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Osteogenic differentiation of dura mater stem cells cultured in vitro on three-dimensional porous scaffolds of poly(ϵ -caprolactone) fabricated via co-extrusion and gas foaming

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Abstract

A novel scaffold fabrication method utilizing both polymer blend extrusion and gas foaming techniques to control pore size distribution is presented. Seventy five per cent of all pores produced using polymer blend extrusion alone were less than 50 μm . Introducing a gas technique provided better control of pore size distribution, expanding the range from 0-50 to 0-350 μm . Varying sintering time, annealing temperature and foaming pressure also helped reduced the percentage of pore sizes below 50 μm . Scaffolds chosen for in vitro cellular studies had a pore size distribution of 0-300 μm , average pore size $66 \pm 17 \mu\text{m}$, $0.54 \pm 0.02\%$ porosity and 98% interconnectivity, measured by micro computed tomography (microCT) analysis. The ability of the scaffolds to support osteogenic differentiation and cranial defect repair was evaluated by static and dynamic ($0.035 \pm 0.006 \text{ m s}^{-1}$ terminal velocity) cultivation with dura mater stem cells (DSCs). In vitro studies showed minimal increases in proliferation over 28 days in culture in osteogenic media. Alkaline phosphatase expression remained constant throughout the study. Moderate increases in matrix deposition, as assessed by histochemical staining and microCT analysis, occurred at later time points, days 21 and 28. Although constructs cultured dynamically showed greater mineralization than static conditions, these trends were not significant. It remains unclear whether bioreactor culture of DSCs is advantageous for bone tissue engineering applications. However, these studies show that polycaprolactone (PCL) scaffolds alone, without the addition of other co-polymers or ceramics, support long-term attachment and mineralization of DSCs throughout the entire porous scaffold.

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Keywords

polycaprolactone; polymer blend; gas foaming; bioreactor culture; dura; bone repair

1. INTRODUCTION

Restoration of form and function to the craniofacial skeleton following trauma, congenital malformations or resection poses a considerable clinical challenge for the plastic surgeon. Materials available to treat these conditions include autografts (tissue from the patient), allografts (tissue from a donor) and synthetic materials such as metals, ceramics and polymers. Although autografts provide the best clinical outcome when compared with other material options, complications, including post-operative pain [1], donor site morbidity [2] and infection [3], affect approximately 10-30% of patients [4]. Furthermore, autologous tissue may not be an option for large defects given the limitation in supply. In contrast, bone allografts are not limited in supply but often result in poor functional outcome due to limited revascularization, low cellularity and higher resorption rates [5]. Alternatively, metal synthetics are permanent fixtures which suffer from fibrous encapsulation [6], device translocation from bone apposition/resorptive patterns [7], hypersensitivity [8] and long-term stress shielding, ultimately resulting in bone failure [9]. A variety of biodegradable polymers in combination with novel fabrication techniques have been explored as alternative solutions to repair large cranial defects.

In cranial bone repair, dural and periosteal tissue layers directly apposed to cranial bone play an important role in the repair process. Recent findings have suggested that the secretion of cytokines and/or migration of osteoprogenitor cells from these layers contribute to the repair process and functional outcome of cranial bone healing [10,11,12,13]. Scaffold parameters such as pore size and pore structure may directly influence the diffusion of factors and migration of progenitor populations from these adjacent tissues into the defect area. Therefore, tailoring such scaffold characteristics to support these activities may significantly improve new bone growth.

Several different techniques have been established to create open pore structures using degradable polymers. The most common three-dimensional (3-D) biodegradable polymer scaffolding fabrication techniques include solvent extraction [14], particulate leaching [15], microsphere sintering [16], gel casting [17], phase separation [18] and 3-D printing [19,20]. Alternatively, polymer leaching is a particularly attractive technique. This cost-effective method yields fully interconnected porous networks where interconnections are characterized by cylinders and not small pores connecting larger ones. However, maximum porosity is usually limited to 50-60%. Alternatively, combination methods using both particulate and polymer leaching techniques have shown dramatic improvement in pore size control and have enabled the fabrication of scaffolds with multimodal pore size distributions [21].

