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Corresponding Author:	Stacey M. S. Gruber University of Cincinnati Cincinnati, OH UNITED STATES
Corresponding Author's Institution:	University of Cincinnati
Corresponding Author E-Mail:	grubersy@mail.uc.edu
Order of Authors:	Stacey M. S. Gruber
	Paulomi Ghosh
	Karl Wilhelm Mueller
	Patrick Whitlock
	Chia Ying James Lin
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TITLE:

Novel Process for 3D Printing Decellularized Matrices

AUTHORS:

Stacey M.S. Gruber[1], Paulomi Ghosh[2], Karl Wilhelm Mueller[2], Patrick W. Whitlock[1,2,3], Chia-Ying Lin[1,3]

[1]Department of Biomedical Engineering, University of Cincinnati, Cincinnati, Ohio, United States

[2]Division of Orthopaedic Surgery, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, United States

[3]Department of Orthopaedic Surgery, University of Cincinnati, Cincinnati, Ohio, USA

CORRESPONDING AUTHOR:

Chia-Ying (James) Lin (linc9@ucmail.uc.edu)

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SHORT ABSTRACT:

This protocol describes the production of polycaprolactone (PCL) filament with embedded polylactic acid (PLA) microspheres which contain decellularized matrices (DM) for 3D printing of structural tissue engineering constructs.

LONG ABSTRACT:

3D bioprinting aims to create custom scaffolds that are biologically active and accommodate the desired size and geometry. A thermoplastic backbone can provide mechanical stability similar to native tissue while biologic agents offer compositional cues to progenitor cells, leading to their migration, proliferation, and differentiation to reconstitute the original tissues/organs[1,2]. Unfortunately, many 3D printing compatible, bioresorbable polymers (such as polylactic acid, PLA) are printed at temperatures of 210 °C or higher — temperatures that are detrimental to biologics. On the other hand, polycaprolactone (PCL), a different type of polyester, is a bioresorbable, 3D printable material that has a gentler printing temperature of 65 °C. Therefore, it was hypothesized that decellularized extracellular matrix (DM) contained within a thermally protective PLA barrier could be printed within PCL filament and remain in its functional conformation. In this work, osteochondral repair was the application for which the hypothesis was tested. As such, porcine cartilage was decellularized and encapsulated in polylactic acid (PLA) microspheres which were then extruded with polycaprolactone (PCL) into filament to produce 3D constructs *via* fused deposition modeling. The constructs with or without the microspheres (PLA-DM/PCL and PCL(-), respectively) were evaluated for differences in surface features.

INTRODUCTION:

Current tissue engineering techniques for clinical applications such as bone, cartilage, tendon, and ligament reconstruction use auto- and allografts to repair damaged tissue. Each of these techniques is performed routinely as a "gold standard" in clinical practice by first harvesting the donor tissue either from the patient or a cadaveric match, and then placing the donor tissue into the defect site[2]. However, these strategies are limited by donor site morbidity, donor site scarcity for large defects, risk of infection, and difficulty finding grafts that match the desired geometry. In addition, studies have shown that allografts used for reconstruction have reduced mechanical and biologic properties when compared with native tissue[3]. With these considerations in mind, tissue engineers have recently turned to three dimensional (3D) bioprinting to produce custom, complex geometries that are biologically active and designed to accommodate defect size and shape while providing sufficient mechanical properties until biologic remodeling is complete.

Ideally, a 3D-printed scaffold would consist of a polymeric backbone that can retain the required mechanical stability of native tissue while the incorporated biologics offer biochemical cues to surrounding cells, leading to their migration, proliferation, differentiation, and tissue production[2,5]. Unfortunately, most constructs that contain biologic components are made with gels or polymers that are too weak to withstand *in vivo* forces experienced by the targeted tissues for auto/allograft reconstruction. Other polymers such as polylactic acid (PLA) are bioresorbable, 3D printable and structurally sound, but are printed at temperatures at or above 210 °C – making it impossible for biologics to be co-printed during fabrication. Polycaprolactone (PCL) is another FDA-cleared, bioresorbable polymer that can be 3D printed at a lower temperature (65 °C), which has become increasingly popular in fabricating patient-specific implants with complex morphologies[5–9]. However, most bioprinters using pneumatic technology make it impossible to print PCL at lower temperatures where biological activities can remain unharmed. To date, the integration of these polymers with auto/allografts into a novel printable biomaterial has yet to be accomplished. In the absence of such a material, a true tissue engineered approach to tissue reconstruction is unlikely. Therefore, we have sought to combine PLA, PCL, and decellularized allograft matrices (DM) to utilize the advantages of each material in order to manufacture a viable construct capable of reconstructing complex tissues. This process would provide the initial mechanical strength necessary to resist *in vivo* forces and the thermal stability to accommodate additive manufacturing in a construct that induces formation of the desired tissue.

In a recent attempt to address the aforementioned hurdles, we showed that it is feasible to encapsulate decellularized cartilage extracellular matrix within a thermally protective PLA barrier that can be extruded within PCL filaments, maintaining the ability of DM to influence surrounding host cells[2]. This has inspired us to seek clinically effective approaches for tissue reconstruction. In the current study, we utilize the platform technology to build all-in-one scaffolds that include PLA, DM, and PCL (PLA-DM/PCL).

Our goal is to improve the efficacy and utility of allografts using the proposed novel biofabrication technique to more accurately recapitulate native tissue, to ultimately use them in various applications.

