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TITLE:

Pancreatic Tissue-Derived Extracellular Matrix Bioink for Printing 3D Cell-Laden Pancreatic Tissue Constructs

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Bioink, 3D cell printing, pancreas, decellularized extracellular matrix, Islet transplantation, Type 1 diabetes

SUMMARY:

Decellularized extracellular matrix (dECM) can provide suitable microenvironmental cues to recapitulate the inherent functions of target tissues in an engineered construct. This article elucidates the protocols for the decellularization of pancreatic tissue, evaluation of pancreatic tissue-derived dECM bioink, and generation of 3D pancreatic tissue constructs using a bioprinting technique.

ABSTRACT:

The transplantation of pancreatic islets is a promising treatment for patients who suffer from type 1 diabetes accompanied by hypoglycemia and secondary complications. However, islet transplantation still has several limitations such as the low viability of transplanted islets due to poor islet engraftment and hostile environments. In addition, the insulin-producing cells differentiated from human pluripotent stem cells have limited ability to secrete sufficient hormones that can regulate the blood glucose level; therefore, improving the maturation by

culturing cells with proper microenvironmental cues is strongly required. In this article, we elucidate protocols for preparing a pancreatic tissue-derived decellularized extracellular matrix (pdECM) bioink to provide a beneficial microenvironment that can increase glucose sensitivity of pancreatic islets, followed by describing the processes for generating 3D pancreatic tissue constructs using a microextrusion-based bioprinting technique.

INTRODUCTION:

Recently, pancreatic islet transplantation has been considered a promising treatment for patients with type 1 diabetes. The relative safety and minimal invasiveness of the procedure are great advantages of this treatment[1]. However, it has several limitations such as the low success rate of isolating islets and the side effects of immunosuppressive drugs. Furthermore, the number of engrafted islets decreases steadily after transplantation due to the hostile environment[2]. Various biocompatible materials such as alginate, collagen, poly(lactic-co-glycolic acid) (PLGA) or polyethylene glycol (PEG) have been applied to pancreatic islet transplantation to overcome these difficulties.

3D cell printing technology is emerging in tissue engineering due to its great potential and high performance. Needless to say, bioinks are known as important components for providing a suitable microenvironment and enabling the improvement of cellular processes in printed tissue constructs. A substantial number of shear-thinning hydrogels such as fibrin, alginate, and collagen are widely used as bioinks. However, these materials show a lack of structural, chemical, biological, and mechanical complexity compared to the extracellular matrix (ECM) in native tissue[3]. Microenvironmental cues such as the interactions between islets and ECM are important signals for enhancing the function of islets. Decellularized ECM (dECM) can recreate the tissue-specific composition of various ECM components including collagen, glycosaminoglycans (GAGs), and glycoproteins. For example, primary islets that retain their peripheral ECMs (e.g., type I, III, IV, V, and VI collagen, laminin, and fibronectin) exhibit low apoptosis and better insulin sensitivity, thus indicating that tissue-specific cell-matrix interactions are important for enhancing their ability to function similarly to original tissue[4].

In this paper, we elucidate protocols for preparing pancreatic tissue-derived decellularized extracellular matrix (pdECM) bioink to provide beneficial microenvironmental cues for boosting the activity and functions of pancreatic islets, followed by the processes for generating 3D pancreatic tissue constructs using a microextrusion-based bioprinting technique (**Figure 1**).

PROTOCOL:

Porcine pancreatic tissues were collected from a local slaughterhouse. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Asan Medical Center, Seoul, Korea.

1. Tissue decellularization

1.1. Prepare the solutions for decellularization.

NOTE: 1x phosphate-buffered saline (PBS) used in all solution preparations is diluted by adding distilled water to 10x PBS.

1.1.1. For the 1% Triton-X 100 solution, dissolve 100 mL of 100% Triton-X 100 solution in 900 mL of 1x PBS using a magnetic stir bar with the stirring at 150 rpm for 6 h. Make 400 mL of 1% Triton-X 100 solution with 40 mL of 10% Triton-X 100 solution and 360 mL of 1x PBS prepared just before use.

NOTE: The 10% Triton-X 100 solution can be stored at room temperature until needed.

1.1.2. For the 0.1% peracetic acid solution, dilute 8.5 mL of 4.7% peracetic acid into 22.8 mL of 70% ethanol with 368.7 mL of distilled water just before use.

1.2. Remove the peripheral tissues of the pancreas and slice the tissue before decellularization.

1.2.1. Wash the resected porcine pancreas with running tap water and remove the peripheral tissues using sterilized scissors.

1.2.2. Transfer the pancreas into a plastic bag with forceps and freeze at -80 °C for 1 h to help cut the pancreas effectively for the next step.

1.2.3. Slice the frozen pancreas into 1 mm thick pieces using a grater.

1.2.4. Transfer 50 g of the sliced tissue into a 500 mL plastic container.

NOTE: A plastic container with a lid is recommended here to protect tissues from contamination and prevent solution evaporation.

1.3. Treatment with reagents.

NOTE: The entire decellularization process should be carried out at 4 °C on a digital orbital shaker at 150 rpm. In all decellularization steps, physical detachment using forceps is required to prevent the slices of pancreas from sticking together. Washing the container with distilled water is necessary to remove the residual reagents completely in the container.

1.3.1. Before any reagent treatment, wash 50 g of sliced pancreas with 300 mL of distilled water using a shaker.

1.3.2. Stir the tissue continuously at 150 rpm using a magnetic stirrer until the cloudy water disappears (after approximately 12 h). Replace the distilled water every 2 h.

NOTE: Changing the distilled water every hour is recommended for efficiency to remove the cloudy water more quickly.

1.3.3. Discard the water and treat the 50 g of tissues with 400 mL of 1% Triton-X 100 in 1x PBS solution for 84 h. Refresh the solution every 12 h.

NOTE: At this point, the amount of tissue will decrease because the cellular components start being removed.

1.3.4. Treat with 400 mL of isopropanol (IPA) for 2 h to remove the remaining fat from the pancreas.

NOTE: It is normal for the tissue to become tough due to the removal of fat in this process.

1.3.5. After 2 h, remove the IPA and wash the tissue with 400 mL of 1x PBS for 24 h. Refresh the 1x PBS every 12 h.

1.3.6. To sterilize the decellularized tissue, discard the previous solution and treat with 400 mL of 0.1% peracetic acid in 4% ethanol for 2 h.