More recently, Washburn et al. published a co-polymer extrusion technique using two immiscible polymers, where one is water-soluble [22]. In this study, a new scaffold fabrication technique is developed where this polymer blend extrusion method is combined with gas foaming to create scaffolds with optimal pore size distribution. Compared with polymer leaching techniques used to create porous structures, this method uses a water-soluble polymer, poly(ethylene oxide) (PEO), which does not require potentially toxic organic solvents for dissolution. Additionally, PEO itself is non-toxic to cells; therefore residual polymer should not affect cell viability. This technique allows for scaffolds to be fabricated into thin disks (<1 μm) using a microtome, an important feature for cranial defect implants where both rat and mouse skull anatomy dictates thicknesses <1 μm .

The blending process developed by Washburn et al is accomplished using a twin-screw extrusion compounder. The polymers are added simultaneously to the compounder, heated to the operating temperature (above the melting temperature of both polymers) and the blend is mechanically mixed. Factors that alter the resulting blended morphology include polymer ratios, polymer viscosities and mixing conditions (temperature and shear) [20]. Here, poly(ϵ -caprolactone) (PCL) a Food and Drug Administration (FDA)-approved biodegradable polyester, is blended with PEO, which is also biocompatible and water-soluble. Following blending of PCL with PEO using a twin-screw extruder, a gas foaming technique is applied to create an open pore structure. Subsequently, the water-soluble PEO is dissolved to enhance the overall pore size.

Utilizing PCL to heal large cranial defects may be particularly advantageous given what is already known about this synthetic polymer. PCL degrades by hydrolytic scission with resistance to rapid hydrolysis via its hydrolytic aliphatic-ester linkage [23]. Degradation times can extend for up to 24 months. PCL scaffolds alone, without co-blending of other polymers, yield mechanical properties adequate for craniofacial bone repair [24]. Additionally, PCL scaffolds support mesenchymal stem cell attachment, proliferation, osteogenic differentiation, and aid in bone repair of critical sized rabbit cranial defects [25,26]. Its degradation, mechanical strength and biocompatibility properties make PCL an excellent polymer for long-term cranial bone applications.

Cells proposed for bone regeneration have been isolated from a range of different sources including bone, bone marrow, skin, adipose tissue and dental pulp [27,28,29,30,31]. Within the craniofacial community, it is widely accepted that progenitor cells located in underlying dural tissue play a crucial role in cranial skull morphogenesis and repair. Strategies to recruit these populations into damaged cranial bone areas have been a focus within our laboratory. Initial studies characterizing dura mater stem cells (DSCs), isolated from local stem cell niches underlying host cranial bone, indicate that these cells show promise as cellular agents for defect repair. Specifically, our group was the first to show cells from the dura mater tissue can be isolated, cultured and expanded in vitro up to passage 60 without changes in morphology or doubling time (2.5 days) [32]. Furthermore, this multipotent population demonstrates robust mineralized matrix deposition following 28 days induction using an osteogenic media [33]. Cells cultured long term on both 2-D films and 3-D microsphere-based scaffolds of 85:15 PLGA also demonstrate robust matrix deposition [34]. More research is needed to optimize the intended in vivo delivery construct. Others have previously shown that PCL is an excellent synthetic dural substitute showing no infection or significant adhesion to the underlying cortex [35]. Therefore, delivery of transplanted DSCs on a dural substitute material may encourage host DSCs to migrate to the wound bed to participate in tissue repair. Furthermore, this polymer has been shown to exhibit mechanical properties near the range of cranial bone [22], demonstrating its ability to provide structural integrity during the repair process.