PROTOCOL:

1. Obtaining and Preprocessing Microspheres

1.1. Produce microspheres with the desired matrix encapsulated (PLA-DM)[2].

Note: It is imperative that the microspheres are of uniform size. For this reason, sieving the microspheres prior to use is essential. Although matrix decellularization and encapsulation have been detailed in previous publications[2], a brief summary of the process follows.

1.1.1. First, harvest cartilage plugs from porcine hind limbs. Decellularize the cartilage in a series of washes with 0.05% trypsin/0.5 mm tetrasodium ethylenediaminetetraacetic acid (EDTA), Dulbecco's modified Eagle's medium (DMEM), and 1.5% peracetic acid and 2.0% Triton X-100 for 4 h each with distilled water washes before and after each step[2].

1.1.2. Drain the decellularized matrix, freeze it, lyophilize, grind, and dissolve into pepsin solution. Following dissolution, mix the pepsin solution with PLA which has been dissolved in dichloromethane.

1.1.3. Add the mixture dropwise into a 3% polyvinyl alcohol in water solution. Centrifuge the resulting microspheres, rinse, drain, and lyophilize again.

Note: For full details on the process see the previously published protocol[2].

1.2. Sieve the microspheres.

1.2.1. Ensure that all sieve plates have been thoroughly cleaned and are dry prior to use. If necessary, clean sieves using ultrasonic cleaner to ensure that all spheres are removed from the sieve.

1.2.2. Assemble the sieve shaker with the 106 µm sieve tray at the top, the 53 µm tray after that, and the sieve pan at the bottom.

1.2.3. Place dry microspheres in the topmost sieve tray and place the lid on the top tray. Turn on coarse sieving for 8 to 10 min. Repeat on fine for 8 to 10 min.

Note: The sieve times may need to be increased or decreased depending on the batch.

1.2.4. Carefully remove the sieve plates one by one and place them upside down on a large weigh paper. Tap the sides gently to ensure that most of the spheres have fallen out of the sieve and onto the paper.

1.2.5. Discard the oversized spheres (>106 µm) and undersized spheres (<53 µm). Add spheres that are in the 53 to 106 µm size range to a labeled centrifuge tube with the type and batch number then place in a -20 °C freezer until further use.

2. Microsphere Quality Control Assessments

Note: See **Figure 1**.

2.1. Perform macroscopic/visual assessment to check that the microspheres are uniform and spherical, with no aggregates present.

2.2. Assess the microspheres using a scanning electron micrograph (SEM).

2.2.1. For this, place microspheres onto an SEM chuck and sputter coat with gold-palladium in argon atmosphere using a sputter coater to a thickness of 4 nm.

2.2.2. Observe surface features, morphology, and diameters of the microspheres using a 10 kV accelerating voltage and a 10 mm working distance to ensure that production and sieving of the microspheres was successful.

3. Filament Creation for 3D Printing

3.1. Measure and record the mass of the microspheres obtained from steps 2 and 3; at least 25 g is needed.

3.2. Add polycaprolactone (PCL) powder to the microspheres for a 1:4 weight ratio of microspheres to PCL.

3.3. Mix the powder mixture on a miniature rolling mixer at 20 rpm for 5 min then flip the container and mix at 20 rpm for an additional 5 min (see **Figure 2**).

3.4. Many commercially available extruders (see the **Table of Materials**) have insulating jackets because their intended working temperatures are for traditional fused deposition modeling (FDM) filaments. Modify the extruder (if necessary) by removing the insulating material and use it in combination with desktop fans (which blow ambient air onto the extruder and extruded filament) to use of the extruder at lower temperatures.

Note: Desktop fans which blow ambient air to cool the extruder and filament are useful for this procedure.

3.5. Setup the equipment setup for extrusion. See **Figure 3**.

3.5.1. Setup the extruder so that its outlet is ~60 cm from the inlet to the spooler, with a direct path from the extrusion outlet to the spooler inlet.

Note: The spooler can optionally be raised 3–4 inches from the bench if it is found that the filament is drooping to the point of touching the benchtop.

3.5.2. Place a desktop fan ~15 cm from the heating jacket and direct it towards the heating jacket to offer cooling with ambient air throughout filament production. Place a second cooling fan approximately halfway between the extruder and spooler and direct it towards the extrudate to assist in cooling the filament with ambient air.

3.5.3. Adjust the positioning as needed throughout the process.

3.6. Set the modified extruder heating element to 52 °C, turn on the desktop cooling fans, and allow the instrument to come to equilibrium for 20 to 30 min. Ensure that the proper nozzle is attached to the extruder.

3.7. Just before beginning, fill the extruder hopper with the microsphere/PCL mixture from step 3.3. Turn on the spooler and the extruder auger to initiate extrusion of filament.

3.8. When the initial filament is extruded, manually pull the extrudate from the extrusion outlet with forceps and feed it to the filament spooler.

3.9. The desired filament will take some time to come out of the spooler. Using separate spools or tape, clearly mark when the filament composition visually appears uniform.

3.10. Monitor the process closely and modify parameters as necessary. Adjust the extruder temperature, extrusion auger speed, and spooler speed to obtain a 1.75 mm diameter filament as measured by calipers. Adjust the fans as needed to cool the filament properly to avoid non-circular filament cross-sections. Mix and refill the hopper as necessary.

Note: Close attention is required during this process to obtain adequate filament for subsequent 3D printing. The above parameters will change depending on the ambient conditions, the fill level and uniformity of the mixture in the hopper, and the thermodynamics and flow dynamics of the specific batches of PCL and microspheres.

3.11. Continue extruding until all of the powder has been used and the hopper is almost empty. Add PCL powder (without microspheres) to the hopper to flush out the microsphere mixture that is currently in the extruder. Continue adding PCL powder to the hopper until no more microspheres are visible in the extrudate.

