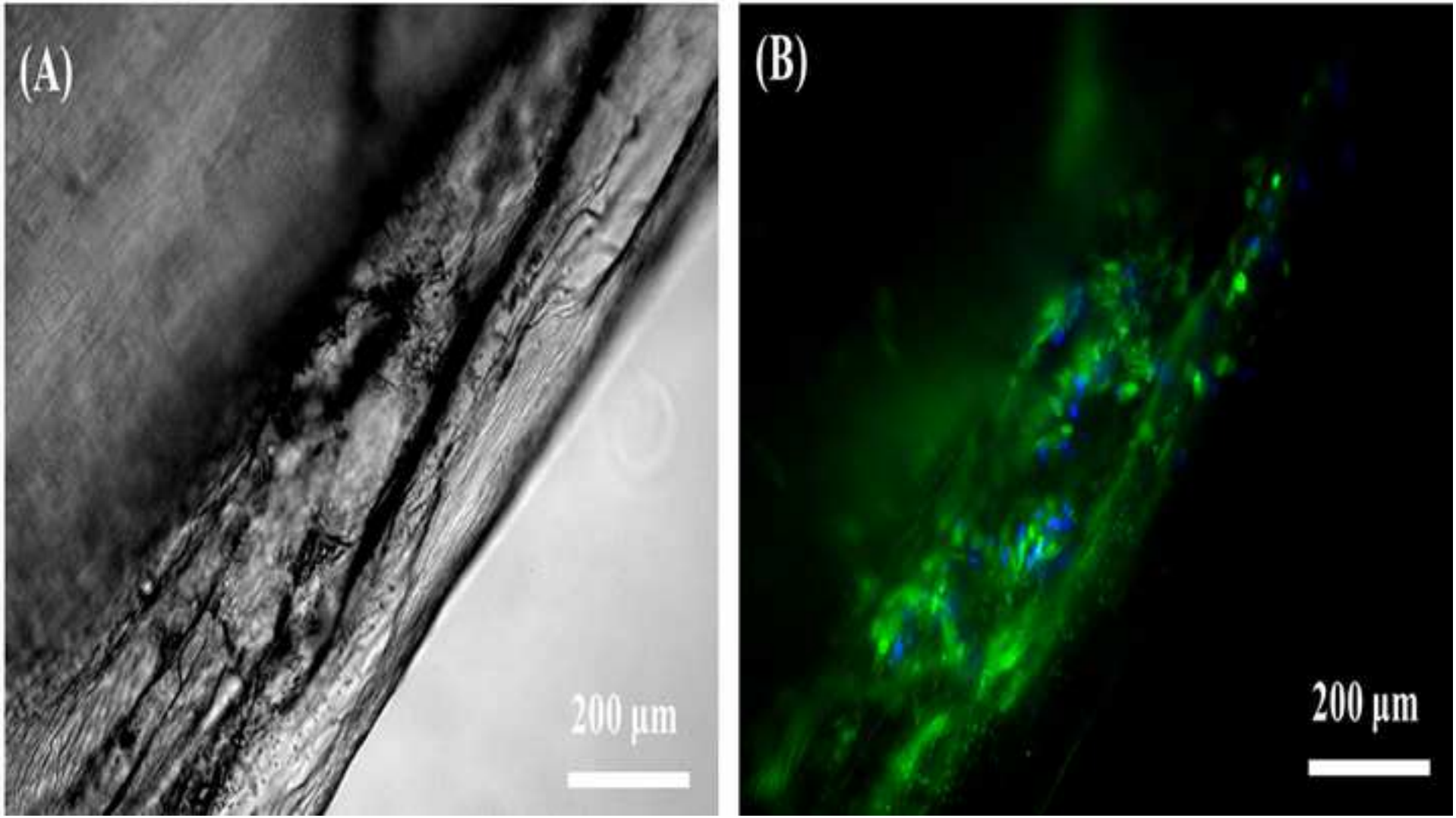

Figure 5