In this study, we hypothesize that PCL scaffolds, fabricated using both gas foaming and extrusion techniques to achieve optimal pore size distributions, will permit long-term expansion and cultivate significant mineralization of DSCs in vitro. Sintering time and temperature will also be varied to understand how these parameters alter pore size distributions of 3-D PCL scaffolds. Concurrently, the ability of DSCs to attach, grow and differentiate on these porous biodegradable 3-D scaffolds will also be evaluated using histochemical staining and 3-D reconstructed micro computed tomography (microCT) techniques.

2. MATERIALS AND METHODS

2.1 PEO/PCL extrusion and gas foaming technique

PCL ($M_N = 80K \text{ g mol}^{-1}$, $\rho = 1.15 \text{ g cm}^{-3}$; Sigma Aldrich) is blended with PEO ($M_N = 100K \text{ g mol}^{-1}$, $\rho = 1.20 \text{ g cm}^{-3}$; Polysciences) via an extrusion technique. The Cox-Merz rule is used to determine the appropriate polymer ratios to ensure a co-continuous structure. This rule generates predictions of blending behavior at high shear rates. From the viscosity values of PCL and PEO, 4700 and 4200 Pa s, respectively, the viscosity ratio is determined as 1.1, suggesting that a co-continuous structure should be observed with approximately 50% PCL composition. Therefore, a mass ratio blend of 50:50 PCL:PEO is added simultaneously to the inlet of a twin-screw mini-compounder (Daca Instruments, Goleta, CA), and mixed at 50 rpm, 100°C, for 10 min. The blends of polymer are recovered at the exit port and collected as tubes 8 mm in diameter. The 8 mm diameter PCL/PEO polymer tubes are cut into cylinders 4 mm long and heated in a vacuum oven at either 80, 100, 110 or 120°C, above the melting temperatures of both PCL and PEO. Once heated, the polymers are foamed using ambient gas at a pressure of 20 mmHg (2.67 kPa) or 10 mmHg (1.34 kPa). This change in pressure causes nucleation and creation of gas bubbles, hence new pore volumes, within the PCL/PEO polymer tubes. The samples are then transferred to individual containers filled with deionized water and agitated on a rotator (New Brunswick Scientific) at 100 cycles min^{-1} to leach out the water-soluble PEO. The purpose of leaching away the PEO is to increase existing pore interconnections and create new ones throughout the scaffold of sufficient size to allow for tissue infiltration. The space occupied by the PEO within the polymer blend becomes a network of connected pores once the PEO is leached away. Following 48 h of agitation, the samples are removed, washed in methanol and returned to the vacuum oven at room temp for 8 h. The samples are weighed to confirm complete removal of the PEO.

2.2 Pore size distribution calculations

Mercury porosimetry (Micromeritics Autopore III porosimeter; Micromeritics, Norcross, GA) was used to quantitatively assess pore size distribution of the extruded gas foamed scaffolds. Empty 5 ml penetrometers were weighed to determine the initial apparatus weight. Then six samples per group were sealed into individual penetrometers, the apparatus was weighed again, and mercury intrusion was initiated at 0.5 psia up to a maximum of 50 psia. Micromeritics Software analysis, which reports pore diameter as a function of the external pressure necessary to force liquid mercury into the existing porous structure, reported total pore area, percentage porosity, bulk density, median pore diameter and pore size distribution following completion of the experiment (Table 1). The original pore size data represented was binned into size ranges of 50 μm increments shown on graphs in **Figs. 1-3**. The mercury porosimetry apparatus used in these studies can measure pore size distributions with 0.003 μm sensitivity. Pore size distributions calculated using mercury porosimetry also matched well with scanning electron microscopy (SEM) images. SEM analysis was used to obtain representative images of pore size distributions within each experimental group.