3.12. Be sure to label and separate the filament which contains the microspheres in the desired concentration, as after the filament is cooled it is harder to distinguish the uniform filament from non-uniform filament.

3.13. Continue extruding until there is minimal powder left in the hopper, then turn off the spooler, extruder auger, extruder heating element, and fans.

4. Printing with the Filament

4.1. Design a geometry of the desired shape and form using a computer aided design software. Then slice the model and dictate the toolpath using slicing software that is compatible with the 3D printing machine being used.

4.2. Load the filament from step 3 onto any standard FDM printer, fitted with standard nozzles of the desired diameter (typically 0.4 mm). Begin the print (typically at 65–70 °C and 300 mm/min linear speed) as the custom filament is deposited layer-by-layer by the machine.

4.3. Make sure to pay special attention to the first layer and adjust settings as needed to get a good quality print.

Note: Adjustments may be made to the print speed, print temperature, platform temperature, extrusion multiplier, and other parameters. Refer to the printer and slicing manufacturer's troubleshooting guide for further assistance.

5. **Quality Control Assessment**

5.1. Place the printed constructs on SEM chucks and sputter coat with gold-palladium in argon atmosphere using a sputter coater to a thickness of 4 nm.

5.2. Observe under the microscope using a 10 kV accelerating voltage and a 10 mm working distance to check surface features and for the presence or absence of microspheres if applicable.

6. **Functional Testing of the Printed Constructs**

Note: Alkaline phosphatase (ALP) can be used as a surrogate for decellularized matrix to determine if encapsulated proteins are biologically active after the filament production process. ALP is used because it catalyzes a reaction from a substrate, p-nitrophenyl phosphate, to change from colorless to yellow byproducts, p-nitrophenol and inorganic phosphate, but only if ALP is in the functional conformation.

6.1. Print a geometry (n = 3) that has an end mass of at least 400 mg with the ALP microsphere filament (PLA-ALP/PCL) using identical print parameters as the PLA-DM/PCL scaffolds. Also print PCL-only (PCL(-)) scaffolds of the same geometry as the PLA-ALP/PCL scaffolds. Submerge them in 1 mL Tris-HCl buffer and incubate for 24 h at 37 °C and 110 rpm rotation to allow enzyme diffusion.

6.2. Add 1 mL of 1 mg/mL p-nitrophenyl phosphate, disodium hexahydrate in Tris-HCl. Incubate at 37 °C, 110 rpm for an additional 10 h. Read the supernatant absorbance at 415 nm.

REPRESENTATIVE RESULTS:

After sieving, microspheres should appear uniform and be free from aggregates. Under SEM, the sieved microspheres may have small pores on their surface, but will otherwise be spherical and smooth, as shown in **Figure 1**. All extruded filaments should be of uniform diameter and circular

cross-section. A filament that contains microspheres (PLA-DM/PCL) will have a slightly more matte finish while a PCL-only (PCL(-)) filament would look more glossy. The PLA-DM/PCL filament would also feel coarser to the touch than the PCL(-) filament. Scaffolds should be printed in the desired geometry which was dictated by the software in step 4.1. The scaffold quality and shape should be repeatable and uniform from one print to another. After printing, scaffolds with and without microspheres will be difficult to distinguish macroscopically, but under SEM, microspheres should be visible on the surface and throughout the constructs. Under SEM, PCL(-) filament will appear smooth, with some striations as an artifact of the extrusion process (**Figure 4B)**. Microspheres should be visible both protruding through and under the surface of the PLA-DM/PCL scaffolds (see **Figure 4C**). When using ALP as a surrogate for DM, the functionality of the enzyme within the scaffold should be maintained with significantly higher absorbance (*t*-test, $p < 0.05$) at 415 nm than those of blank PCL(-) scaffolds, 0.297 ± 0.023 and 0.166 ± 0.012, respectively, **Figure 5**.

FIGURE LEGENDS:

Figure 1. Representative macroscopic (left) and SEM (right) images of microspheres after preparation and sieving[2]. Note that the microspheres are spherical and in the appropriate size range (53–106 μm diameter).

Figure 2. Custom made rolling mixer. The custom-made rolling mixer is used for combining the microspheres with PCL powder.

Figure 3. Filament production setup. The outlet of the extruder is set approximately 60 cm from the inlet of the spooler. Desktop fans are located near the heating element and approximately half way between the extruder and spooler. The spooler can optionally be elevated 3–4 inches above the benchtop.

Figure 4. Quality Assessments. (A) PCL(-) (left) and PLA-DM/PCL (right) scaffolds are difficult to distinguish macroscopically. **(B)** Under SEM, the PCL(-) scaffold appears mostly smooth, with a few striations as artifacts of the printing process. **(C)** Under SEM, microspheres are visible in the PLA-DM/PCL samples. Some of the microspheres are indicated using arrows.

Figure 5. Representative results of an ALP colorimetric assay. The absorbance of ALP-containing scaffolds (PLA-ALP/PCL) is significantly higher than that of the PCL-only (PCL(-)) scaffolds, indicating that the ALP enzyme catalyzed the reaction from colorless p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate. This demonstrates that the ability to print functional proteins with the process described in this manuscript. * significantly different ($p < 0.05$) from all other groups. Error bars indicate standard deviation.