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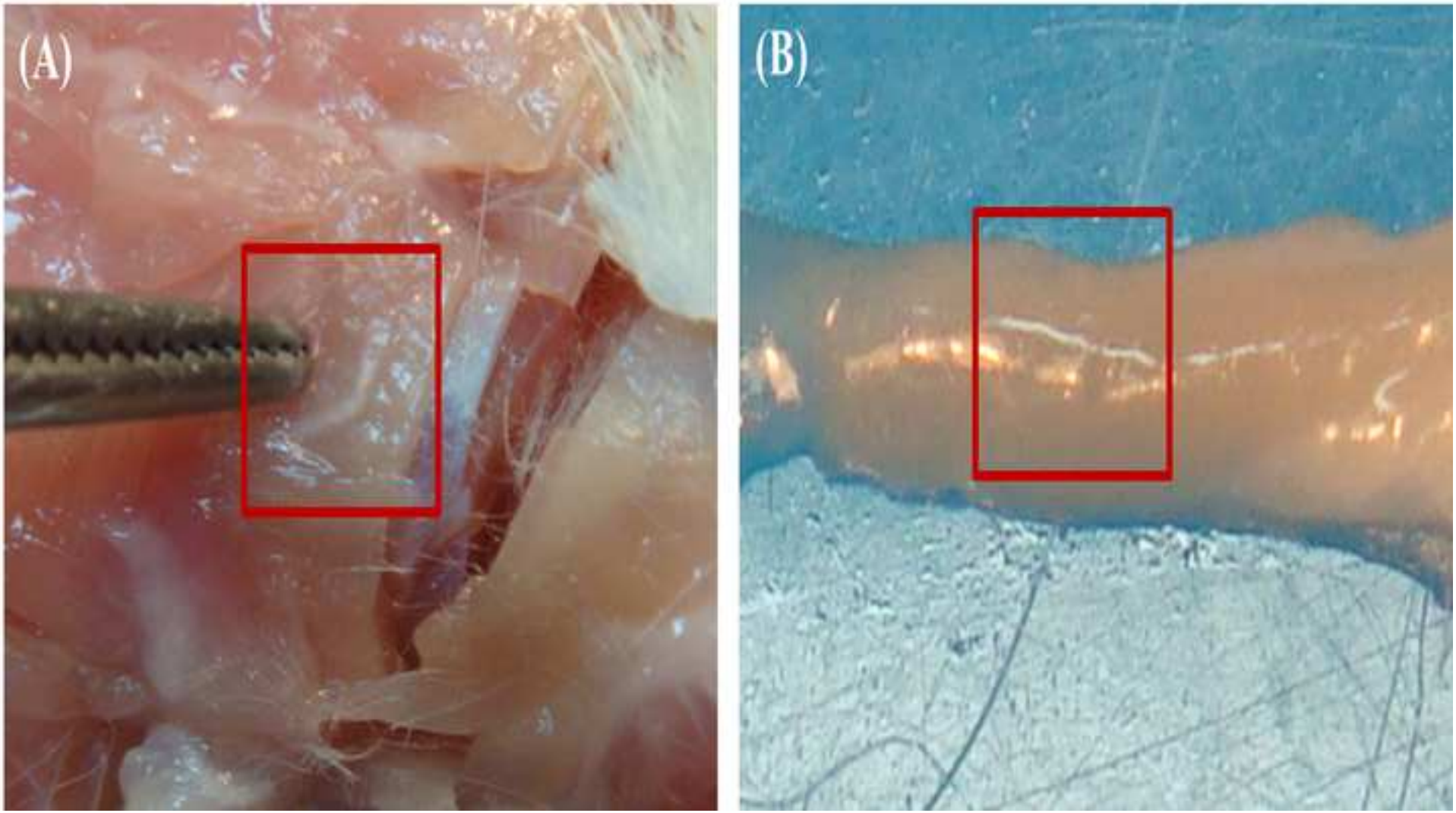