2.3 Cell isolation, culture, characterization

Primary DSCs were isolated using explant culture as previously described [32]. NIH guidelines for the care and use of laboratory animals were observed and all isolation procedures were performed according to a protocol approved by the Institutional Animal Care Committee at the University of Virginia [36]. Briefly, the dural tissue lying beneath the frontal and parietal bones was removed aseptically from rat fetuses at 19 days. The tissue was washed in a physiological buffer and plated in tissue culture dishes with basal media (10% FBS, 1% L-glutamine, 0.5% gentamycin in DMEM) to allow outgrowth of DSCs from these explants. DSCs were maintained at subconfluent levels, cultured at 37°C with 5% CO_2 , and only passage 5 or less were used for these experiments.

In vitro scaffolds were placed individually into wells of a 24-well plate. Each scaffold was seeded with 37,000 DSCs suspended in 50 μ l of media. Following 2 h of incubation, wells were filled with 1 ml of basal media. Following 2 days in culture, basal media was exchanged for osteogenic media (10% FBS, 1% L-glutamine, 0.5% gentamycin, 50 μ M ascorbic acid, and a mix of organic/inorganic phosphates). Half the DSC-seeded scaffolds remained in the 24-well plate. The other half was moved to a high aspect ratio vessel (HARV) and cultured dynamically for the remainder of the experiment. Osteogenic media was exchanged every 3-4 days in both static and dynamic culture systems.

During the 28 day culture period, DSC-seeded PCL scaffolds were evaluated for proliferation, alkaline phosphatase expression and mineralized matrix production at 7, 14, 21 and 28 days following osteogenic induction as previously described [32]. Briefly, proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) mitochondrial reduction kit. At each time point, fresh media was incubated for 2 h at 37C with the MTT solution (2:10 ratio) and the absorbance of the resulting supernatant was read with a spectrophotometer at 570 nm. A standard curve to relate cell number to absorbance was established to determine cell numbers in the unknown samples. Alkaline phosphatase expression was quantified using an enzymatic reaction where the presence of alkaline phosphatase dephosphorylates p-nitrophenyl phosphate, resulting in a product that can be quantified on a spectrophotometer at 415 nm.

Mineralized matrix deposition was analyzed by two different methods. First, an alizarin red staining method specific for calcium deposition was used to qualitatively and quantitatively assess mineralized matrix. This assay uses a colorimetric analysis technique based on solubilizing the red matrix precipitate with cetylpyridinium chloride to yield a purple solution quantified on a spectrophotometer at 620 nm. Second, a quantitative microCT specimen scanner (VivaCT 40, Scanco, Basserdorf, Switzerland) was used to measure and assess the spatial distribution of matrix deposition. A high-resolution 45 kVp specimen scan was performed on each scaffold to determine appropriate threshold values of scaffold material and new mineralization. Following 2-D reconstruction, the upper threshold defined grayscale values equal to new mineralization and the lower threshold defined grayscale values equal to scaffold material. Bone volume, as defined by a 100-1000 threshold, was reported for each scaffold in mm³. The percentage of new bone volume was calculated by dividing the reported bone volume by the known volume of the original scaffold.

2.4 Statistical significance

Results are presented as mean $\pm$ SEM. All comparisons were made using a one-way ANOVA, followed by Tukey's test for pairwise comparisons. Significance was set at $P < 0.05$.

3. RESULTS

3.1 Pore diameter distributions

The PCL/PEO extrusion fabrication method alone was evaluated for pore size distributions. Variations in annealing times (15, 30, 60 min) at constant temperature (100°C) were evaluated to determine the effects of sintering time on pore size. All three annealing times using extrusion alone yielded large pore areas but low porosity (35-62%) and low pore size distributions with median pore diameters between 6.031 and 14.030 μ m (Fig. 1, Table 1). SEM micrographs confirm the small pore diameters. Furthermore, SEM revealed smooth circular pores with poor connectivity between neighboring pores.