DISCUSSION:

Both decellularized matrices and 3D printed PCL scaffolds have independently been shown to allow adhesion and proliferation of cells, validating their use for osteochondral repair[10–12]. The use of decellularized matrix in engineering approaches to tissue repair has been a subject of much

interest and success in the recent past[2, 3, 14, 15]. We have previously noted the increased migration, adhesion, proliferation, and overall maintenance of resulting tissues when compared to traditional techniques[2, 15–18]. Many have attributed these desirable results to the process of dynamic reciprocity through which the host cells receive cues from the decellularized matrix, dynamically respond, and replicate the cues for new cells by laying more extracellular matrix that typically resembles what is already present[19–22]. While this has been studied for many applications, many of the processes are not easy to replicate and cannot be adapted for different uses, unable to successfully create highly patient-specific constructs, unable to create complex morphologies, and unable to withstand *in vivo* forces[2-4, 13-16].

The innovative approach proposed herein avoids both transient and prolonged exposure to high temperatures that are typically required by 3D printing when using traditional mechanical extrusion-based FDM printers with a new carrier vehicle. Moreover, the carrier vehicle (PLA microspheres) helps protect the encapsulated biologic for the relatively short period of time it is exposed to heat and provides an all-in-one treatment option for fast turnover in the clinic[2]. The methods proposed herein demonstrate how to create biologically active filaments for 3D printing and scaffolds via 3D printing where a critical step is the extrusion of the filament and the printing of those filaments at low temperatures (65 °C). The ability of the encapsulated proteins to remain functional was demonstrated by using ALP as a surrogate for DM throughout the process. ALP was used as the enzyme must be in a very specific functional conformation in order to catalyze the colorimetric reaction assessed in this protocol[23]. If the filament is not extruded with careful attention to diameter, temperature, and speed, the biologic activity and utility for 3D printing would be sacrificed.

In this protocol, microspheres containing decelluarized matrices (PLA-DM) were co-extruded with PCL to make 3D printable filaments and 3D printed scaffolds for osteochondral repair (PLA-DM/PCL). As mentioned in the protocol steps, continuous monitoring of the filament production process is essential for high quality of the filament. Adjustments must be made to extrusion speed, spooler speed, and extrusion temperature in order to maintain the desired filament diameter (typically 1.75 mm). The presence of the microspheres in the scaffolds is confirmed by SEM imaging and the maintenance of enzyme functionality is demonstrated by an alkaline phosphatase assay. Note that this protocol is limited by the large amount of microspheres required for production and the relatively lower resolution of fused deposition modeling to other 3D printing modalities. Nevertheless, the increased biologic activity is a major advancement. Although not the focus of this protocol, subsequent studies will concentrate on the impact of the microspheres on mechanical strength, cell migration and differentiation, and further characterizations of the scaffolds. Overall, the technique described herein allows decellularized matrix and other proteins to be printed at lower temperatures than previously allowed and in thermally protective barriers in order to maintain function and mechanical strength[2,3].

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DISCLOSURES:

The authors have nothing to disclose.

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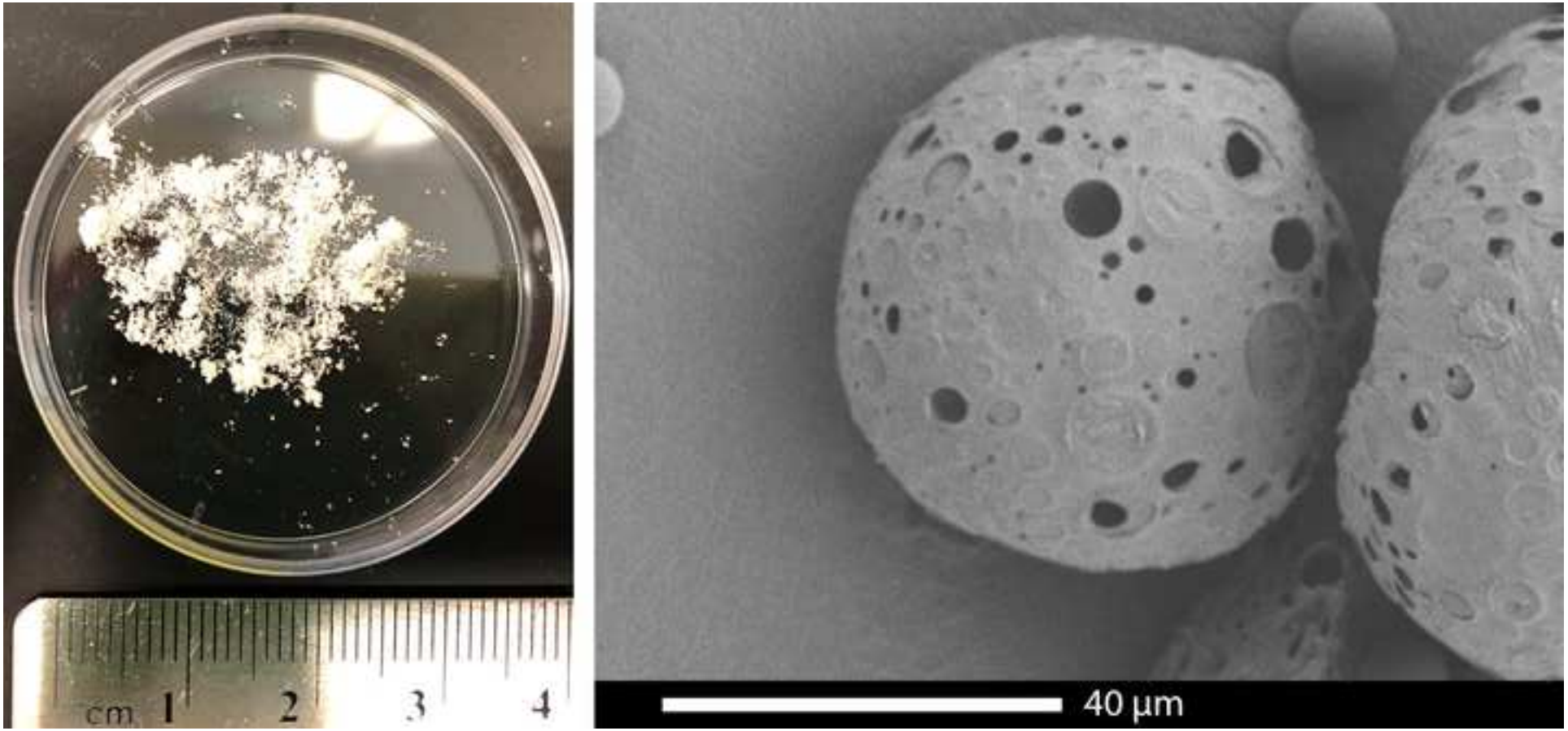