Item	Company	Catalog Number	Comments
Solution preparation:			
Gelatin type B (porcine)	Ferak	Art. -Nr. 10733	500 g vial
Wet spinning process:			
Peristaltic pump	Gilson	Model M312	Minipuls*3
Plastic tube connector	World Precision Instruments	14011	1 box
Syringe	Sterican	5A06258541	26Gx1/2"(0.45 x 12mm)
Acetone	Ferak	Art. -Nr. 00010	2.5 L vial
Polycaprolactone CAPA 6500	Perstorp	24980-41-4	-
Dichloromethane	Scharlau	CL03421000	1 L vial
Hemostat	Shinetec instruments	ST-B021	-
Peripheral venous catheter (Introcan Certo)	B. Braun	1B03258241	24Gx3/4"(0.7 x 19mm)
Morphology of the gelatin tube:			
Ion sputter coater machine	Hitachi	e1010	-
Scanning electron microscopy	Hitachi	S-3000N	-
Cultivation of cells on tube:			
Trypsin-EDTA	Gibco	488625	100 mL vial
Fetal bovine serum	Gibco	923119	500 mL vial
Dulbecco's modified Eagle's medium	Gibco	31600-034	Powder
T25 culture flask	TPP	90025	VENT type
6-well plate	Falcon	1209938	-
Immunocytochemistry:			
Phospate-buffered saline	Gibco	654471	500 mL vial
Acetic acid glacial	Ferak	Art. -Nr. 00697	500 mL vial
NP-40 surfactant (Tergitol solution)	Sigma	056K0151	500 mL vial
Normal goat serum	Vector Laboratories	S-1000-20	20 mL vial, concentrate
Nestin (primary antibody)	Santa Cruz Biotechnology	SC-23927	-
Donkey anti-mouse-fluorescein isothiocyanate (secondary antibody)	Santa Cruz Biotechnology	SC-2099	-
Hoechst 33342	Anaspec	AS-83218	5 mL vial
***In vivo* biocompatibility test:**			
Tiletamine+zolazepam	Virbac	BC91	5 mL vial
Xylazine	Bayer korea	KR03227	10 mL vial
Ketoprofen	Astar	1406232	2 mL vial
Povidone-iodine solution	Everstar	HA161202	4 L barrel

Cefazolin	China Chemical & Pharmaceutical	18P909	1 g vial
Scalpel Blade	Shinetec instruments	ST-B021	-

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Title of Article: Wet Spinning in Gelatin for Tissue Regeneration

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CORRESPONDING AUTHOR:

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Institution:	Tri-Service General Hospital, National Defense Medical Center
Article Title:	Wet Spinning in Gelatin for Tissue Regeneration
Signature:	Sheng-Tang Wu
Date:	10-08-2018

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2018.10.24

Dear Editors,

We thank the reviewers for their generous suggestions regarding our manuscript JoVE58932-Wet Spinning of Gelatin for Tissue Regeneration and we have modified the manuscript to address their concerns. In this revision, we also want to declare that the title of the manuscript was revised into "**Wet Spinning-based Molding Process of Gelatin for Tissue Regeneration**".

We hope that the revised manuscript may suitable for the publication in JoVE. We are looking forward to hear from you.

Thank you in advance.

Sincerely,

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Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   Answer: Noted.

2. Please note that numbering of institutional affiliation should follow the order of authors. First author gets 1, next author with different affiliation gets 2, etc., following from first to last.
   Answer: Noted.

3. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
   Answer: Already added. Page 5 line 187-188 and page 7 line 279-281.

4. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:
   Answer: Revised, and the numbering of protocol steps was changed due to the revision.

5. 2.1.1: A scheme showing the spinning setup would be helpful.
   Answer: Already added.

6. 2.1.3: What volume of acetone solution is needed? What container is used?
   Answer: Revised. Beaker glass containing 100 mL of 99.5% acetone solution.

7. 2.1.4: What is the injected solution?
   Answer: Revised. Gelatin solution.

8. 2.1.5: What fibers? This step is unclear.
   Answer: Revised. Gelatin fibers.

9. 3.1: Please mention how to coat the surface with gold. What is the unit for 60? How to prepare the sample for SEM?
   Answer: Revised. Coating with gold for 60 seconds by using ion sputter coater

machine.

10. Lines 151-152: Please move the ethics statement before your numbered protocol steps.
    Answer: Revised.

11. 4.1: Please specify the source/type of fat tissues. What container is used? What volume of the transfer solution is needed?
    Answer: Revised. The source of fat tissue is oral adipose tissue, use petri disk containing a 10 mL of transfer solution.

12. 4.2: What is used to cut?
    Answer: Revised. Scalpel blade.

13. 4.4: What container is used? Please specify throughout.
    Answer: Revised. 15 mL plastic tube.

14. 4.5: What is the temperature? What container is used?
    Answer: Revised. 15 mL plastic tube, stand for 1 day at 37℃ in humidified atmosphere containing 95% air and 5% CO_2.

15. 4.6: Please provide centrifugation parameters and composition of stem cell medium.
    Answer: Revised. Centrifuge the sediment at 500xg for 5 min, transfer it into T25 culture flask containing stem cell medium (consisting DMEM, 5% of FBS, N-acetyl-L-cysteine, ascorbic acid-2-phosphate, 1% antibiotic/antimyotic, and insulin).

