Novel Fully Biodegradable Biomimetic Scaffolds for Bone Regeneration and Repair

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Statement of Purpose

Restoring lost function to failed or diseased tissues remains a major clinical challenge. Traditional biomaterials and current treatments are often ineffective for tissue regeneration and/or repair. Therefore, there is a great demand for innovative biomaterials to solve these problems. Highly bioactive and readily available tissue substitutes would revolutionize the current treatments for bone tissue loss and failure due to trauma, disease, or congenital defects, as well as significantly reduce annual health care costs. Two of the most critical factors of a scaffold in promoting tissue regeneration and/or repair are a scaffold's porous structure and its biological properties. To this end we used proprietary 3D Precision Microfabrication Technology to fabricate a biodegradable polymer scaffold with well controlled porous structures that are optimized for bone and cartilage regeneration and/or repair. To further render desired biological properties to the porous polymer scaffolds, we are further developing a bio-mimetic coating process to coat the porous scaffolds with bone matrix-like extra-cellular matrix (ECM) by culturing osteogenic cells (including normal and genetically engineered cells). Living cells and residual DNAs are removed after the ECM coating process. These biomimetic scaffolds will have a cell derived ECM coating enriched with proper growth factors, such as BMPs, to recruit stem cells to defect sites for tissue repair. These novel hybrid biomimetic scaffolds are tissue equivalents that can be mass produced without the risk of disease transmission and immune responses that are associated with allograft. These biomimetic scaffolds can be terminally sterilized and ready to use. We believe that this biomimetic coating process can be used to create superior and ready-to-use tissue equivalents for bone regeneration/repair.

Methods

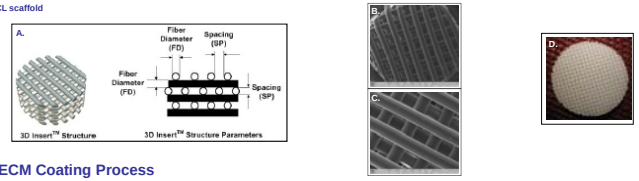
Cells

Human mesenchymal stem cells (hMSCs) and human dermal fibroblast cells (hFB) were purchased from Lonza (Walkersville, MD) and LifeLine Cell Technologies (Walkersville, MD), respectively. According to manufacturer's instructions, hMSCs and hFB were maintained in growth media. Cells were maintained in a humidified tissue culture incubator at 5% CO_2 and 37°C.

Polycaprolactone (PCL) Scaffold Microfabrication (3D Insert™-PCL)

Porous polycaprolactone (PCL) scaffolds were engineered using 3D Biotek's Proprietary 3D Precision Microfabrication Technology **(Figure 1A, D)**. Uniquely, fiber diameter is controlled by nozzle diameter while spacing between fibers is controlled by a motion control system. The struts of each layer are oriented 90° relative to the struts of the layer immediately below **(Figure 1B-C)**. Before use, scaffolds are tissue culture surface treated and γ-ray radiation sterilized. This study implemented 96-well compatible 3D Insert™-PCL scaffolds of 5 mm in diameter, 1.5 mm in thickness, and a configuration of 300 µm fiber diameter and 300 µm pore size (PCL3030). The total cell growth area of a 96-well 3D Insert™-PCL3030 is 3.3 cm^2 compared with 0.32 cm^2 of total growth area in a traditional 96-well 2D tissue culture plate (TCP).