1.3.7. To remove residual detergent, wash the tissue with 400 mL of 1x PBS for 6 h. Refresh the solution every 2 h.

1.3.8. Collect the decellularized tissues in a 50 mL conical tube with forceps.

1.3.9. Freeze the sample at -80 °C for 1 h. Cover the conical tube with a lint-free wipe instead of the lid and fix with a rubber band for efficient lyophilization.

1.3.10. Lyophilize the decellularized tissue at -50 °C for 4 d.

NOTE: For step 1.3, 1 g of non-decellularized tissue should also be freeze-dried under the same conditions.

2. **Assessment of decellularized tissues**

NOTE: To evaluate the residual amount of dsDNA, glycosaminoglycans (GAGs), and collagen in the decellularized tissue compared to native tissue, at least 1 g of each of the non-decellularized tissue (native tissue) and decellularized tissue are required for one batch of assessment. The amount of dsDNA, GAGs, and collagen can be calculated based on the dry weight of the tissue.

2.1. Prepare solutions for biochemical assays.

2.1.1. Prepare papain solution for sample digestion.

NOTE: The amount of buffer to be made can be adjusted according to the number of samples.

2.1.1.1. Dissolve 119 mg of 0.1 M sodium phosphate (monobasic), 18.6 mg of 0.5 mM Na2-EDTA, and 8.8 mg of 5 mM cysteine-HCl in 10 mL of autoclaved water.

2.1.1.2. Adjust the pH of the solution to 6.5 by adding 10 M NaOH solution.

2.1.1.3. Add 125 µL of 10 mg/mL papain stock solution to the above solution and vortex, allowing each element to mix evenly.

2.1.2. Prepare solutions for dimethyl-methylene blue (DMMB) assay.

2.1.2.1. To make DMMB dye, dissolve 8 mg of 1,9-dimethyl-methylene blue zinc chloride double salt, 1.52 g of glycine, and 1.185 g of NaCl in 500 mL of autoclaved water. Adjust the pH to 3 by adding 0.5 M HCl solution while measuring the change of pH using a bench-top pH meter. Then, filter this through a 500 mL bottle-top vacuum filter.

2.1.2.2. Make 15 µL of 10 mg/mL chondroitin sulfate A solution for standard.

2.1.3. Prepare solutions for hydroxyproline assay.

2.1.3.1. For the chloramine working solution, dissolve 2.4 g of sodium acetate, 1 g of citric acid, and 0.68 g of sodium hydroxide in 24 mL of distilled water and add 240 µL of glacial acetic acid, 10 µL of toluene, and 6 mL of IPA.

NOTE: Dissolve all powders in solution using a vortex mixer. Chloramine working solution can be stored at 4 °C for up to three months.

2.1.3.2. For chloramine T solution, dissolve 0.35 g of chloramine T in 20 mL of chloramine working solution and add 2.5 mL of IPA. Vortex to mix all components.

NOTE: Prepare immediately before use.

2.1.3.3. For P-DAB solution, put 3.75 g of P-DAB into 6.5 mL of perchloric acid and 15 mL of IPA; wrap it in aluminum foil.

NOTE: Prepare immediately before use.

2.2. Add 1 mL of papain solution to 10 mg of lyophilized tissue in a 1.5 mL microcentrifuge tube and vortex the tube to better digest the sample.

2.3. Place the 1.5 mL microcentrifuge tube in a rubber rack and digest the samples in a 500 mL beaker that contains 300 mL of water at 60 °C for 16 h.

2.4. Centrifuge at 9,500 x *g* for 20 min, collect the supernatant, and transfer it into a new tube.

2.5. Quantify the residual DNA and major proteins in the decellularized tissue.

2.5.1. Load 1 µL of digested sample into the spectrometer and measure the amount of dsDNA according to the manufacturer's instructions.

NOTE: The experimenter should tie their hair back and wear a mask to avoid contaminating the sample.

2.6. Perform a DMMB assay to quantify the amount of GAGs that remains in the decellularized tissue.

2.6.1. Mix 1 µL of chondroitin sulfate A solution and 499 µL of 1x PBS to make standards. Dilute the chondroitin sulfate A solution with distilled water at concentrations of 0, 4, 8, 12, 16, and 20 µg/mL.

2.6.2. Load triplicates of 50 µL of each concentration of the standard and digested samples into a 96-well plate.

2.6.3. Add 200 µL of the DMMB dye to each well using a multi-channel pipette.

2.6.4. Immediately read the absorbance at 525 nm on a microplate reader.

2.7. Perform a hydroxyproline assay to quantify the amount of collagen.

2.7.1. To conduct a hydroxyproline assay, incubate 250 µL of digested sample with equal volumes of HCl at 120 °C for 16 h.

2.7.2. Dry the residues at room temperature for 3 h to cool the samples and then re-dissolve the samples in 1 mL of 1x PBS.

2.7.3. Centrifuge at 2,400 x *g* for 10 min at 4 °C.

2.7.4. Prepare 100 µg/mL hydroxyproline solution as standard.

2.7.5. Dilute hydroxyproline solution with distilled water at a concentration of 0, 1, 2, 3, 4, 5, 6, 8, 10, 15, 20, 30 µg/mL for a standard solution.

2.7.6. Load triplicates of 50 µL of samples and standard solution into a 96-well plate.

2.7.7. Add 50 µL of Chloramine T solution then incubate for 20 min at room temperature.

2.7.8. Add 50 µL of p-DAB solution and incubate for 30 min at 60 °C.

NOTE: Aliquot in a dark room. After the addition, wrap the plate with aluminum foil.

2.7.9. Incubate at room temperature for 30 min.

2.7.10. After cooling, measure the absorbance at 540 nm on a microplate reader.

3. Bioink preparation

NOTE: pdECM powder can be stored stably at -80 °C for at least one year. Before pH adjustment, the digested pdECM solution can be stored at -20 °C for one month. Prior to use, thaw the sample of frozen pdECM solution at 4 °C overnight. The pH-adjusted pdECM solution can be stored at 4 °C for up to one week. The digested pdECM solution can be stored at 4 °C for at least a few days but should not exceed 1 week.

3.1. Digest the freeze-dried pdECM with pepsin.

3.1.1. For effective digestion of bioink, pulverize the lyophilized pdECM with liquid nitrogen using a mortar and pestle.