16. 4.7, 5.2.2: What volume of trypsin-EDTA is added?
    Answer: Revised. 1 mL.

17. 4.8, 5.2.3: What volume of FBS is added?
    Answer: Revised. 1 mL.

18. 4.9, 5.2.4: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm). Please specify the volume of stem cell medium.
    Answer: Revised.

19. 5.1: Please specify which tube is used here.
    Answer: Revised. Gelatin tube.

20. 5.3, 6.4, 6.11: What is the incubation temperature? Please specify throughout.
    Answer: Already specified.

21. 6.11: What volume of Hoechst 33342 is added?
    Answer: Revised. 1 mL.

22. 7.1.1: Please specify the age, gender and type of rat. Please mention how proper anesthetization is confirmed.
    Answer: Revised.

23. 7.3.1: Please specify the surgical instruments used. How large is the incision?
    Answer: Revised. 2 cm of wound.

24. 7.3.2: What material is placed?
    Answer: Revised. Gelatin tube.

25. 7.5.1: What is the incubation temperature? What is the concentration of CO_2?
    Answer: Revised.

26. 7.5.2: How to remove the implanted tissue? What tool is used?
    Answer: Revised.

27. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
    Answer: Noted.

28. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
    Answer: Noted.

29. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Answer: Noted.

30. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
    Answer: Noted.

31. Figure 1: Please indicate the magnification used in panel C.
    Answer: Revised the figures in this manuscript. The figure 1C become the figure 2C.

32. Figure 3: Please indicate what the blue and green colors stand for in the figure legend.
    Answer: Revised.

33. Figure 4: Please explain what the red rectangle stands for.
    Answer: Revised.

34. Please provide figures with higher resolution.
    Answer: Noted.

35. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Answer: Revised.

Reviewer #1:

1. The title is incorrect - as the authors are not 'wet spinning in gelatin...' or is 'of' gelatin, not 'in'. Seems to be confusion in the script as to how fibres and tubes were spun, more specifically the link between them; was it that the fibres were made and then a tube dipped into a fibre solution or that fibres is one thing and tubes are another?
   Answer: The title of this manuscript was currently revised. The fabrication steps of gelatin fibers and gelatin tube are two different things. The gelatin fibers were made by wet spinning and then by employing the basic concept of the wet spinning method, we developed the gelatin tube with another protocol (molding process), which utilize the shape of the material as desired to increase the functionality of its surface and to mimic the features of human tissues. The confusion part of the manuscript regarding to this issue was already revised.

2. The term 'pure gelatin' should be changed to 'gelatin' as there are always impurities and nearly always endotoxins contained in gelatin solutions.
   Answer: Revised.

3. If tubes are the desired form then why were tubes not directly extruded? Does the rolling up and the fact that gelatin can be remodeled quickly in vivo affect tube patency? For cardiovascular applications burst strength will be low for rolled up tubes.
   Answer: The idea of tube fabrication is coming from the concept of wet spinning of gelatin fibers, thus we firstly explored about the wet spinning of gelatin fibers then the gelatin tube one. As tissue regeneration application, we chose the gelatin tube form in the case of its functionality surface and to mimic the features of human tissues. However, intrinsically, we also can develop any shape of material from gelatin fibers, *e.g.* gelatin conduit that rolled-up from several gelatin fibers, for the other desired application.

4. Part of the last sentence '...and its implementation in the laboratories is expected to greatly benefit the enhancement of tissue regeneration' is conjecture and should be removed.
   Answer: Revised.

5. The first paragraph of the introduction is misleading, in fact autograft for nerve for some injuries is gold standard and does function. Revise.
   Answer: Revised.

6. The source of the gelatin needs defining - porcine? Bloom strength? Gelatin A or B? At least this should be put in supporting information.
   Answer: Gelatin type B derived from porcine. Already added in supporting information.

7. The 5% concentration is that w/v%?. Define.
   Answer: 5% weight per volume solution. Revised.

8. Gelatin is thermoreversible, dependant on concentration too - at what temperature was the gelatin spun at and indeed comment should be made about the temperature range/concentrations that are suitable for this spinning.
   Answer: The fabrication of gelatin-based material in this protocol was done in the room temperature (22-26°C). Already added this condition in the discussion part.