Figure 1. 3D PCL scaffold

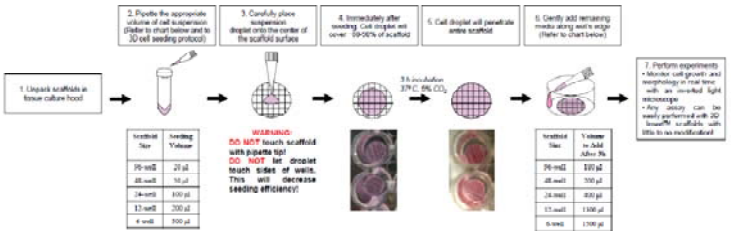


Biomimetic ECM Coating Process

3D Cell Seeding

hFB & hMSC were statically seeded onto 3D Insert™-PCL scaffolds for 24 h according to 3D Biotek's 3D cell seeding protocol **(Figure 2)**. Cells were resuspended in 1 ml of media using 3.1x10^3 cells/cm^2 (1.0x10^4 cells/PCL scaffold). To seed each scaffold, 20 µl of the cell suspension was slowly pipetted onto the top surface of each 3D Insert™-PCL. To ensure high seeding efficiency, the cell suspension droplet was not allowed to contact the sides of the wells. After a 3 h incubation in 5% CO_2 at 37°C, 180 µl of media was added to the wells containing 3D Insert™-PCL scaffolds.

Figure 2. Cell Seeding Flow-Chart



Stem Cell Differentiation

Twenty-four hours after seeding, hMSC stem cell growth media was replaced with osteoblastic differentiation media. hMSC osteoblastic differentiation was performed according to manufacturer's instructions. Every second day, hMSC and hFB cells were replenished with fresh hMSC osteogenic differentiation and hFB growth media, respectively.

Alkaline Phosphatase Activity Assay

hMSC lysates were prepared using M-PER (Pierce) followed by a centifugation at 14,000 rpm for 5 min. The lysate in supernatant was collected and analyzed using p-Nitrophenyl Phosphate Liquid Substrate System (pNPP) (Sigma) and 4-nitrophenol solution. Alkaline phosphatase activity was normalized to DNA concentration.

Von Kossa Staining

Scaffolds containing hMSC-osteo and hFB cells were fixed with 10% formalin for 0.5 h. Cultures were rinsed with ddH_2O, incubated with 2% silver nitrate, and covered. Scaffolds were incubated for 10 min and then rinsed again with ddH_2O, exposed to bright light for 15 min, dehydrated in 100% EtOH for 1 min, and then dried. Scaffolds with cells were imaged using a digital camera.

Scanning Electron Microscopy

Scaffolds containing hMSC-osteo and hFB cells were fixed with 2.5% glutaraldehyde overnight at 4°C. Cultures were rinsed well with ddH_2O and then immersed in a 1% osmium tetroxide solution, covered, and incubated for 1 h. Scaffolds with cells were rinsed well with ddH_2O and air-dried in a fume hood before imaging.

Generation of Stable Biomimetic Coating

At weeks 1, 2, 3, and 4 of culture, fibroblastic- and osteoblastic-derived ECM coated PCL scaffolds were lyophilized to destroy live cells and residual DNAs to create a stable, biomimetic coating.

hMSC Reseeding

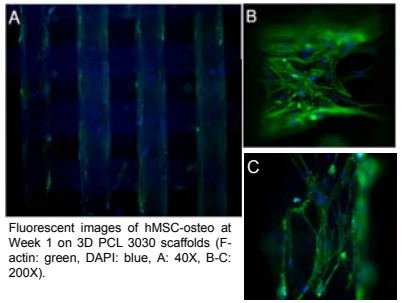
After lyophilization, fresh hMSCs were reseeded onto week 1, 2, 3, and 4 fibroblastic- and osteoblastic-derived ECM coated PCL scaffolds using 1x10^4 cells/96-well-3D PCL scaffolds resuspended in 20 µl of hMSC or hFB growth media. After 24 h, hMSC media was changed to contain osteoblastic differentiation media. hFB and hMSC osteoblastic differentiation media were changed every second day.

Results

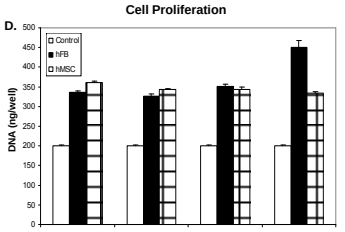
Cell Proliferation

Fibroblastic and osteoblastic cell growth on 3D Insert™-PCL was monitored using an inverted light and fluorescent microscope. Fluorescent images show that osteoblastic cells are viable and grow along PCL fibers **Figure 3A, C)** and within the pores **(Figure 3B)**. Fluorescent DNA assays (Sigma) performed at weeks 1-4 confirmed increases in fibroblastic cell proliferation and a plateau of osteoblastic proliferation, indicative of their differentiation into osteoblasts **(Figure 3D)**. Ultimately, 3D PCL scaffolds support human cell proliferation that can be easily monitored.

