Video Article

Novel Process for 3D Printing Decellularized Matrices

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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/58720/

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[5,6,7,8,9]. 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. 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. 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].
   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.
   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].

2. Sieve the microspheres.
   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.
   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.
   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.
   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.
   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**.

1. Perform macroscopic/visual assessment to check that the microspheres are uniform and spherical, with no aggregates present.
2. Assess the microspheres using a scanning electron micrograph (SEM).
   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. 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

1. Measure and record the mass of the microspheres obtained from steps 2 and 3; at least 25 g is needed.
2. Add polycaprolactone (PCL) powder to the microspheres for a 1:4 weight ratio of microspheres to PCL.
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**).
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.
5. Setup the equipment setup for extrusion. See **Figure 3**.
   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.
   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. Adjust the positioning as needed throughout the process.
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.
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.

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

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.
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.
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

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.
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.

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.
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**.
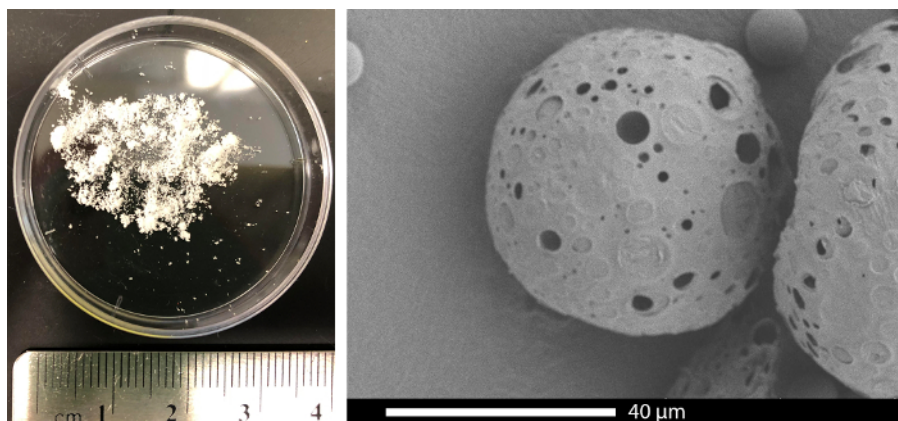
Figure 1. **Representative macroscopic (left) and SEM (right) images of microspheres after preparation and sieving**[2]. Note that the microspheres are spherical and in the appropriate size range (53-106 µm diameter). Please click here to view a larger version of this figure.
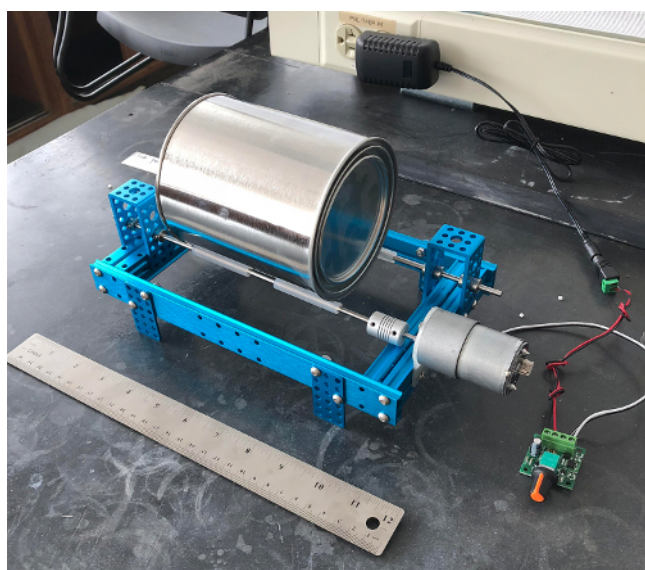

Figure 2. Custom made rolling mixer. The custom-made rolling mixer is used for combining the microspheres with PCL powder. Please click here to view a larger version of this figure.
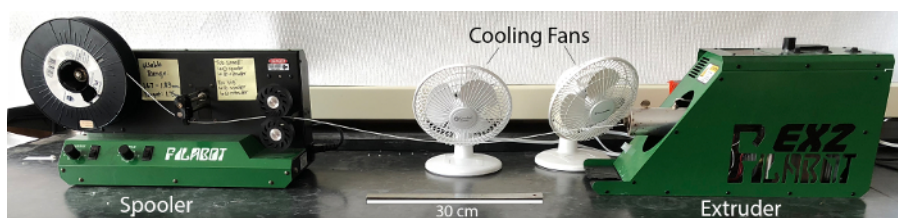