9. Section 2.1.3. 'proper tube connector....' Makes no sense. Revise.
   Answer: Revised.

10. The peristaltic pump rpm is given however better would be to declare the solution flux coming out of the needle e.g. ml/min or micro L/h whichever makes most sense.
    Answer: Understood. However, the machine didn't give an information about how much rpm thus how much the solution flux coming out. So, we just declared the rpm and the type of the machine, and the flow rate depends on the rpm we used.

11. Most importantly - when gelatin is in fibre form and immersed in water (solvent for gelatin) the fibres will often reorganise and form a film. Especially above their sol-gel transition temperature. Comment should be made on this. Ergo - how stable were the fibres and is there evidence that the fibres maintained their form, else they may just have conglutinated into a tube-like structure with little fibre-like morphology.
    Answer: Gelatin fibers and tube were stable in their shapes after spun or molded in the acetone solution and then we immersed it into polycaprolactone/dichloromethane (PCL/DCM) solution to maintain their shapes for the *in vitro* and *in vivo* examination. We have added these new steps in the protocol.

12. The link between fibre formation 2.1 and tube formation is unclear - it seems that

tubes are formed from gelatin solution and not fibres. Revise for clarity.

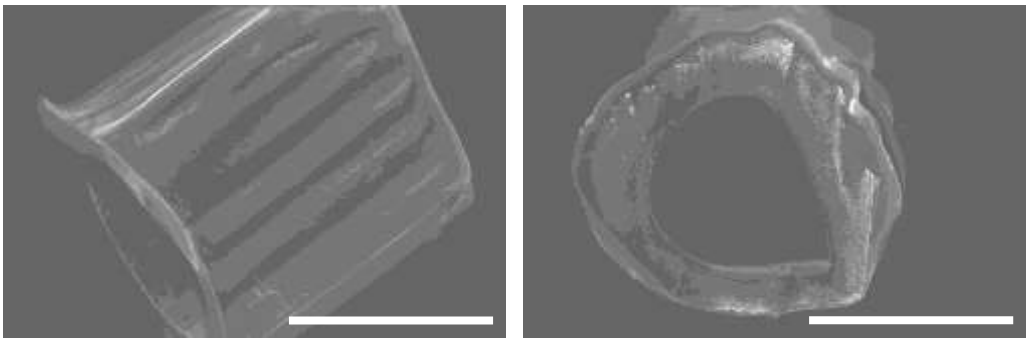
Answer: Revised. The fabrication of gelatin fibers and tube are two different things.

13. There are details missing in 3.1 - 60 what? Also dry fibers when immersed in water will likely re-swell so how relevant is the SEM? Would the tubes be dried before use?

    Answer: 60 seconds, already revised. The SEM test didn't require the water immersion of gelatin materials. Right after spun or molded, the gelatin materials were dried in the room temperature and followed the SEM test protocol.

14. SEM of the tube after immersion in PBS - similar to the time points of the cell culture study should be shown, to evidence how the fibres reorganise and if the tube collapses and fibres conglutinate forming a film/solid gel mass.

    Answer: The fabrication of gelatin fibers and tube are two different things. The idea of tube fabrication is coming from the concept of wet spinning of gelatin fibers, and then by employing this basic concept, we developed the gelatin tube with another protocol (molding process). Hereby we attached the SEM of the tube after immersion in PBS (scale bar: 500 μm), but we didn't perform SEM for the fibers one.



15. The discussion section: '...including ant semi-crystalline or amorphous state of collagen' makes little sense - may be an English issue here; in any case there will indeed likely be helical structure formation. Revise.

    Answer: Already revised based on reference.

16. The endotoxin issues around gelatin are not mentioned. They should be as this is one disadvantage to using gelatin.

    Answer: Gelatin has been well-known and widely used in the food, pharmaceutical, and cosmetic industries due to their excellent biocompatibility

weak antigenicity, so we thought that the endotoxin issues around gelatin is the thing need to be focused to reduce, such as the usage of gelatin powder with high quality, but not the part of disadvantage.

17. The discussion section mentions work by Kulkarni et al. however there are numerous examples of biopolymers being formed into fibres using simple equipment. The statements in the discussion are inflated and inaccurate and should make mention of other biopolymers that are spun, such as cellulose (including Lyocel), cellulose nanocrystal and fibrils, - alginate (also commonly made as fibres), and there are quite a number of examples with gelatin and gelatin/alginate e.g. CY Yang 2009. https://doi.org/10.1080/10731190903041022 also see: ADVANCED FUNCTIONAL MATERIALS 2013 Volume: 23 Issue: 3 Pages: 346-358 DOI: 10.1002/adfm.201201212
    Answer: Revised.