Gas foaming was introduced following co-extrusion of the two polymers. The gas pressure, 20 mmHg (2.67 kPa), and annealing time, 60 min, were held constant while variations in

annealing temperature were evaluated. Introducing gas foaming into the fabrication process encouraged a larger spread of pore sizes, predominantly between 0 and 250 μm (Fig. 2, Table 1). Increases in annealing temperature (80, 100, 110, 120°C) caused large increases in the median pore size diameter (64.181- μm to 110.298 μm) and total porosity (41-48%). The highest temperature, 120°C, yielded the largest and most even spread of diameters, with 10-20% within each size range, except 0-50 μm . SEM confirmed that the lowest temperature, 80°C, yielded the smallest pore size distribution, whereas increases in temperature led to increased pore size. Again, interconnectivity between existing pores was poor, as assessed by SEM.

The effects of annealing time (15, 30, 60 min) on pore structure were investigated while holding gas pressure (1.34 kPa) and annealing temperature (110°C) constant. Each group yielded similar pore size distributions, with a bell curve centered at the 50-100 μm range (Fig. 3, Table 1). Similar to changes in annealing temperature, increases in annealing time increased the median pore size diameter (70.248-110.298 μm). In contrast, total porosity (49-59%) and total pore areas (0.042-0.092 $\text{m}^2 \text{g}^{-1}$) decreased with increased annealing times. SEM confirms the size distributions and also demonstrates significant interconnectivity between neighboring pores.

Overall bulk density measurements of foamed samples following leaching (obtained using mercury porosimetry) ranged from 0.563 to 0.777 g ml^{-1} . Gas foamed co-extruded scaffolds with 10 mmHg (1.34 kPa) gas pressure, 15 min annealing at 110°C were used for in vitro cell culture studies to test the hypothesis that constructs with smaller pore size ranges and significant interconnectivity between pores may be more permissive to local progenitor migration and support subsequent osteogenic differentiation within cranial defect areas.

3.2 Scaffold characterization

Unseeded scaffolds were scanned at high resolution using a microCT scanner. 2-D reconstructed grayscale images confirm porous structure throughout the thickness of the scaffolds (Fig. 4). Using appropriate thresholds to define the radio opacity of PCL, 3-D evaluation software calculated the total pore volume to be 0.54 ± 0.02 , 98% interconnectivity, with an average pore size of $66 \pm 17 \mu\text{m}$, values in close agreement with mercury porosimetry results. Motion analysis of porous scaffolds in the HARV rotating bioreactor was described previously by Botchwey et al. [37,38]. Two distinct regions of dynamic fluid flow are present during 3-D dynamic culture in the HARV: (1) an exterior region directly exposed to scaffold motion through the fluid, and (2) an interior region exposed only to fluid perfusion through the porous scaffold network. Exterior fluid flow is equal to the free-fall speed of scaffold through the fluid, which is primarily determined by the net gravitational force acting on the construct. Previous experimental and numerical modeling studies have shown that internal fluid perfusion is approximately 1% of the exterior fluid flow rate. PCL scaffolds utilized in this study exhibited a free fall speed of $0.035 \pm 0.006 \text{ m s}^{-1}$.

3.3 In vitro cell culture studies

DSCs were seeded onto PCL scaffolds at an initial seed density of 25,000 cells cm^{-2} . Following 2 days in base media, scaffolds were transferred into osteogenic media. Half of the scaffolds were cultured dynamically in a HARV system to evaluate the effects of flow on DSC osteogenic differentiation. Quantitative proliferation results showed minimal increases in proliferation over 28 days of culture with no statistical difference between static and dynamic culture (Fig. 5A). Quantitative alkaline phosphatase staining exhibited constant expression levels over 28 days with no statistical difference between static and dynamic culture (Fig. 5B). Total alizarin staining moderately increased from day 7 to day 28. Although dynamic mineralization values were higher than static at all time points, these differences were not statistically different (Fig. 5C). Qualitative microscopic images of alizarin staining showed robust mineralization by day

21, with moderate increases in staining intensity at day 28. Again, dynamic staining appears greater than static at both time points (Fig. 6). Mineralization was further characterized using high-resolution microCT scans following 28 days' culture in osteogenic media. 3-D reconstructed images show remaining scaffold material in translucent blue and new mineralization in solid white (Fig. 7). MicroCT imaging dissection allowed for evaluation of spatial distribution of matrix deposition throughout the scaffold interior.