Figure 3. Filament production setup. The outlet of the extruder is set approximately 60 cm from the inlet of the spooler. Desktop fans are located near the heating element and approximately half way between the extruder and spooler. The spooler can optionally be elevated 3-4 inches above the benchtop. Please click here to view a larger version of this figure.
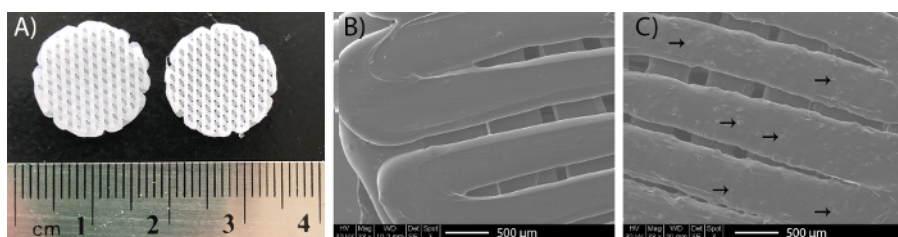

Figure 4. Quality Assessments. (A) PCL(-) (left) and PLA-DM/PCL (right) scaffolds are difficult to distinguish macroscopically. **(B)** Under SEM, the PCL(-) scaffold appears mostly smooth, with a few striations as artifacts of the printing process. **(C)** Under SEM, microspheres are visible in the PLA-DM/PCL samples. Some of the microspheres are indicated using arrows. Please click here to view a larger version of this figure.
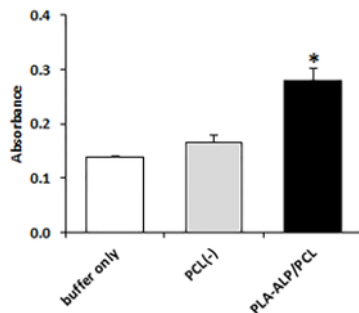
Figure 5. **Representative results of an ALP colorimetric assay.** The absorbance of ALP-containing scaffolds (PLA-ALP/PCL) is significantly higher than that of the PCL-only (PCL(-)) scaffolds, indicating that the ALP enzyme catalyzed the reaction from colorless p-nitrophenyl phosphate to p-nitrophenol 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. Please click here to view a larger version of this figure.

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[10,11,12]. 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[2,15,16,17,18]. 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[19,20,21,22]. 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[2,3,4,13,14,15,16].

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].

Disclosures

The authors have nothing to disclose.

Acknowledgements

References

1. Hutchmaker, D., Teoh, S., Zein, I., Ng, K. W., Schantz, J. -T., Leahy, J. C. Design and Fabrication of a 3D Scaffold for Tissue Engineering Bone. *Synthetic Bioabsorbable Polymers and Implants*. **15** (2), 845-847 (1988).
2. Ghosh, P., Gruber, S. M. S., Lin, C. -Y., Whitlock, P. Microspheres containing decellularized cartilage induce chondrogenesis and remain functional after incorporation within a poly(caprolactone) filament useful for fabricating a 3D scaffold. *Biofabrication*. (2018).