3.1.2. Collect 200 mg of the pdECM powder in the 50 mL conical tube and add 20 mg of the pepsin and 8.4 mL of the 0.5 M acetic acid (final concentration is 2 w/v%).

3.1.3. Place the magnetic stir bar in the 50 mL conical tube and stir at 300 rpm for 96 h.

3.2. Adjust pH of digested pdECM solution.

NOTE: In order to avoid gelation before pH adjustment, this process should be conducted on ice.

3.2.1. Filter out the undigested particles in the pdECM solution using a 40 µm cell strainer using a positive displacement pipette on ice to obtain the optimal digestion of parts.

3.2.2. Add 1 mL of the 10x PBS and vortex before using NaOH.

3.2.3. Adjust the pH to 7 with 10 M NaOH checking the pH with pH indicator strips.

NOTE: Vortex each time NaOH is added so that the bioink is thoroughly mixed with the other reagents.

4. Rheological analysis

4.1. Experimental setup

4.1.1. Prepare the 1.5% (w/v) of pdECM bioink to assess the rheological properties.

4.1.2. Establish a 20 mm cone plate geometry (cone diameter of 20 mm with a 2° angle) in rate-controlled mode of a rheometer.

4.1.3. Create experimental sequences in the installed software (TRIOS) to measure the viscosity, gelation kinetics, and dynamic modulus of the pdECM bioink.

4.1.3.1. Viscosity: Place the pdECM bioink on the plate. Measure complex viscosity (Pa·s) of pdECM bioink under an increasing shear rate from 1 to 1,000 s^{-1} at a constant temperature of 15 °C.

4.1.3.2. Gelation kinetics: Place the pdECM bioink on the plate. Calculate the complex modulus (G*) by measuring the storage and loss modulus of pdECM bioink at 4–37 °C with an incremental increase rate of 5 °C/min (time-sweep mode).

4.1.3.3. Dynamic modulus: Place the pdECM bioink on the plate at 37 °C for 60 min prior to measurement. Measure the frequency-dependent storage modulus (G') and loss modulus (G'') of the pdECM bioink in the range of 0.1–100 rad/s at 2% strain.

5. 3D cell printing of pancreatic tissue constructs using islet

5.1. Preparation of isolated islets

5.1.1. Isolate primary islets from a rat according to the protocols described in a previous work[5].

5.1.2. To separate debris and dead cells from the isolated islet, pass the cell suspension through a 70 μm cell strainer. Islets with a diameter smaller than 70 μm are considered dead or abnormal.

5.1.3. Suspend the isolated islets in RPMI-1640 medium and place them on the petri dish. Remove islets larger than 300 μm in diameter by using a P200 volume pipette under the microscope (4x objective lens) in the biosafety cabinet.

5.2. Islet encapsulation into pdECM bioink

5.2.1. Prepare pH adjusted pdECM bioink and isolated islet.

NOTE: To avoid gelation before pH adjustment, this process should be performed on ice.

5.2.2. Gently mix the the pdECM bioink and the media suspended with islets (ratio 3:1) using a positive displacement pipette until uniformly mixed.

NOTE: The final concentration of the pdECM bioink is 1.5% and the cell density in the pdECM bioink is 3,000 IEQ/mL.

5.3. 3D cell printing of pancreatic tissue constructs

5.3.1. Prepare a sterilized syringe and 22 G nozzle.

NOTE: This gauge was selected for printing islets with a diameter of 100–250 µm.

5.3.2. Load islet-laden pdECM bioink into the syringe.

5.3.3. Print the bioink with the optimized printing condition (feed rate: 150 mm/min; pneumatic pressure: 15 kPa) at 18 °C in the shape of a lattice.

5.3.4. To crosslink the bioink, place the printed construct in the incubator for 30 min.

5.3.5. Immerse the printed construct into the islet culture media which is RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin.

6. 3D cell printing of pancreatic construct with patterned structure

6.1. Preparation of two types of the bioink

6.1.1.To validate the printing versatility using multiple bioinks, prepare two sets of pdECM bioinks and stain them by adding 0.4% Trypan Blue and Rose Bengal solution into each pdECM bioink at a ratio of 1:20, respectively.

NOTE: To avoid gelation before pH adjustment, this process should be conducted on ice.

6.1.2.Gently mix the the pdECM bioink and the media suspended with islets (ratio 3:1) using a positive displacement pipette until uniformly mixed.

NOTE: The final concentration of the pdECM bioink is 1.5% and the cell density in the pdECM bioink is 3,000 IEQ/mL.

6.2. 3D cell printing of multimaterial-based pancreatic tissue constructs

6.2.1. Prepare sterilized syringes and a 25 G nozzle.

6.2.2. Load each bioink (blue and red) into two different syringes, respectively.

6.2.3. Print the bioink with optimized printing condition (feed rate: 150 mm/min; pneumatic pressure: 15 kPa) at 18 °C in a shape of a lattice with alternating lines of blue and red.

6.2.4. To crosslink the bioink, place the printed construct in the incubator for 30 min.

6.2.5. Immerse the printed construct into islet culture media which is RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin.

REPRESENTATIVE RESULTS:

Decellularization of pancreatic tissues

We developed the process for preparing pdECM bioink to provide pancreatic tissue-specific microenvironments for enhancing functionality of islets in a 3D bioprinted tissue construct (**Figure 2A**). After the decellularization process, 97.3% of dsDNA was removed and representative ECM components such as collagen and GAGs remained at 1278.1% and 96.9% compared to that of the native pancreatic tissue, respectively (**Figure 2B**).

Bioink preparation

To apply the pdECM in the printing process, the pdECM powder was solubilized in weak acid with pepsin and neutralized using 10 M NaOH solution. The digested pdECM solution could then be diluted through mixing with a cell culture medium or 1x PBS. In this study, we prepared pdECM bioink at a final concentration of 1.5% for further study. The pdECM bioink maintained a solution phase when it was placed under room temperature and instantly converted into a gel phase after incubation at 37 °C for 30 min. To investigate the effect of the pdECM bioink on islets, isolated islets were encapsulated in the pdECM, alginate and collagen bioinks at a concentration of 1.5%. The result of the glucose-stimulated insulin secretion test showed islets in the pdECM bioink represented the highest index (approximately 3.174) among the experimental groups, indicating higher functionality over the widely applied hydrogels for islet encapsulation[5].