4. DISCUSSION

In this study, a novel scaffold fabrication method is developed to optimize pore size distribution by combining two existing methods: co-polymer extrusion and gas foaming. Co-polymer extrusion is an ideal method for creating porous structures intended for implantation in vivo. This method creates an open, porous structure by leaching out water-soluble PEO from its non-soluble counterpart, PCL. This technique avoids the use of harsh organic solvents or salt solutions common in other polymer processing techniques where residual amounts of these solutions can remain after processing and have an adverse affect on cell-material interactions and in vivo healing.

Although pore size does not appear to influence in vitro osteogenesis [39], it remains unclear how pore size distribution affects osteogenesis in vivo. Hulbert et al. first reported a minimal pore size requirement of 100 μm in vivo [40] for cellular infiltration into the implanted construct. Others have reported 300-400 μm as the critical threshold above which capillaries could infiltrate constructs [41,42] thereby establishing nutrient blood flow within the scaffold, a process that is critical to the long-term viability of implanted biomaterials. Studies using PLGA microsphere-based scaffolds to heal cranial defects found 56% of the void volume filled with new bone by 90 days using scaffolds with average pore sizes of 120 μm [32]. Still others have observed no differences in cranial bone regeneration using scaffolds with pore sizes of 179, 218 and 353 μm [43]. In this study, co-polymer extrusion alone is not sufficient to produce optimal pore structures above 100 μm , the minimal requirement according to Hulbert. Close to 75% of all pores generated by co-polymer extrusion alone were less than 50 μm . Incorporating a gas foaming technique provided better control of pore size distribution by expanding the range in pore sizes from 0-50 to 0-350 μm . Varying these parameters also appeared to enhance the interconnectivity of the existing pore structure visualized by SEM. Combining these techniques not only increases the diameter of individual pores but also provides better control over the distribution of pore size ranges, providing for wider application of this scaffolding method.

In cranial bone repair applications, migration of progenitor populations from both the cranial sinus and underlying dural tissue are known to play dominant roles in the repair process. Therefore, a key strategy to improving functional outcome is to promote cellular infiltration from progenitor sources proximal to this defect. As previously mentioned, most studies using synthetic bone repair constructs report the greatest amount of regeneration for median pore diameters of 100-300 μm . However, in terms of supporting stem cell attachment and infiltration, nanofibrous meshes of 20-45 μm in diameter have proved successful using mesenchymal stem cells (MSCs) [44]. Other studies have observed 50% healing of critical sized cranial defects using median pore sizes of 16 μm [45]. Therefore, the optimal pore size for promotion of meningeal stem cell migration and differentiation in cranial bone defects has yet to be established. In this study, as an initial design goal, scaffolds with a smaller pore size range were used to test the ability of DSCs to infiltrate and mineralize these scaffolds. This scaffold group had pore sizes ranging from 0 to 300 μm with $0.54 \pm 0.02\%$ porosity, 66 ± 17 μm average pore size and 98% interconnectivity. MicroCT measurements of pore size are in close agreement with averages calculated using mercury porosimetry. These results were similar to those previously published [46].

Although not directly measured in this experiment, mechanical testing on 50% PCL scaffolds was previously performed and presented [22]. Briefly, it was shown that 50% PCL scaffolds had moduli that measured 1.5 MPa. This value is lower than values for cranial bone (3-10 MPa) but in the case of this defect model, appropriate scaffold thickness, flexibility to conform to the skull shape and an open porous structure may be more important parameters for successful tissue integration compared with exact matches to mechanical properties. Mechanical testing of these samples will be further explored in future studies comparing this method with other processing techniques.