3. Partington, L. *et al.* Biochemical changes caused by decellularization may compromise mechanical integrity of tracheal scaffolds. *Acta Biomaterialia.* **9** (2), 5251-5261 (2013).
4. Hutmacher, D. W. Scaffolds in tissue engineering bone and cartilage. *Biomaterials.* **21** (24), 2529-2543 (2000).
5. Kang, H., Hollister, S. J., La Marca, F., Park, P., Lin, C. -Y. Porous biodegradable lumbar interbody fusion cage design and fabrication using integrated global-local topology optimization with laser sintering. *Journal of biomechanical engineering.* **135** (10), 101013-8 (2013).
6. Kang, H., Lin, C. Y., Hollister, S. J. Topology optimization of three dimensional tissue engineering scaffold architectures for prescribed bulk modulus and diffusivity. *Structural and Multidisciplinary Optimization.* **42** (4), 633-644 (2010).
7. Lin, C.-Y. *et al.* Functional bone engineering using *ex vivo.* gene therapy and topology-optimized, biodegradable polymer composite scaffolds. *Tissue Engineering.* **11** (9-10), 1589-1598 (2005).
8. Lin, C. -Y., Hsiao, C. -C., Chen, P. -Q., Hollister, S. J. Interbody Fusion Cage Design Using Integrated Global Layout and Local Microstructure Topology Optimization. *Spine.* **29** (16), 1747-1754 (2004).
9. Zopf, D., Hollister, S., Nelson, M., Ohye, R., Green, G. Bioresorbable Airway Splint Created with a Three-Dimensional Printer. *New England Journal of Medicine.* **368** (21), 2043-2045 (2013).
10. Pati, F., Song, T. H., Rijal, G., Jang, J., Kim, S. W., Cho, D.W. Ornamenting 3D printed scaffolds with cell-laid extracellular matrix for bone tissue regeneration. *Biomaterials.* **37** (October), 230-241 (2015).
11. Zhang, W. *et al.* The effect of interface microstructure on interfacial shear strength for osteochondral scaffolds based on biomimetic design and 3D printing. *Materials Science and Engineering C.* **46** (September), 10-15 (2015).
12. Williams, J. M. *et al.* Bone tissue engineering using polycaprolactone scaffolds fabricated *via.* selective laser sintering. *Biomaterials.* **26** (23), 4817-4827 (2005).
13. Monibi, F. A., Cook, J. L. Tissue-Derived Extracellular Matrix Bioscaffolds: Emerging Applications in Cartilage and Meniscus Repair. *Tissue Engineering Part B: Reviews.* (2017).
14. Wiles, K., Fishman, J., Coppi, P., Birchall, M. The Host Immune Response to Tissue-Engineered Organs: Current Problems and Future Directions. *Tissue Engineering Part B: Reviews.* **22** (3), (2016).
15. Sutherland, A. J., Detamore, M. S. Bioactive Microsphere-Based Scaffolds Containing Decellularized Cartilage. *Macromolecular Bioscience.* (2015).
16. Whitlock, P. W., Smith, T. L., Poehling, G. G., Shilt, J. S., Van Dyke, M. A naturally derived, cytocompatible, and architecturally optimized scaffold for tendon and ligament regeneration. *Biomaterials.* (2007).
17. Whitlock, P. W. *et al.* Effect of cyclic strain on tensile properties of a naturally derived, decellularized tendon scaffold seeded with allogeneic tenocytes and associated messenger RNA expression. *Journal of surgical orthopaedic advances.* **22** (3), 224-232, (2013).
18. Whitlock, P. W. *et al.* A novel process for optimizing musculoskeletal allograft tissue to improve safety, ultrastructural properties, and cell infiltration. *Journal of Bone and Joint Surgery - Series A.* **94** (16), 1458-1467 (2012).
19. Schultz, G. S., Davidson, J. M., Kirsner, R. S., Herman, I. M. Dynamic Reciprocity in the Wound Microenvironment. *Wound Repair Regeneration.* **19** (2), 134-148 (2012).
20. Benders, K. E. M., van Weeren, P. R., Badylak, S. F., Saris, D. B. F., Dhert, W. J. A., Malda, J. Extracellular matrix scaffolds for cartilage and bone regeneration. *Trends in Biotechnology.* **31** (3), 169-176 (2013).
21. Crapo, P., Gilbert, T., Badylak, S. An overview of tissue and whole organ decellularization processes. *Biomaterials.* **32** (12), 3233-3243 (2011).
22. Guan, Y. *et al.* Porcine kidneys as a source of ECM scaffold for kidney regeneration. *Materials Science and Engineering C.* **56**, 451-456 (2015).
23. Dean, R. L. Kinetic studies with alkaline phosphatase in the presence and absence of inhibitors and divalent cations. *Biochemistry and Molecular Biology Education.* **30** (6), 401-407 (2002).