Rheological analysis

Viscosity is one of the critical characteristics when considering a printable biomaterial. We measured viscosity of the pdECM bioink at a frequency ranging from 1 to 1,000 Hz at 15 °C for printing various dECM bioinks[6-8]. The pdECM bioink showed shear-thinning behavior and the value was approximately 10 Pa·s at the shear rate of 1/s, indicating the pdECM bioink had appropriate rheological characteristics for extrusion through a nozzle (**Figure 3A**). The gelation kinetics at a temperature ranging from 4 to 37 °C indicated the gelation behavior of the pdECM bioink at physiologically relevant temperatures. The complex modulus started to increase when the temperature reached 15 °C, and it increased rapidly when the temperature was maintained at 37 °C, indicating the sol-gel transition of the pdECM bioink (**Figure 3B**). The dynamic G' and G" of pdECM bioink were investigated at physiologically relevant temperatures to ensure its stability after the printing process, which resulted in having a stable modulus under the frequency sweep condition (**Figure 3C**).

3D cell printing

3D cell-laden pancreatic tissue constructs were fabricated by using a microextrusion-based printing process. To build a construct containing at least 3,000 Islet equivalents (IEQ), that corresponds to the tissue volume of a perfectly spherical islet with a diameter of 150 µm[9], we designed the construct with a dimension of 10 mm x 10 mm x 3 mm (**Figure 4A**). The process

parameters and conditions for printing pancreatic islets were selected to encapsulate islets, which are large cellular clusters in sizes ranging 100–250 µm in diameter (**Figure 4B**). Using a multi-head printing system, various types of 3D constructs—such as the shape of the lattice having alternate lines of blue and red—were fabricated by using the developed pdECM (**Figure 4C**), indicating the versatility of pdECM for the purpose of 3D bioprinting to harmonize two or more types of living cells in a tissue-like arrangement.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the development of decellularized pancreatic tissue, evaluation of pdECM bioink and fabrication of 3D pancreatic tissue constructs.

Figure 2: **Representative images of the decellularization process and biochemical characterization of pdECM.** (**A**) Overview of the decellularization of porcine pancreatic tissue. (**B**) Results of biochemical assays of native tissue and pdECM. Error bars show standard deviation. Copyright (2019) The Royal Society of Chemistry[5].

Figure 3: Rheological analysis of pdECM bioink. (**A**) Viscosity of pdECM and collagen bioinks that exhibited shear thinning behavior. (**B**) Gelation kinetics of pdECM and collagen bioinks during temperature change. (**C**) The complex modulus of crosslinked pdECM and collagen bioinks. Copyright (2019) of The Royal Society of Chemistry[5].

Figure 4: 3D cell printing of cell-laden pdECM bioink for 3D pancreatic tissue constructs. (**A**) The dimensions of 3D pancreatic tissue constructs. (**B**) Pancreatic islet-laden and (**C**) multimaterial-based 3D pancreatic tissue constructs. Copyright (2019) of The Royal Society of Chemistry[5].

DISCUSSION:

This protocol described the development of pdECM bioinks and the fabrication of 3D pancreatic tissue constructs by using 3D cell printing techniques. To recapitulate the microenvironment of the target tissue in the 3D engineered tissue construct, the choice of bioink is critical. In a previous study, we validated that tissue-specific dECM bioinks are beneficial to promote stem cell differentiation and proliferation[10]. Compared to synthetic polymers, dECM can serve as a cell-favorable environment because of the tissue-specific composition and architecture[11]. Therefore, the decellularization process should be seriously considered for the high retention of major components in the dECM.

The selection of different detergents for decellularization of pancreatic tissue varies the residual ECM constituent[12]. In the process of decellularization, we noticed that the use of sodium dodecyl sulfate (SDS) can affect loss of the ECM proteins[13]. Thus, we modified our previous protocol by eliminating the step for the treatment of SDS solution, which is an ionic surfactant used in many cleaning and decellularization processes featuring relatively harsh characteristics compared to the others such as Trion-X 100, or 3-[(3-cholamidopropyl) dimethyl-lammonio]-1-propanesulfonate (CHAPS). In this protocol, we used 1% Triton-X 100 solution for 84 h instead of SDS solution, which was able to remove the cellular components effectively while preserving

GAGs and collagenous proteins. In addition, we noted that removal of residual lipids by treating with IPA is also a very crucial process for inducing the crosslinking of pdECM bioink and it can be understood in the same context as a previously published article[4]. Treatment with peracetic acid solution was also applied for the sterilization of decellularized tissue. In addition, removal of the remaining detergents and chemicals in the decellularized tissue is a crucial step to prevent the inflammatory host response. However, we did not discuss that issue in this protocol. Protocols that include a sanitization process at the end of decellularization will improve the biocompatibility of the decellularized material. Furthermore, standards for evaluation criteria should be considered to ensure that detergents and chemicals are completely removed.

The digestion of pdECM bioink with pepsin was performed to achieve the homogenous mixing of pdECM powder in the acidic solution by cleavage of the telopeptide region in the collagenous protein. In the pH adjustment process, keeping the pdECM bioink on ice is critical for the preservation of gelation. Afterward, we can produce physically crosslinkable pdECM pre-gel bioinks that can enter a gel state by incubating at 37 °C, which is one of the main advantages of dECM-based bioinks. Selection of the proper concentration of the pdECM bioink is also important[10]. An ideal bioink should protect cells from external damage that occurs during the printing process such as pneumatic pressure and temperature change. It is known that the applied shear force may cause damage to the cells and reduce the cell viability in the printed constructs[10]. Also, enhancing concentrations of bioink could induce cell death[5]. In contrast, low concentrations of bioink induces low viscosity which means poor printability and shape-fidelity during printing. It is necessary to check the viscosity of bioink and optimize its concentration.

Currently, researchers are actively studying the development of various types of tissue-derived bioinks for printing 3D tissue constructs[14-16]. The results of these studies indicate that the bioink could provide tissue-specific microenvironments for cells. These unique conditions can promote the differentiation or maturation of stem cells and the proliferation of cells. Moreover, utilizing the multi-head equipped 3D cell-printing system makes it possible to print multiple types of bioinks with high precision simultaneously. Using this technique, a structure with a specific pattern can be produced, thus showing design versatility. In addition, it is feasible to encapsulate different types of cells into each bioink to mimic native cell arrangement[17]. These patterned structures can be utilized in the induction of vascularization or co-culture effect by improving cell-to-cell interactions, which can be key factors in the long-term survival of specific cells[18,19].

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DISCLOSURES:

None.