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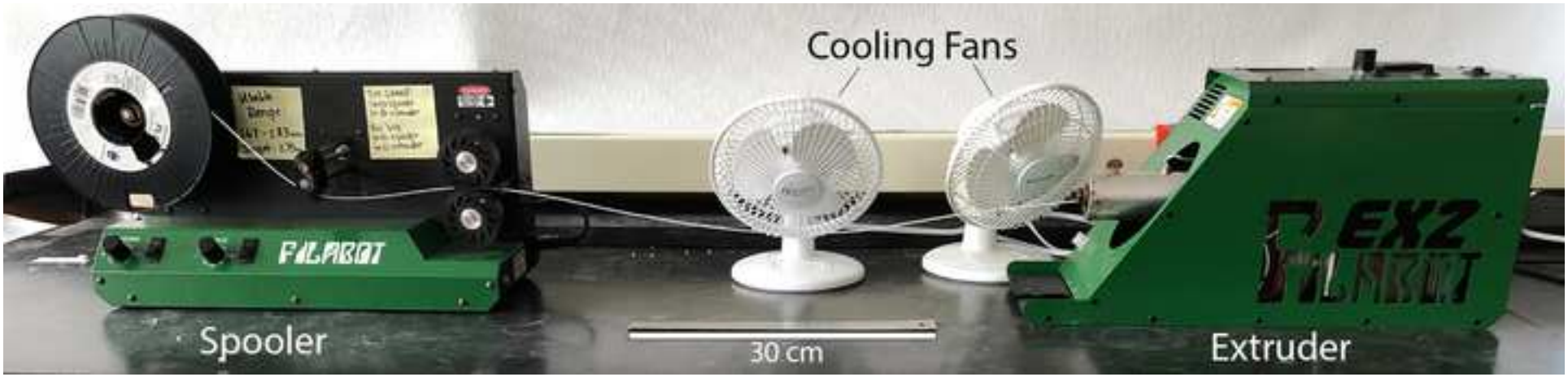

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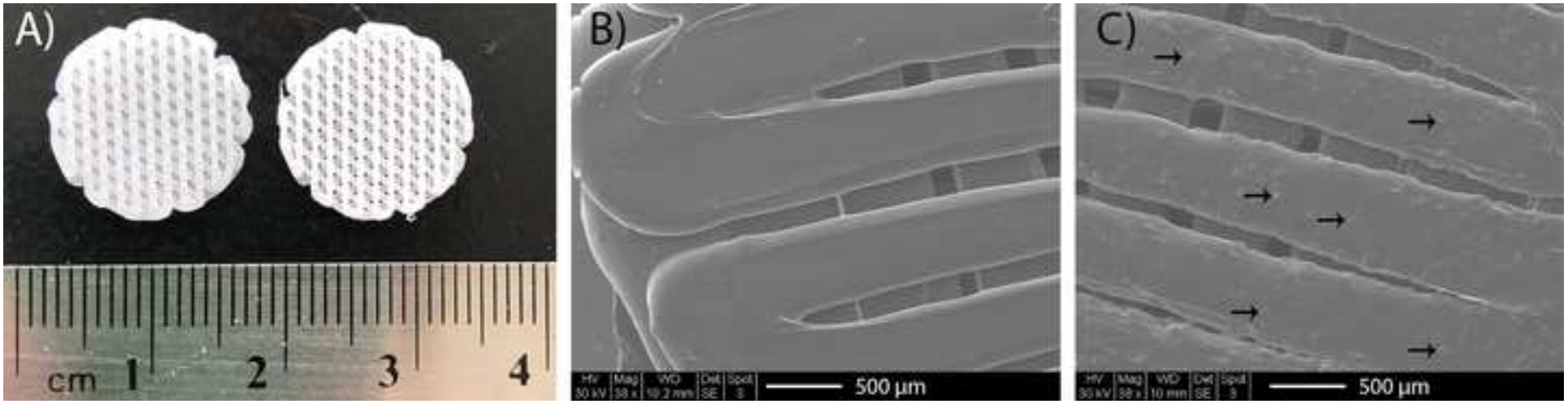