Following initial cell seeding, a small drop in proliferation was observed at initial time points in dynamic culture. This decrease in proliferation is observed in other bioreactor studies [47], and is most likely the result of shear forces. Osteoblastic differentiation of DSCs on PCL scaffolds was characterized using several different methods. Alkaline phosphatase expression, an early marker of osteoblast maturation which acts as a phosphate donor during the mineralization process, was quantified by histochemical staining. Dynamic culture demonstrated minimal increases by day 28, whereas expression levels remained constant across all static time points. The results from this study were different compared with typical expression curves, which demonstrate peak staining between 7 and 14 days [48]. Micrograph images show intense alizarin red staining at day 21 with moderate increases at day 28. Quantitative values of mineralization were measured using both histochemical staining and microCT analysis. Staining results show increasing levels of mineralization across all time points, with greater mineralization at day 28. MicroCT results at day 28 revealed new bone volume of 0.4 mm³, which represents approximately 2% of the void volume. In vitro osteogenesis can be enhanced by decreasing scaffold porosity [33]. However, these scaffolds are intended for in vivo studies where maximal porosity is advantageous. Future studies will continue to alter fabrication parameter to maximize the percent porosity. 3-D reconstructed images of scaffold mineralization demonstrate matrix deposition across outer surfaces. Although the majority of matrix deposits were found on the outer surfaces, microCT imaging dissection allowed for spatial visualization of mineralization throughout the scaffold thickness. Using this feature, significant matrix deposition at center of the scaffold was confirmed, although spatial deposition throughout the scaffold was inhomogeneous. This non-uniform distribution of matrix is most likely due to non-uniform seeding densities, an issue often confronted in studies where cells are seeded statically onto constructs, as was the case in this experiment. Future studies using dynamic seeding techniques will be used to improve cell distribution issues.

To our knowledge, this is the first study to investigate the responsiveness of DSCs to flow stimulus in a HARV bioreactor. Dynamic culture was introduced to evaluate the effects of flow on DSC osteogenic differentiation. Many bone tissue engineering studies have shown that mechanical stimulation via flow enhances seeding uniformity [49] and osteoprogenitor differentiation [50]. Furthermore, dynamic culture also facilitates a well-mixed culture environment, thereby improving nutrient/waste mass transport throughout the structure [51] and minimizing local decreases in pH caused by polymer acidic degradation [52]. In this study, alizarin red staining following 21 and 28 days of osteogenic induction appear to show greater staining in scaffolds cultivated in dynamic culture. Quantitative measurements of matrix deposition, both by histochemical staining and microCT evaluation, show greater mineralization of dynamically cultured scaffolds. However, these trends are not statistically significant. Furthermore, alkaline phosphatase expression profiles show greater activity in dynamic culture across all time points, but the trend is not significant. Based on these results, it remains unclear whether bioreactor culture is necessarily beneficial for DSC osteogenic cultivation in vitro. Our original hypothesis, which predicted dynamic culture would further enhance mineralized matrix staining of DSCs cultured on PCL scaffolds, was not supported here. This may suggest that DSCs are unique to other osteoprogenitors in that flow stimulation

does not further enhance osteogenic differentiation over static culture. In this case, DSCs may be more sensitive to media supplementation rather than flow stimulus.

Furthermore, while the subtle differences between static and dynamic culture could be related to limited interconnectivity and therefore limitations in flow penetration through the scaffold, we believe that the small scaffold pore size is not the decisive factor. Others have shown that cells cultured on PCL nanofibers with micron-sized pores were able to support cellular infiltration and differentiation in both static and dynamic culture regimes [53]. Additionally, Chastain et al. demonstrated that MSCs adhere to PCL primarily via vitronectin and that this integrin-mediated adhesion was responsible for lower levels of alkaline phosphatase expression compared to MSCs cultured on PLGA, a type-I collagen-mediated event [54]. Because this is the first report of DSCs cultured on PCL scaffolds, as well as the first investigation on the role of dynamic culture of DSCs, further studies are necessary to elucidate the role of DSC adhesion to PCL scaffolds in promoting osteogenic differentiation.