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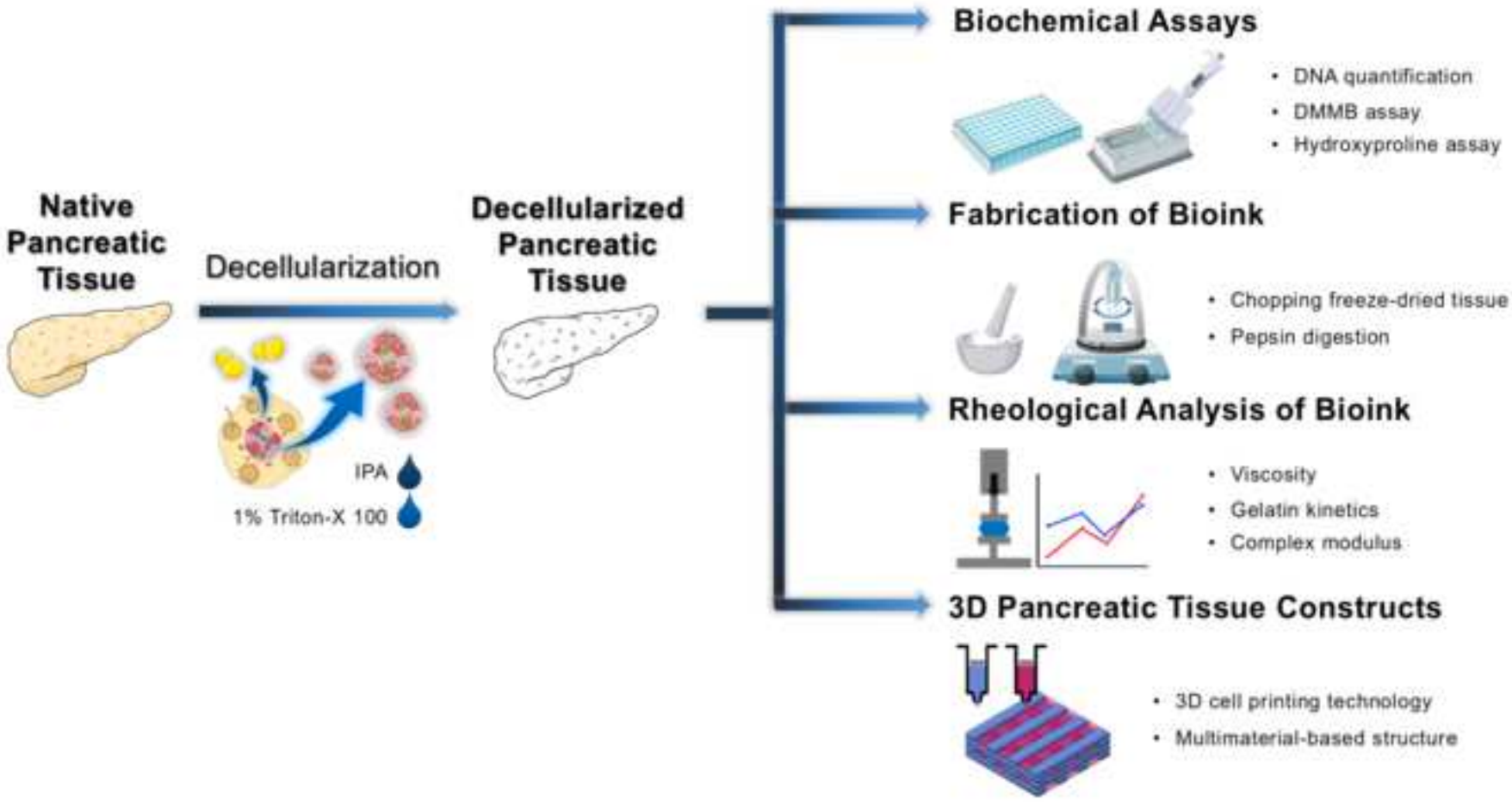
Figure



Figure

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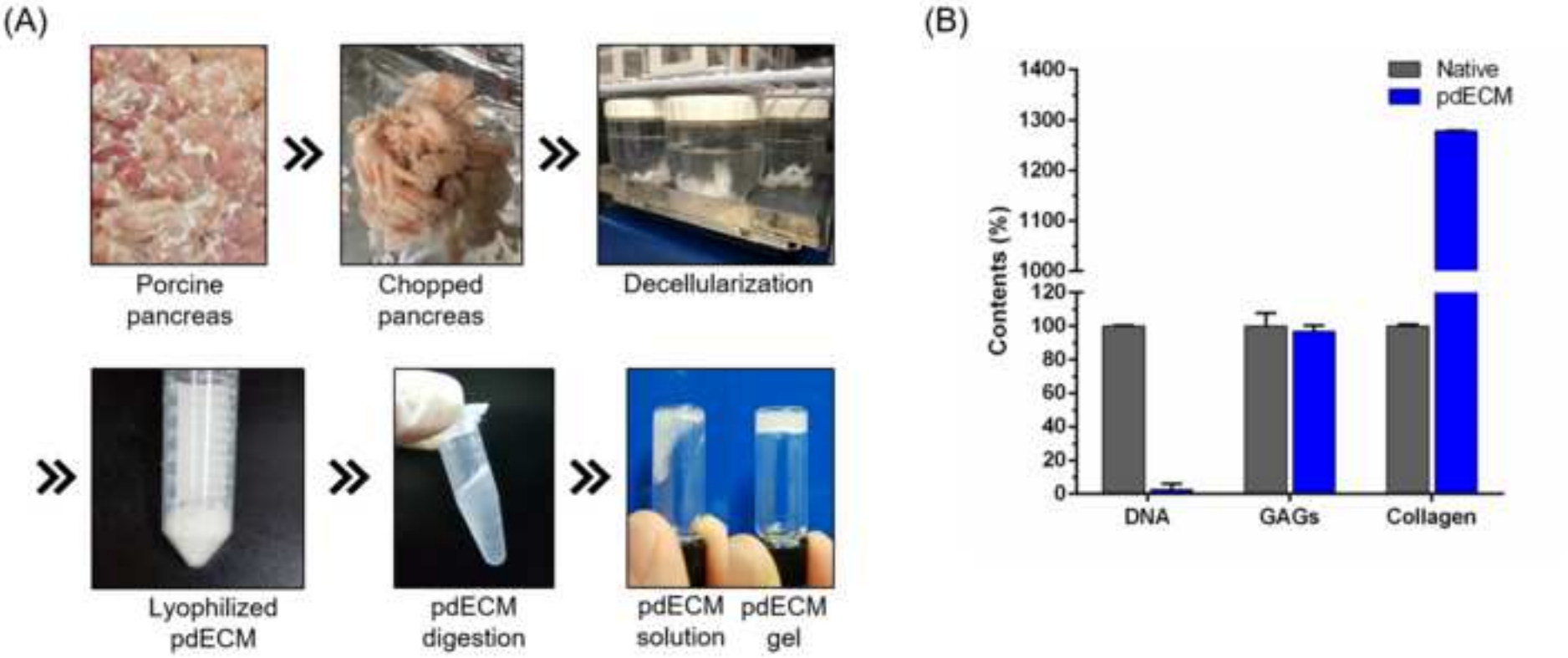