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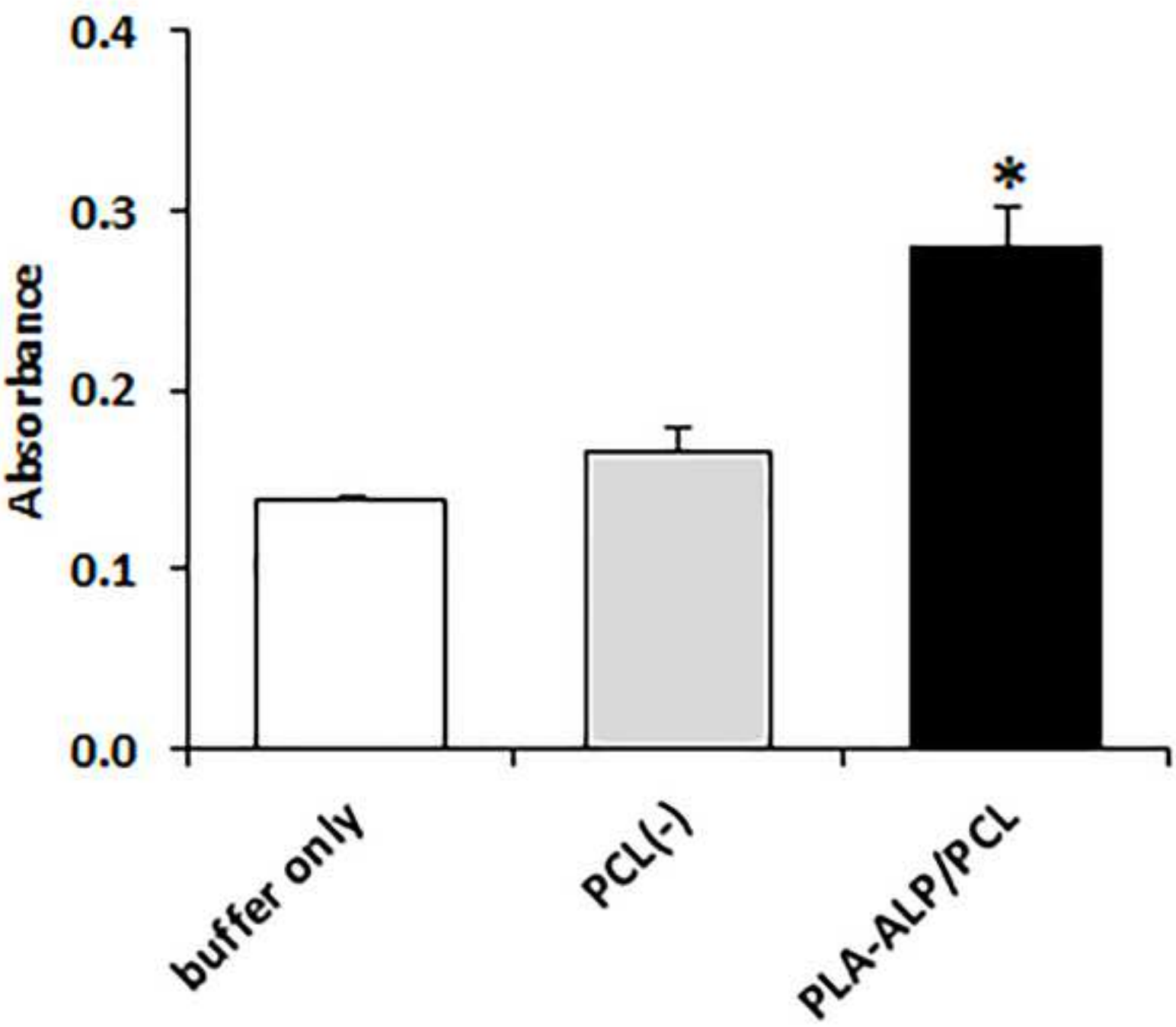

Click here to access/download;Table of Materials;JoveEquipmentListRevised (1).xlsx

Name of Reagent/ Equipment	Company
Sieve machine	Haver & Boecker Tyler
Sieve 90 um	Fisherbrand
Sieve 53 um	Fisherbrand
Sieve 106 um	Fisherbrand
Sputter coater	Leica
Scanning Electron Microscope	Hitachi, USA
Filabot EX2	Filabot.com
Filabot Spooler	Filabot.com
CAPA 6506	Perstorp
Phosphate buffered saline, PBS	Gibco
6" Fan	Comfort Zone, Amazon
Ultrasonic Water Bath	Cole Parmer
Dreamer	FlashForge
Drum Mixer	Custom made
Micro Balance	Mettler Toledo, Fisher Scientific
Simplify3D	Simplify3D
SolidWorks	SolidWorks
Microspheres	Produced in-house, see concurrently submitted JoVE submission
p-nitrophenyl phosphate, disodium	Millipore
Phosphatase, alkaline	Roche Diagnostics GmbH
Absorbance Reader	Tecan
Tris-HCl Buffer	Sigma-Aldrich
Heated shaker	New Brunswick Scientific

Catalog Number	Comments/Description
Ro-Tap RX 29-E Pure	
170328156	No. 170
162513588	No. 270
162018121	No. 140
n/a	
n/a	
FB00061	
FB00073	
24980-41-4	
10010023	
n/a	

SK-08895-13

n/a

n/a Similar piece of equipment: https://www.coleparmer.com/i/argos-technologies-flexi

01-913-851

n/a

n/a

4876-5GM

10 713 023 001

Sunrise

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tel. 617.945.9051
www.jove.com

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Title of Article: Novel Process for 3D Printing Decellularized Matrices

Author(s): Stacey M. S. Gruber, Paulomi Ghosh, Karl Wilhelm Mueller, Patrick W. Whitlock, Chia-Ying James LIn

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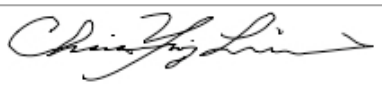
CORRESPONDING AUTHOR:

Name:	Chia-Ying James Lin
Department:	Department of Orthopaedic Surgery
Institution:	University of Cincinnati
Article Title:	Novel Process for 3D Printing Decellularized Matrices
Signature:	 Date: 06/12/2018

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August 2, 2018

Re: Resubmission of manuscript *Novel Process for 3D Printing Decellularized Matrices*

The Editors
Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Editors,

Thank you for the opportunity to revise our manuscript, *Novel Process for 3D Printing Decellularized Matrices.* We appreciate the careful review and constructive suggestions.