18. Often gelatin should be cross-linked to maintain its form, yet no crosslinking has been performed here - therefore did the fibre structure reorganise to form a film/solid mass.
    Answer: The polycaprolactone/dichloromethane (PCL/DCM) solution post-treatment was done so gelatin-based materials made by this protocol can maintain their shapes without any re-organization. We have added these new steps in the protocol.

Reviewer #2:

Major Concerns:

Generally, the present research work is appropriate, innovative and could be acceptable. However, some modifications are necessary to be made which some of the most important issues are mentioned below and it would be acceptable and publishable after doing the modifications.

1. Page 2, Line 62: Title "1" should be put for introduction.
   Answer: Based on the journal's template, there's no numbering order for each section.

2. Page 2, Lines 85 and 86: This sentence is not correct in general, it should be corrected and a reference(s) should be added.
   Answer: Already revised and added reference.

3. Page 3, Paragraph 1, Lines 88, 89, 90 and …: In general, solution spinning processes like wet spinning are more difficult and more expensive in fiber production industry compared to melt spinning and if there is a possibility of melt spinning for a specific polymer, industrialists always prefer melt spinning for producing fibers. Therefore, the pointed matter from the previous page to here, is necessary to be corrected in terms of contents and according to the mentioned point.
   Answer: Understood and revised.

4. As it is mentioned in the article title, the present research is based on wet spinning, thus, some points in wet spinning logic and principles should be mentioned in introduction so that the readers of the article have an accurate understanding from this section. Since the respected authors are the researchers in medical and pharmaceutical field, they could use the following papers for better guidance:

1. "Exploring the effects of non-solvent concentration, jet-stretching and hot-drawing on microstructure formation of poly (acrylonitrile) fibers during wet-spinning." Journal of Polymer Research 18, no. 6 (2011): 1343-1351.

2. "Simultaneous effects of polymer concentration, jet-stretching, and hot-drawing on microstructural development of wet-spun poly (acrylonitrile) fibers." Polymer Bulletin 66, no. 9 (2011): 1267-1280.

3. "Designing index of void structure and tensile modulus in wet-spun poly (acrylonitrile) proto-fibres. Part II: synergistic effect of dope non-solvent concentration and jet draw ratio." Iranian Polymer Journal 17, no. 3 (2008): 227-235.

4. "The synergistic effect of dope concentration and jet drawing on structure development of wet-spun poly (acrylonitrile)." e-Polymers 8, no. 1 (2008).
5. Designing index of void structure and tensile properties in wet - spun polyacrylonitrile (PAN) fiber. I. Effect of dope polymer or nonsolvent concentration." Journal of applied polymer science 109 (6), 3461-3469 (2008).

Answer: Revised based on the suggestion.

5. Page 3, Line 104: It is better to use "Experimental" instead of "Protocol".
6. Page 3, Line 104: 2. Experimental.
   Answer: The use of “Protocol” is based on the journal’s template.

7. Page 3: Specifications of the used Instruments for samples characterization such as the manufacturer company, the device model, etc. for all devices like SEM are necessary to be mentioned precisely in Experimental section.
   Answer: Revised.

8. Page 3:
   2.1. Solution preparation, 2.2. Wet spinning, 2.2.1. Fiber formation, 2.2.2. Tube formation …
   Answer: Revised.

9. Page 3, Line 115: The schematic shape of the spinning machine with the mentioned specifications is necessary to be added to this section.
   Answer: Revised by adding the scheme of wet spinning setup.

10. Page 7, Line 271: What is the purpose of showing A, B and C in Figure 1? While A, B and C are not referred in the Results section.
    Answer: Revised.

11. Page 7, Figure 2: The SEM images do not have any scale in order to investigate the accuracy of the mentioned claims in Figure 2.
    Answer: Revised the SEM images.

12. Page 6, Line 289: The place of Figure caption is not here, the end of the article after the references section is appropriate.
    Answer: Revised.

13. Page 7, Results section: Little and limited information is available, however further discussion is possible.
    Answer: Revised.

14. Page 8, Lines 310-333: This is a part of the introduction and should be transferred to introduction section.
    Answer: Revised based on suggestion.

15. Page 8, Lines 335-351: Regarding to the figures and the results contents, more comprehensive discussion could be made here.
    Answer: Revised.