Figure

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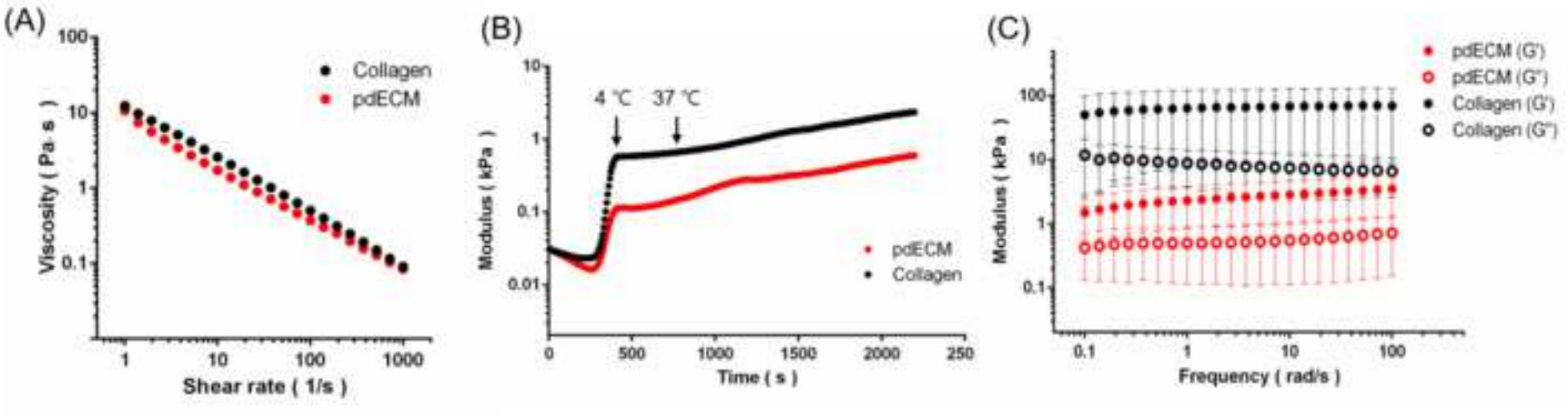

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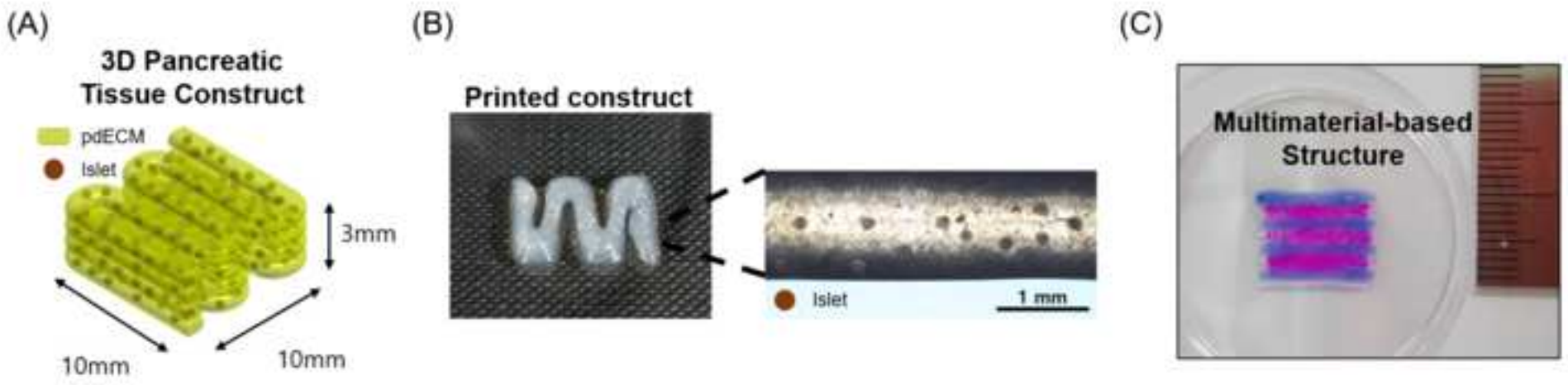

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Equipment			
Biological Safety Cabinets	CRYSTE	PURICUBE 1200	
Deep Freezer	Thermo Scientific Forma	957	
Digital orbital shaker	DAIHAN Scientific	DH.WSO04010	
Dry oven	DAIHAN Scientific	WON-155	
Freeze dryer	LABCONCO	7670540	
Fridge	SANSUNG	CRFD-1141	
Grater	ABM	1415605793	
Inverted Microscopes	Leica	DMi1	
Microcentrifuge	CRYSTE	PURISPIN 17R	
Microplate reader	Thermo Fisher Scientific	Multiskan GO	
Mini centrifuge	DAIHAN Scientific	CF-5	
Multi-Hotplate Stirrers	DAIHAN Scientific	SMHS-6	
Nanodrop	Thermo Fisher Scientific	ND-LITE-PR	
pH benchtop meter	Thermo Fisher Scientific	STARA2110	
Rheometer	TA Instrument	Discovery HR-2	
Vortex Mixer	DAIHAN Scientific	VM-10	
Cirurgical Instruments			
Operating Scissors	Hirose	HC.13-122	
Forcep	Korea Ace Scientific	HC.203-30	
Materials			
1.7 mL microcentrifuge tube	Axygen	MCT-175-C	
10 ml glass vial	Scilab	SL.VI1243	
40 μm cell strainer	Falcon	352340	
5 L beaker	Dong Sung Science	SDS 2400	
50 mL cornical tube	Falcon	352070	
500 mL beaker	Korea Ace Scientific	KA.23-08	
500 mL bottle-top vacuum filter	Corning	431118	
500 mL plastic container	LOCK&LOCK	INL301	