Following this letter are the referee comments with our responses in italics, including how and where the manuscript was modified if applicable. Changes made in the attached manuscript are marked using track changes.

Sincerely,

Chia-Ying Lin, PhD

Thank you for your thorough review of our manuscript and your thoughtful questions. We appreciate all of the reviewer comments and have addressed them below.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Thorough review has been completed.

• Please avoid use of the pronouns "you" and "your" throughout the manuscript.

All instances have been removed.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) 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. Some examples:

1) 1.1: What is the size (or size range) of the microspheres used? What is their composition (other than the encapsulated matrix)?
   The size range used is 53 – 106 µm. This has been clarified.
   2) 2.2.1,5.1: Mention sputtering settings, coating thickness etc. Observe on an SEM? Mention magnification and all additional settings.
   The sputtering settings and SEM settings have been clarified.

• **Protocol Numbering:** All steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

This has been addressed.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

1) The highlighting must include all relevant details that are required to perform the step. 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 included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail (3-6 paragraphs) : modifications and troubleshooting, limitations of the technique, significance with respect to existing methods, future applications and critical steps within the protocol.

The authors have added details about modifications, troubleshooting, limitations, and critical steps.

• **Figures:** Please add scale bars to all micrographs.

• **Figure/Table Legends:** Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

The legends have been modified.

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All instances have been removed.

• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as microspheres.

Software has been added to the table.

• Please define all abbreviations at first use.

This is done.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

This has been addressed.

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Not applicable.

Comments from Peer-Reviewers:

Reviewer #1:
Major Concerns:
My chief concern is that the authors do not discuss any methodology or provide data that detail whether the composite filament is biologically active. The authors state that the purpose of the composite filament is for use in osteochondral applications, but do not show any direct data that provide evidence for the mammalian cells to adhere, migrate, survive, or differentiate on the composite filament. Data showing that the DM is biologically active and/or that cells adhere to the filament is essential for showing that this methodology can be used for osteochondral, and more broadly tissue engineering applications.

A protocol and results for a functional alkaline phosphatase (ALP) test has been added to show that encapsulated materials remain biologically active. While in vitro studies were not the focus of this protocol, it is a future aim and has been added as such in the discussion section. In addition, our previous study and a protocol submitted concurrently with this one shows the

increased cellular activity, migration, and differentiation that resulted from decellularized matrix encapsulated in PLA microspheres.

Minor Concerns:
1. General
a. The authors state in the abstract that the melting temperature of PCL is 60 °C, whereas in the introduction the authors state "Polycaprolactone (PCL) is another FDA-cleared, bioresorbable polymer that can be 3D printed at a lower temperature (65 C)..." (Pg. 3, Paragraph 2, Lines 70-71). Which is correct? Please make sure values are consistent throughout the manuscript.

In 3D printing, filament is usually printed just above the melting temperature of the material. Therefore, there is usually a slight increase from melting temperature to printing temperature. However, to maintain clarity, the language in the protocol was changed to only address the temperature at which the filament is printed to avoid confusion and since the print temperature is more critical.

b. The use of imperative tense is not consistent throughout the Protocol Section of the Manuscript. There are several instances where the authors shift from imperative tense to second-person, which is distracting. Please make sure the use of imperative tense is uniform throughout the manuscript. Instances of second-person (You, your) are listed below:
i. Ln 111
ii. Ln 126
iii. Ln 135
iv. Ln 140
v. Ln 141
vi. Ln 143
vii. Ln 148
viii. Ln 154
ix. Ln 164
x. Ln 169
These instances have been changed.

c. Please double-check grammar throughout manuscript.
Grammar has been double checked.

2. Methods
a. Very little information is provided about the settings used for imaging via SEM. Only the brand of the SEM is provided. Information regarding voltage, magnification, and vacuum is

revealed on the SEM micrograph in Figure 1 (Right Panel), but is otherwise not explicitly stated or referenced. Additional information would be greatly appreciated for replicating the protocol such as the model of the SEM and the detector that was used. Mentioning the SEM settings used in the Protocol would help strengthen the manuscript and increase the reproducibility.

While the use of commercial language (such as detector and model of the SEM) was not allowed by JoVE, other settings for the sputter coating and imaging have been added to the protocol.

3. Figures
a. Figure 2 could be enhanced with some annotations such as arrows that explicitly point out where the microspheres are in the filament, as it very difficult to distinguish microspheres from structural artifacts in the filament.
b. Figure 2 could also be enhanced by labeling the components of the equipment displayed.
c. Figure 2 could be enhanced by including an image that shows what a filament without microspheres looks like.
d. I do not understand the purpose of Figure 3. Please provide more information in the figure legend, and annotations on the panels for

The figures have been modified.
The intention for Figure 3 was to show examples of what could be created from the 3D printer. The SEM images also show the absence of microspheres in the PCL(-) group and the presence of microspheres in the PLA-DM/PCL group. The legend has been updated.

Reviewer #2:
Manuscript Summary:
This article described a method for producing a PLA-Decellularized matrices (DM)/PCL filament with PLA-DM microspheres as 3D printing ink, and the authors introduced the use of the PLA-DM/PCL filament to print a designed geometry. By using the fused deposition modeling, the protocol prepared the PLA-DM/PCL filament in a gentle temperature. This approach avoids the exposures of DM to the high temperatures in the traditional procedure, which is significant for preserving the bioactivity of the DM and facilitating the tissue reconstruction in vivo. This novel and efficient method protocol may appeal to the readers of JoVE. However, there are some significant concerns to be solved and/or clarified. Therefore, I would recommend major revision for this manuscript.