5. CONCLUSIONS

These studies show that incorporating gas foaming techniques with co-polymer extrusion provides greater control of pore size distribution without the use of salts or harsh chemicals. PCL scaffolds alone, without the addition of other co-polymers or ceramics, support long-term attachment and mineralization of DSCs throughout the entire scaffold. However, it remains inconclusive whether bioreactor culture of DSCs is advantageous for bone tissue engineering applications. Furthermore, additional studies are needed to identify an optimal scaffold pore size range to support meningeal stem cell migration and subsequent osteogenic differentiation, processes crucial for cranial bone repair.

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Figure 1.

Pore diameter distribution percentages across various diameter ranges (A-C). SEM images of pore structure; scale bar = 100 μm (D-F). Temperature held constant at 110°C. Variations in sintering time: (A,D) 15 min sintering; (B,E) 30 min sintering; (C) 60 min sintering.

Figure 2.

Pore diameter distribution percentages across various diameter ranges (A-D). SEM images of pore structure; scale bar = 100 μm (E-H). Variations in temperature with pressure, 20 mmHg (2.67 kPa), and time, 1 h, held constant. (A, E) 80°C; (B, F) 100 °C; (C, G) 110 °C; (D, H) 120 °C.

Figure 3.

Pore diameter distribution percentages across various diameter ranges (A-C). SEM images of pore structure; scale bar = 100 μm (D-F). Pressure and temperature held constant at 10 mmHg (1.34 kPa) and 110°C. Variations in sintering time: (A, D) 15 min/sintering; (B, E) 30 min sintering; (C,F) 60 min sintering.

Figure 4.

2-D reconstructed slice of unseeded scaffold scanned at high resolution in microCT. Scaffold properties such as pore volume, pore size and terminal velocity reported below. The processing conditions used to produce the scaffold shown here are 10 mmHg (1.34 kPa) pressure, 110°C, and 15 min annealing time. Scale bar = 1 mm.

Figure 5. DSCs cultured in vitro on 3-D PCL scaffolds for 4 weeks in osteogenic media. Quantitative comparison of proliferation (A), alkaline phosphatase expression (B) and mineralization (C) between static (■) and dynamic (□) in vitro culture. * $P < 0.05$.

Figure 6. DSCs cultured in vitro on 3=D PCL scaffolds for 3 weeks (A, B) and 4 weeks (C, D) in osteogenic media stained for alizarin red. Qualitative comparison of mineralization between static (A, C) and dynamic (C, D) in vitro culture. Scale bar = 1 mm.

Figure 7.

3-D reconstructed images of DSC-seeded PCL scaffolds following 28 days in osteogenic media scanned at high resolution using the microCT. Mineralized matrix shown in solid white. PCL scaffold shown in transparent blue. (A) Side view. (B) Top view. (C) Quantitative values of mineralization using microCT analysis. Scale bar = 1 mm.

Table 1

Fabrication parameters (annealing temperature, annealing time, foaming pressure) and resulting scaffold characteristics (total pore area, % porosity, bulk density, median pore diameter) outlined for each scaffold group. Scaffold characteristics measured using mercury porosimetry

Group	Anneal Temp (degrees C)	Anneal Time (minutes)	Foam Pressure (kPa)	Total Pore Area (m ² /g)	Total Porosity (%)	Bulk Density (g/mL)	Median Pore Diameter (microns)
1	110	15	none	0.338	35.164	0.563	6.031
2	110	30	none	0.301	49.717	0.687	9.384
3	110	60	none	0.210	62.541	0.777	14.030
4	80	60	2.67	0.067	47.322	0.616	64.181
5	100	60	2.67	0.042	45.544	0.568	110.298
6	110	60	2.67	0.045	48.533	0.612	130.591
7	120	60	2.67	0.031	40.764	0.606	171.984
8	110	15	1.34	0.092	59.114	0.590	70.248
9	110	30	1.34	0.054	57.830	0.723	84.665
10	110	60	1.34	0.042	45.544	0.568	110.298