96well plate	Falcon	353072
Aluminum foil	DAEKYO	
Kimwipe	Kimtech	
Magnetic bar	Korea Ace Scientific	BA.37110-0003
Mortar and pestle	DAIHAN Scientific	SC.MG100
Multi-channel pipettor	Eppendorf	4982000314
Petri Dish	SPL	10100
pH indicator strips	Sigma-Aldrich	1095350001
Sieve filter mesh	DAIHAN Scientific	

Decellularization

10x pbs	Hyclone	SH30258.01
4.7% Peracetic acid	Omegafarm	
70% ethanol	SAMCHUN CHEMICALS	E0220 SAM
Distilled water		
IPA	SAMCHUN CHEMICALS	samchun I0348
Triton-X 100	Biosesang	T1020

Biochemical assay

1,9-Dimethyl-Methylene Blue zinc	Sigma-Aldrich	341088
10 N NaOH	Biosesang	S2018
Chloramine T	Sigma-Aldrich	857319
Chondroitin sulfate A	Sigma-Aldrich	C4384
Citric acid	Supelco	46933
Cysteine-HCl	Sigma-Aldrich	C1276
Glacial acetic acid	Merok	100063
Glycine	Sigma-Aldrich	410225
HCl	Sigma-Aldrich	H1758
Na2-EDTA	Sigma-Aldrich	E5134
NaCl	SAMCHUN CHEMICALS	S2097
Papain	Sigma-Aldrich	p4762
P-DAB	Sigma-Aldrich	D2004
Perchloric acid	Sigma-Aldrich	311421

Sodium acetate	Sigma-Aldrich	S5636
Sodium hydroxide	Supelco	SX0607N
Sodium phosphate(monobasic)	Sigma-Aldrich	RDD007
Toluene	Sigma-Aldrich	244511

Bioink

Charicterized FBS	Hyclone	SH30084.03
Penicillin-Streptomycin	Thermo Fisher Scientific	15140122
Pepsin	Sigma-Aldrich	P7215
Rose bengal	Sigma-Aldrich	198250
RPMI-1640 medium	Thermo Fisher Scientific	11875093
Trypan Blue solution	Sigma-Aldrich	T8154

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Title of Article:	Pancreatic Tissue-derived Extracellular Matrix Bioink for Printing 3D Cell-laden Pancreatic Tissue Constructs
Author(s):	Jaewook Kim*, Myungji Kim*, Dong Gyu Hwang, In Kyoung Shim, Song Cheol Kim, and Jinah Jang

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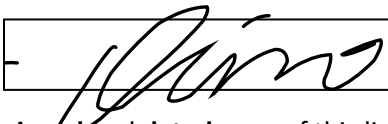
Signature:	
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Response to Editorial Comments

Manuscript ID	JoVE60434
Title	Pancreatic Tissue-derived Extracellular Matrix Bioink for Printing 3D Cell-laden Pancreatic Tissue Constructs
Authors	Jaewook Kim , Myungji Kim , Dong Gyu Hwang , In Kyoung Shim , Song Cheol Kim , and Jinah Jang

Summary of responses:

The authors have carefully read the valuable comments from the editor and considered how they can improve the manuscript. We have tried to find appropriate responses to the editor's meaningful comments and we responded to all comments in a point-wise manner. The responses to comments and revisions made in the manuscript are highlighted in blue and red, respectively. To improve the accuracy of the information on the grant funder, we made some correction and highlighted them in blue.

Editor's comment 1:

Reduce to 50 words.

Response 1:

According to the editor's comment, we have made the following modifications.

Page 1, line 32 in Summary

Decellularized extracellular matrix can provide suitable microenvironmental cues to recapitulate the inherent functions of target tissues in an engineered construct. This article elucidates the protocols for the decellularization of pancreatic tissue, evaluation of pancreatic tissue-derived dECM bioink, and generation of 3D pancreatic tissue constructs using a bioprinting technique.

Editor's comment 2:

Please ensure that the current manuscript supports this claim. I believe this was verified in Ref 5 but not the current manuscript. I suggest adding a few sentences to the results section while referencing ref 5 to address this.

Response 2:

Thank you for your comment regarding with the claim in the Abstract section. The authors modified a sentence that can confuse the readers. Also, we added a few sentences in the results section that can support the claim in abstract.

Page 2, line 45 in Abstract

In this article, we elucidate protocols for preparing a pancreatic tissue-derived decellularized extracellular matrix (pdECM) bioink to provide a beneficial

microenvironment that can increase glucose sensitivity of pancreatic islets, followed by describing the processes for generating 3D pancreatic tissue constructs using a microextrusion-based bioprinting technique.

Page 10, line 402 in Bioink preparation

The pdECM bioink maintained a solution phase when it was placed under room temperature and instantly converted into a gel phase after incubation at 37 °C for 30 min. To investigate the effect of the pdECM bioink on islets, isolated islets were encapsulated in the pdECM, alginate and collagen bioinks at a concentration of 1.5 %. The result of the glucose-stimulated insulin secretion test showed islets in the pdECM bioink represented the highest index (approximately 3.174) among the experimental groups, indicating higher functionality over the widely applied bioink for islet encapsulation[5].

5 Kim, J. et al. 3D cell printing of islet-laden pancreatic tissue-derived extracellular matrix bioink constructs for enhancing pancreatic functions. Journal of Materials Chemistry B. 7 (10), 1773-1781, (2019)

Editor's comment 3:

So far you referred to this as dECM, unclear why you switch after this section. Please maintain consistency across the text and figures.

Response 3:

The authors also agree with the editor's opinion. In the revised manuscript, we modified "dECM" to "pdECM", and highlight the changes in red.

Page 7, line 271 in Bioink preparation

NOTE: pdECM powder can be stored stably at -80 °C for at least one year. Before pH adjustment, the digested pdECM solution can be stored at -20 °C for one month. Prior to use, thaw the sample of frozen pdECM solution at 4 °C overnight. The pH-adjusted pdECM solution can be stored at 4 °C for up to one week. The digested pdECM solution can be stored at 4 °C for at least a few days but should not exceed 1 week.

