Improving cell infiltration and visualization of synthetic scaffolds for osteochondral defect repair

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INTRODUCTION

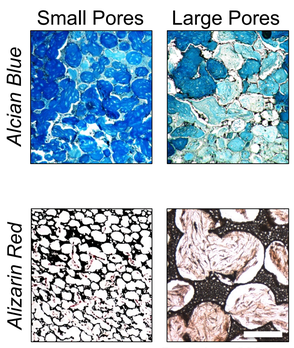
Osteochondral defects affect over 60% of patients that undergo knee surgery and present a major health and mobility concern **[1]**. These defects are characterized by damage to both the articular cartilage and underlying subchondral bone, which often result from either acute trauma, bone disorders, or long-term degeneration **[2]**. Current treatments include microfracture and tissue graft transplant, but can lead to additional complications for patients, such as fibrocartilage formation or donor tissue complexities **[3]**. Thus, tissue engineering represents an exciting alternative. Using knowledge of cell and embryonic differentiation, we designed a synthetic scaffold that can support osteo- *and* chondrogenic cell growth and tissue formation.

Chondrogenesis

Densely Packed Cells → Cartilage Growth → Small pore scaffolds

Osteogenesis

Loosely Spread-out Cells → Bone Growth → Large pore scaffolds

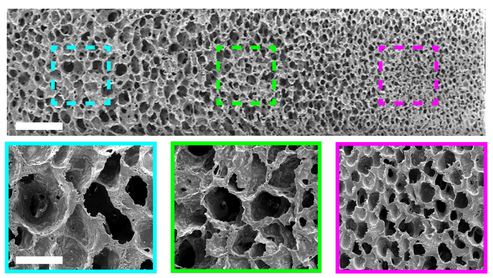


Scale bar: 200μm

SOLUTION: A PLLA GRADIENT POROUS SCAFFOLD MADE WITH THERMALLY INDUCED PHASE SEPARATION

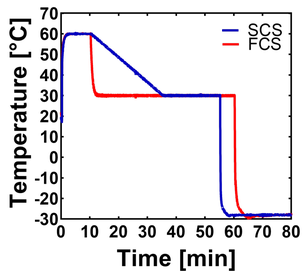
To create a scaffold with a gradient of small to large pore sizes, we used a method known as **Thermally Induced Phase Separation (TIPS)**. In a ternary solution of 4% wt PLLA in 87/13% wt/wt dioxane/water, opposite sides are exposed to different thermal histories or cooling rates. As a result, the PLLA solution forms a controlled pore size gradient scaffold around ice crystals created by the cooling rates (SCS: Slow Cooling Side – small pores, FCS: Fast Cooling Side – large pores) **[4]**. The final scaffold has pore sizes of ~70μm on one side that smoothly transition to ~200μm on the other side.

Scanning Electron Microscopy



Scale bar: 500μm, 200μm inset

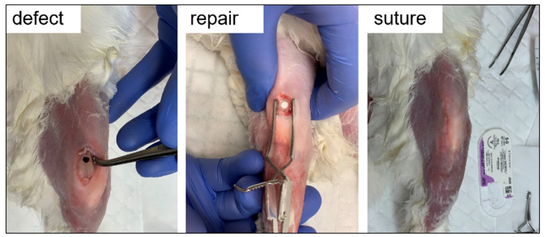
Thermal Histories



BEYOND *IN VIVO* VALIDATION OF SCAFFOLD DIFFERENTIATION AND REPAIR IN RABBIT MODEL OSTEOCHONDRAL DEFECTS

Bilateral osteochondral defect repair was previously performed in an *in vivo* rabbit model to validate the scaffold. Four groups were tested:

1. Empty defect (control)
2. Acellular scaffold (only PLLA scaffold – no cells)
3. Mesenchymal Stem Cell (MSC) seeded scaffold (800K cells/scaffold)
4. Pre-differentiated scaffold (MSC-seeded + 14 days of pre-differentiation in biphasic bioreactor)



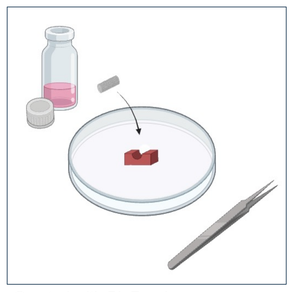
These studies have attained *in vivo* validation of the scaffold's ability to promote localized osteochondral tissue formation and repair post pre-differentiation. My goal was to generate *in vitro* validation in the rabbit model that is comparable to *in vitro* data in the human model.
We proposed main questions:

HOW CAN WE SEED THE PLLA SCAFFOLDS SUCH THAT CELLS ARE MORE ROBUSTLY SEEDED THROUGHOUT?