Editorial comments:

1. Please address reviewer 2's comment below and incorporate relevant references.
   As it is mentioned in the article title, the present research is based on wet spinning, thus, some points in wet spinning logic and principles should be mentioned in introduction so that the readers of the article have an accurate understanding from this section. Since the respected authors are the researchers in medical and pharmaceutical field, they could use the following papers for better guidance:
   1. "Exploring the effects of non-solvent concentration, jet-stretching and hot-drawing on microstructure formation of poly (acrylonitrile) fibers during wet-spinning." Journal of Polymer Research 18, no. 6 (2011): 1343-1351.
   2. "Simultaneous effects of polymer concentration, jet-stretching, and hot-drawing on microstructural development of wet-spun poly (acrylonitrile) fibers." Polymer Bulletin 66, no. 9 (2011): 1267-1280.
   3. "Designing index of void structure and tensile modulus in wet-spun poly (acrylonitrile) proto-fibres. Part II: synergistic effect of dope non-solvent concentration and jet draw ratio." Iranian Polymer Journal 17, no. 3 (2008): 227-235.
   4. "The synergistic effect of dope concentration and jet drawing on structure development of wet-spun poly (acrylonitrile)." e-Polymers 8, no. 1 (2008).
   5. Designing index of void structure and tensile properties in wet - spun polyacrylonitrile (PAN) fiber. I. Effect of dope polymer or nonsolvent concentration." Journal of applied polymer science 109 (6), 3461-3469 (2008).

   Answer: We already addressed this concern with our own relevant reference, mentioned in the line 95-99.

2. Please include the drying step and PCL/DCM step in the scheme.
   Answer: Already added this step in the scheme.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Answer: Already revised.

4. As reviewer 1 has pointed out, it would be to declare the solution flux coming out of the needle e.g. ml/min or micro L/h whichever makes most sense. This can be easily measured, correct?
   Answer: Already added the flux flow rate in the protocol (step 1.2.1.3).

5. Please include the drying step and PCL/DCM step.
   Answer: Already added this step in the scheme.

6. Is the catheter taken out from the acetone or gelatin solution before drying? Please specify. Please indicate the drying step in scheme 1B.
   Answer: Already specified the step.

7. This does not make sense. Why gelatin fibers? Is it the gelatin tube from step 1.2.2.6?
   Answer: Mistaken writing here, already revised.

8. Is the tube taken out from the PCL/DCM solution first before drying? Please specify.
   Answer: Already specified the step.

9. Please remove commercial language throughout the manuscript.
   Answer: Revised.

10. In the rebuttal letter, the authors mentioned that "Right after spun or molded, the gelatin materials were dried in the room temperature and followed the SEM test protocol." Please add relevant details in this step.
    Answer: As we mentioned in the 2.1. step "Mount the piece of dried gelatin tube on a carbon stub.", so before SEM examination the tube should be in dry condition, which is already done in the 1.2.2.8. step.

11. Please define the size of small pieces.
    Answer: Less than 1 mm^2. Revised.

12. Please spell out DMEM and provide its composition.
    Answer: Revised.

13. Is the DMEM medium discarded before transferring? Please specify.
    Answer: Already specified the step.

14. Is supernatant discarded? Please specify.
    Answer: Already specified the step.

15. Please describe any limitations of the technique.
    Answer: Already mentioned the limitations, in the line 393-396.

16. Please address reviewer 1's comment below and incorporate the relevant references.
    The discussion section mentions work by Kulkarni et al. however there are numerous examples of biopolymers being formed into fibres using simple equipment. The statements in the discussion are inflated and inaccurate and should make mention of other biopolymers that are spun, such as cellulose (including Lyocel), cellulose nanocrystal and fibrils, - alginate (also commonly made as fibres), and there are quite a number of examples with gelatin and gelatin/alginate e.g. CY Yang 2009. https://doi.org/10.1080/10731190903041022 also see: ADVANCED FUNCTIONAL MATERIALS 2013 Volume: 23 Issue: 3 Pages: 346-358 DOI: 10.1002/adfm.201201212
    Answer: We no longer cited this reference in the discussion section so we didn't address this concern. However, it's right that numerous examples of biopolymers being formed into fibres using simple equipment, including CY Yang's work, but their works combined the gelatin with another source to build up the material, which is different with our present protocol.

17. Please provide some guidance on the appropriate solution concentration that is suitable for this spinning.
    Answer: Revised.