3.1. Digest the freeze-dried pdECM with pepsin.

3.1.1. For effective digestion of bioink, pulverize the lyophilized pdECM with liquid nitrogen using a mortar and pestle.

3.1.2. Collect 200 mg of the pdECM powder in the 50 mL conical tube and add 20 mg of the pepsin and 8.4 mL of the 0.5 M acetic acid (final concentration is 2 w/v%).

3.2. Adjust pH of digested pdECM solution.

3.2.1. Filter out the undigested particles in the pdECM solution using a 40 µm cell strainer using a positive displacement pipette on ice to obtain the optimal digestion of parts.

Editor's comment 4:

Add an ethics statement at the start of the protocol for this.

Response 4:

We added an ethics statement at the starting point of the Protocol section in page 2, line 80.

Page 2, line 81 in Protocol

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Asan Medical Center, Seoul, Korea.

Editor's comment 5:

Under a microscope? Mention magnification and tools used for picking. Mention media used as well.

Response 5:

We agree with the reviewer's comment that the protocol need additional information for the readers. In this step, we added some details for the readers.

Page 8, line 334 in Section 5.1.3.

Suspend the isolated islets in RPMI-1640 medium and place them on the petri dish. Remove islets larger than 300 μm in diameter by using a P200 volume pipette under the microscope (4X objective lens) in the biosafety cabinet.

In addition, we added information of the biosafety cabinet, petri dish and microscope we used on the material/equipment list.

Editor's comment 6:

At what mixing ratio? What is the bioink concentration? What is the cell density?

Response 6:

Thank you for the valuable comment. There should be more information such as mixing ratio or density. We put the additional information on the revised manuscript.

Page 8, line 342 in Section 5.2.2.

Gently mix the pdECM bioink and the media suspended with islets (ratio 3:1) using a positive displacement pipette until uniformly mixed.

NOTE: The final concentration of the pdECM bioink is 1.5% and the cell density in the pdECM bioink is 3,000 IEQ per ml.

Editor's comment 7:

Briefly describe the staining procedure.

Response 7:

We briefly mentioned some details regarding with the staining.

Page 9, line 366 in Section 6.1.1.

To validate the printing versatility using multiple bioinks, prepare two sets of pdECM bioinks and stain them by adding 0.4% Trypan Blue and Rose Bengal solution into each pdECM bioink at a ratio of 1:20, respectively.

Editor's comment 8:

At what mixing ratio? What is the bioink concentration? What is the cell density?

Response 8:

Thank you for the comment. The authors provided additional information similar with the section 5.2.2.

Page 9, line 371 in Section 6.1.2.

Gently mix the pdECM bioink and the media suspended with islets (ratio 3:1) using a positive displacement pipette until uniformly mixed.

NOTE: The final concentration of the pdECM bioink is 1.5% and the cell density in the pdECM bioink is 3,000 IEQ per ml.

Editor's comment 9:

Functionality and viability of islets was not demonstrated in the current manuscript. Please add a few lines to the end of this section to cover this and cite ref 5.

Response 9:

We thank the editor for giving us a chance to revise this part. In the end of this section, we added a few sentences regarding the functionality and viability of islets.

Page 10, line 413 in Bioink preparation

The pdECM bioink maintained a solution phase when it was placed under room temperature and instantly converted into a gel phase after incubation at 37 °C for 30 min. To investigate the effect of the pdECM bioink on islets, isolated islets were encapsulated in the pdECM, alginate and collagen bioinks at a concentration of 1.5 %. The result of the glucose-stimulated insulin secretion test showed islets in the pdECM bioink represented the highest index (approximately 3.174) among the experimental groups, indicating higher functionality over the widely applied hydrogels for islet encapsulation[5].

5 Kim, J. et al. 3D cell printing of islet-laden pancreatic tissue-derived extracellular matrix bioink constructs for enhancing pancreatic functions. Journal of Materials Chemistry B. 7 (10), 1773-1781, (2019)

Editor's comment 10:

Define.

Response 10:

The islet equivalent (IEQ) is the standard estimate of isolated islet volume and used in both clinic and research to normalize results. According to IEQ calculations, one IEQ corresponds to the tissue volume of a perfectly spherical islet with a diameter of 150 μm[9].

We added the abovementioned answer on the revised manuscript as follows:

Page 10, line 422 in 3D cell printing

To build a construct containing at least 3,000 Islet equivalents (IEQ), that corresponds to the tissue volume of a perfectly spherical islet with a diameter of 150 μm[9], we designed the construct with a dimension of 10 mm x 10 mm x 3 mm (**Figure 4A**).

9 Huang, H.-H., Ramachandran, K. & Stehno-Bittel, L. A replacement for islet equivalents with improved reliability and validity. Acta diabetologica. 50 (5), 687-696, (2013)

Editor's comment 11:

legend does not match the figure. Please fix. Define error bars.

Response 11:

We have revised the manuscript as follows:

Page 10, line 436 in Figure and table legends

Figure 2: Representative images of the decellularization process and biochemical characterization of pdECM. (A) Overview of the decellularization of porcine pancreatic tissue. (B) Results of biochemical assays of native tissue and pdECM. Error bars show standard deviation. Copyright (2019) The Royal Society of Chemistry[5].

In addition, we have modified the legend of figure 1.

Page 10, line 435 in Figure and table legends

Figure 1: Schematic of the development of decellularized pancreatic tissue, evaluation of pdECM bioink and fabrication of 3D pancreatic tissue constructs.

The legends in Figure 1 and Figure 2 have been updated respectively.

5 Kim, J. et al. 3D cell printing of islet-laden pancreatic tissue-derived extracellular matrix bioink constructs for enhancing pancreatic functions. Journal of Materials Chemistry B. 7 (10), 1773-1781, (2019)

Editor's comment 12:

Please also cite the original publication.

Response 12:

We added the reference in the manuscript regarding with the original publication.

Editor's comment 13:

Please also cite the original publication.

Response 13:

Same as Response 12, we added the reference regarding with the original publication.

Editor's comment 14:

define

Response 14:

According to the editor's suggestion, we have included full name of the CHAPS.

Page 11, line 459 in Discussion

Thus, we modified our previous protocol by eliminating the step for the treatment of SDS solution, which is an ionic surfactant used in many cleaning and decellularization processes featuring relatively harsh characteristics compared to the others such as Trion-X 100, or 3-[(3-cholamidopropyl) dimethyl-lammonio]-1-propanesulfonate (CHAPS).

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