Figure 3. 3D Insert-PCL



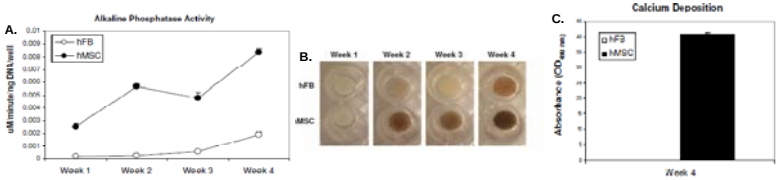
Fluorescent images of hMSC-osteo at Week 1 on 3D PCL 3030 scaffolds (F-actin: green, DAPI: blue, A: 40X, B-C: 200X).



Osteoblastic Differentiation

Compared with fibroblastic cells, osteoblastic cells on PCL scaffolds demonstrated increasing and enhanced alkaline phosphatase (ALP) activity at all time points during culture **(Figure 4A)**. Furthermore, Von Kossa staining **(Figure 4B)** and calcium deposition assay **(Fig. 4C)** reveal that 3D InsertTM-PCL scaffolds support effective hMSC differentiation into the osteoblastic lineage. Finally, cell growth and morphology of freshly seeded hMSCs into decellularized fibroblastic- and osteoblastic-derived ECM coated porous PCL scaffolds was monitored in real-time with a light microscope.

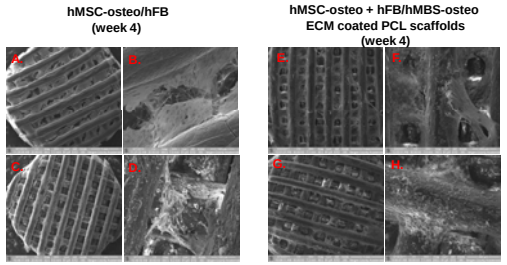
Figure 4. Osteoblastic Differentiation



Biomimetic Coating Development

Scanning electron micrographs show that week 4 hMSC-osteo and hFB cells grow along scaffold fibers and extend across pores. SEM analysis demonstrates that only hMSC-osteoblasts form nodules containing calcium and phosphate **(Figure 5C, D)**, whereas hFBs **(Figure 5A, B)** form 3D sheet-like structures. Further, analysis of week 4 cultures of hMSC-osteo reseeded onto lyophilized week 4 fibroblastic- and osteoblastic-derived ECM coated PCL scaffolds **(Figure 4E-H)** also demonstrated nodule development containing calcium and phosphate on both scaffolds, indicating that both hFB and hMSC-osteo ECM supports and promotes osteoblastic differentiation. However, hMSC-osteo ECM promoted fast mineral nodule formation as compared to hFB ECM coating.

Figure 5. SEM



Discussion

This study demonstrates that it is possible to create an ideal scaffold with desired porous structure and bioactivity through a combination of 3D Precision Micro-fabrication and a cell culture biomimetic coating processes. Specifically, a bone matrix like ECM can be applied to a porous PCL scaffold to create a tissue equivalent for bone repair/regenereation. hMSCs that proliferate and differentiated into the osteoblastic lineage on these PCL scaffolds generated a uniform, cell-derived bone matrix-like ECM coating that is richly incorporated with osteoinductive and osteoconductive substances.

Acknowledgements

This research has been supported by a Small Business Innovative Research Phase I Grant (1R43RR028022-01) from the National Center for Research Resources at the National Institutes of Health and a Edison Innovative R&D grant from New Jersey Commission on Science and Technology (NJCST).

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