Major Concerns:
1. Protocol 1.1
The microspheres are the important materials in the preparation of filament, and the

authors indicated that it is imperative to control the microspheres in uniform sizes. The protocol indicated that 53-micron sieve tray is final tray, so is it the microspheres smaller than 53 micron is usable? Or is there a limited range of the microspheres diameter?

The authors have clarified in the manuscript the desired microsphere size range in the protocol steps. The desired size range is 53 – 106 µm. While hypothetically smaller microspheres could be used, it will change the flow dynamics of the molten powder. Smaller microspheres were not used in our studies, so the effects were not thoroughly studied or included.

2. Protocol 3.4 Creating filament for 3D printing
As the author mentioned, a "modified Filabot EX2" is used in the protocol, are there any necessary modification procedures should be introduced in the article to help the readers repeat the filament extruding process?

Modification steps have been added to the protocol which include removing the insulating jacket and using desktop fans blowing ambient air for cooling.

3. Protocol 4. Printing with the filament
What is the temperature when the authors performing the 3D printing? As the authors mentioned, controlling low temperature is important for protecting the biological cues of the decellularized matrices. So, is it the printing temperature also gentle enough for the decellularized matrices? It is necessary to make it clear to the readers.

The print temperature (65 – 70 ^{o}C) was added to those specific steps of the protocol. Also, a functional test and the results from that test were added to show that biologic activity is maintained.

4. Discussion
In the discussion section, the authors described a series of limitations of the decellularized matrices in the tissue engineering development so far. However, the judgements and language are subjective and ambiguous to the existing progress (eg. 215-218). The authors should clearly indicate the compared objects and describe their advantages and disadvantages. Also, it would be necessary for the authors to provide corresponding references.

The discussion has been enhanced.

5. Discussion

As a method article, the authors should discuss the critical step of the protocol, and indicated clearly how would it affect the success of the experiment.

A discussion of the critical step of the protocol (filament production) and how it could impact the success of the experiments (by producing non-uniform or low quality filament) was added.

Minor Concerns:
1. The figures provided in the manuscript are in low quality. Please provide the scale bars in figures 1 and 3.

The figures have been modified.

2. Please check the reference are given in the correct output style. For example, there are a bunch of gibberish in ref 2.

This has been addressed.

Reviewer #3:
Manuscript Summary:
Gruber et al describe a processing to make a 3D print filament with decellularized matrix.

Major Concerns:
The protocol title is "novel process for 3D printing decellularized matrices" but the authors only focused on the filament creation step and not detailed much about the procedures of decellularization and sphere creation from mixture of decellularized matrix and PLA. In order to grant publication of the methodology, it should have careful description of all steps involved, adding special attention to details.

The decellularization and encapsulation steps have been detailed in a protocol that was co-submitted at the same time as this protocol, and therefore deemed unnecessary to include in this protocol. The authors have added summary steps at the beginning of the protocol.

Furthermore, answers to the following points would be relevant:

1. in protocol 1.1, it will help readers to understand how the decellularized matrix was encapsulated with brief description even that technique was published previously.
Although the matrix decellularization and encapsulation steps are detailed in a manuscript co-submitted with this one, the steps have been summarized at the beginning of the protocol.

2. In protocol 2, the particle size evaluation should be also taken into consideration in QC step to ensure the step of sieve is successful.

We typically use SEM imaging (detailed in this protocol) and SEM measurements to determine that the spheres are of adequate size for our procedure.

3. In protocol 3.1, what size of microspheres is used in filament creation?

This has been clarified in the protocol document (53 – 106 microns).

4. In protocol 3.3, what's the model and manufacturer of the "miniature drum mixer"?

The miniature drum mixer was custom built for our application by a graduate student. A machine which has a similar design has been added to the list of materials, and an image of the custom mixer was added to the protocol.

5. In step 3 (creating filament), how do you ensure the sterility during the filament fabrication? Shouldn't you also consider the sterility evaluation in quality control?

It is very difficult to ensure sterility in this process which involves such high surface area powders and microspheres. Instead, the scaffolds are disinfected after production with UV and ethanol.

6. The key part of the protocol is filament creation and it will be good to have the layout of whole system and label each components of the system.

An image of the layout has been added.

7. Does the mixture of PLA-PLA affect the strength of printed products?

This is currently being studied by our group, but was not the focus of this protocol. This has been added as a future direction in the discussion section.

8. Do the encapsulated matrices affect the cell move in, and bio-recognition?

This is also being studied by our group currently. This has been added as a future direction in the discussion section.

9. What are the characteristics of encapsulated DM? Like pole size, surface, ….

This is also being studied by our group currently. This has been added as a future direction in the discussion section.

10.3.10, please define the exact temperature needed to cool down the filaments.

The exact temperature to cool the filaments is unknown. The cooling fans are desktop units that blow ambient air over the filament to increase the rate of cooling to room temperature. More detail about the fan placement and clarification of the ambient air which is blown has been added to the protocol.

Minor Concerns:
In Figure 1, the scale bar is needed even in macroscopic observation

This has been added.

In Figure 3, the scale bar is needed for all sub-figures and the scale bar in SEM images is not clear.
The picture of SEM on PLC(-) is missed.

These concerns have been addressed. The PCL(-) image was present, but the labeling of the image was clarified to make it more clear.