Dynamic Culture Using Novel Slow Perfusion Biroeactor and Porous Polycaprolactone (PCL) Scaffolds for Bone Regeneration and Repair

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Abstract

In the United States nearly 20 million people have osteoarthritis, a disease which is characterized by the degeneration of joints, including bone and cartilage. As a result, back pain is the second leading cause of sick leave. Further, osteoporotic fractures have doubled in the last decade, and 40% of all women over the age of 50 will suffer from an osteoporotic-related fracture in their life-time. Traditional biomaterials and current treatments for bone and cartilage regeneration and/or repair are unsatisfactory, and have lead to a great demand for innovative biomaterials and scaffolds.

Porous structure and biological properties of the scaffolds are two of the most important factors in promoting tissue regeneration. Using proprietary 3D Precision Microfabrication Technology, we are able to fabricate porous biodegradable polymer scaffolds with well controlled porous structures that are readily compatible with our novel slow perfusion bioreactor system.

Therapies today use bone harvesting, demineralized freeze-dried cadaver bones, metals, or plastic. Current growth factor delivery to an injured/diseased tissue results in serious systemic side effects associated with high doses of these factors necessary for therapeutic effects. When combined with our bioreactor, these porous PCL scaffolds will promote human stem cell proliferation, osteoblastic differentiation, and the generation of a uniform, cell-derived extracellular matrix (ECM) coating richly incorporated with osteoinductive and osteoconductive factors. Using the microenvironment created by these naturally coated PCL scaffolds, it is now possible to localize and enhance growth factor delivery to specific sites for bone regeneration/repair.

Combined, these therapeutic technologies will reduce long-term costs associated with current therapies. They will also increase functional tissue availability, eliminate host immune responses and disease transmission, as well as reduce repeat surgeries, long hospital stays, and rehabilitation.

Materials and Methods

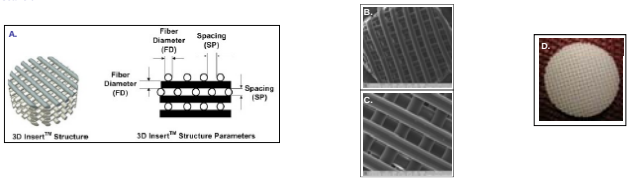
Cells

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 Precision Microfabrication Technology **(Figure 1A)**. Uniquely, fiber diameter is controlled by nozzle diameter while spacing between fibers is controlled by a motion control system. Scanning electron micrographs demonstrate that 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-radiation sterilized. This study implemented 24-well compatible 3D Insert™-PCL scaffolds, 1.9 cm^2 in diameter mm in diameter and 1.6 mm in thickness, with a configuration of 300 µm fiber diameter and 300 µm pore size (PCL3030). The total cell growth area of a 24-well 3D Insert™-PCL3030 is 18.28 cm^2 compared with 1.9 cm^2 of total growth area in a traditional 24-well 2D TCP **(Figure 1D)**.

Figure 1. 3D PCL scaffold



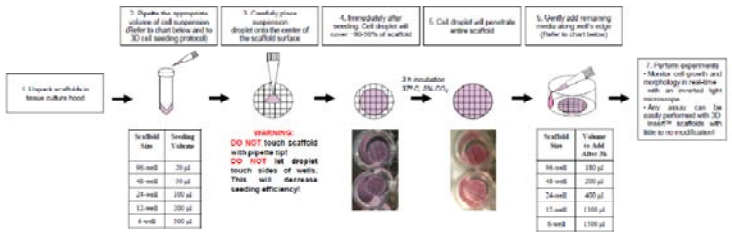
3D Cell Seeding

Cells were initially 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 growth media using $5x10^3$ cells/scaffold. To seed each scaffold, 100 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, 400 µl of media was added to the 3D wells. After 24 h, scaffolds containing cells were loaded into the slow perfusion system according to 3D Biotek's protocol.

Stem Cell Differentiation

Immediately upon loading scaffolds containing hMSCs into the bioreactor, hMSC osteoblastic differentiation was begun. hMSC osteoblastic differentiation was performed according to manufacturer's instructions.

Figure 2. Cell Seeding Flow-Chart



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.

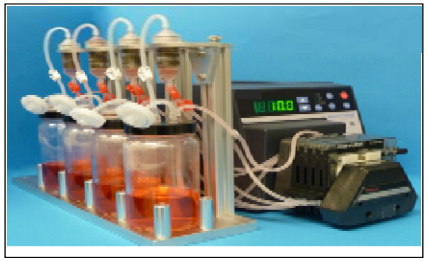
Alkaline Phosphatase Activity Assay

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 with a digital camera.

Slow Perfusion Bioreactor Culture

Scaffolds containing hMSC and hFB were loaded into 3D Biotek's proprietary slow perfusion bioreactor system **(Figure 3)**. The bioreactor consists of four independent, autoclavable polycarbonate chambers that hold up to 10 24-well scaffolds each. The chambers are interchangeable and are compatible with 3D Insert™-PCL scaffolds ranging in size from 96-well to 6-well. Within each chamber there is a 1.5 mm distance separating each scaffold. This distance, combined with the offset fiber engineering ensures perfusion of media. Slow perfusion of sterile CO_2 and media is achieved with a pump speed of 12 rpm through silicone tubing. Medias for hMSC-osteoblastic differentiation and hFB culture were changed every week of culture. At day of assay, chambers containing scaffolds were easily removed. The entire unit is autoclavable and can be used as a single-use bioreactor system.

Figure 3. 3D PCL scaffolds are compatible with slow perfusion bioreactor system

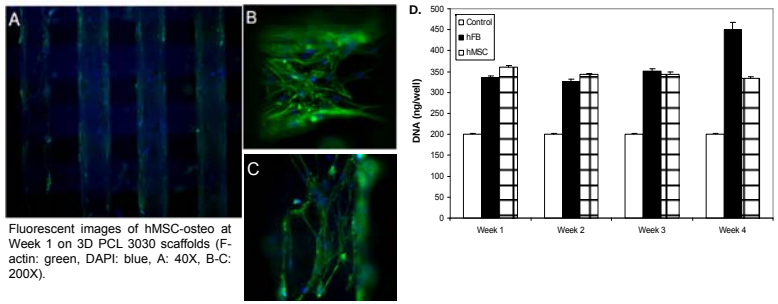


Results

DNA 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 4A, C)** and within the pores **(Figure 4B)**. 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 4D)**. Ultimately, 3D PCL scaffolds support human cell proliferation that can be easily monitored.

Figure 4. 3D Insert-PCL

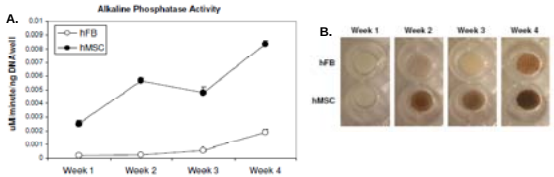


Fluorescent images of hMSC-osteo at Week 1 on 3D PCL 3030 scaffolds (F-actin: green, DAPI: blue, A: 40X, B-C: 200X).

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 5A)**. Furthermore, Von Kossa staining **(Figure 5B)** and calcium deposition assay (Fig. 3C) 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 5. Osteoblastic Differentiation



Conclusion

This study demonstrates the intention of combining 3D Insert™-PCL scaffolds with 3D Biotek's new Slow Perfusion Bioreactor System, to engineer a powerful therapeutic technology for bone regeneration and/or repair.

Acknowledgements

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