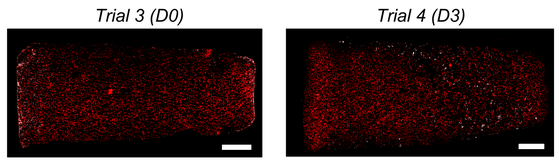
HOW CAN PRESERVATION OF NON-DECALCIFIED TISSUE SECTIONS IMPROVE STAINS FOR VISUALIZATION?

SEEDING PLLA SCAFFOLDS WITH VOCAL FOLD FIBROBLASTS AND SUBSEQUENT VISUALIZATION

To achieve a more robust infiltration of cells into the scaffolds, we first seeded with **Vocal Fold Fibroblasts (VFFs)** which have been immortalized to be tissue cultured for a long time. On the day of seeding, cells are detached from flasks/plates and split to be resuspended (800k cells/40 μL/scaffold). Scaffolds are placed on their side in a rubber holder and 20 μLs of cell solution are pipetted onto the surface in two intervals. The solution is left to saturate the scaffold for 15 mins, and the process is repeated on the opposite side. We collected scaffolds at D0, 3, and 7.



Created with BioRender.com

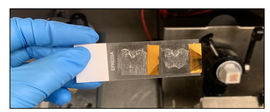


The figures above have been adjusted in ImageJ for enhanced visualization. The blue DAPI fluorescent channel is thresholded to red. Scale bars: 1 mm

To visualize cells within the scaffold, we cryo-embed and sectioned the scaffolds before staining with DAPI to visualize. Trial 5 of our scaffold seeding depicted a most improved and saturated cell infiltration into the scaffold, images not processed to be shown as of this submission.

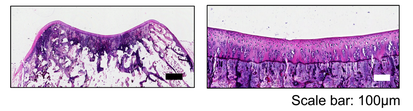
IN VITRO VISUALIZATION OF NATIVE RABBIT KNEE SAMPLES USING HISTOLOGICAL ANALYSIS

We performed histological and immunofluorescent stains at the **patellofemoral groove** to attain a more optimal visualization of osteochondral morphology. We opted for cryo-embedded samples instead of paraffin-embedded ones to avoid a decalcification process that would remove minerals or other calcified tissues from the sample.



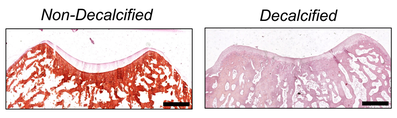
Cryosections of "non-decalcified" native rabbit knees were taken with cryo-tape helping to adhere and preserve tissue structure. The tape was mounted onto fresh slides using 0.75% chitosan in 0.375% acetic acid solution.

Hematoxylin & Eosin (H&E)
Hematoxylin (purple): nuclei + GAGs
Eosin (pink): cytoplasm + matrix



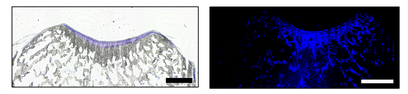
Scale bar: 100μm

Alizarin Red stains strongly for calcium, representative of bone regions.

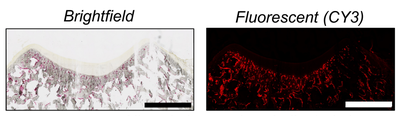


Toluidine Blue stains for nuclei + GAGs (cartilage).

Calcein Blue stains for minerals in the bone front.



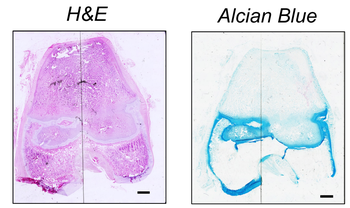
Alkaline Phosphatase (AP/ALP) is a major regulator of bone mineralization.



All scale bars unless indicated: 1 mm

CONCLUSIONS AND NEXT STEPS

Opting for cryo-embedded sections helped to avoid decalcification of samples which allowed for better histological and immunofluorescent staining options for visualization. Usage of cryo-tape helped to preserve tissue and sample structure.



Scale bars: 1 mm

Next steps:
- Continuing to seed PLLA scaffolds with VFFs.
- Validating rabbit MSCs to use for PLLA seeding and visualization.
- Testing seeding and post-scaffold repair in larger animal models with regeneration capabilities and anatomy closer to humans (i.e., see left for native pig knee samples).

ACKNOWLEDGEMENTS

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References: **[1]** Graham, Orthop. Nurs., 237-239, 2017. **[2]** Gottardi, Nat. Biomed. Eng., 502-503, 2019. **[3]** Howel et al., Curr. Rev. Musculoskelet. Med., 60-66, 2021. **[4]** Manella et al., Mater. Lett. 1-4, 2015.