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Effects of Scaffold Architecture on Mechanical Characteristics and Osteoblast Response to Static and Perfusion Bioreactor Cultures

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Running title: Scaffold architecture static perfusion bioreactor
Abstract
Tissue engineering focuses on the repair and regeneration of tissues through the use of biodegradable scaffold systems that structurally support regions of injury whilst recruiting and/or stimulating cell populations to rebuild the target tissue. Within bone tissue engineering, the effects of scaffold architecture on cellular response have not been conclusively characterized in a controlled-density environment. We present a theoretical and practical assessment of the effects of polycaprolactone (PCL) scaffold architectural modifications on mechanical and flow characteristics as well as MC3T3-E1 preosteoblast cellular response in an in vitro static plate and custom-designed perfusion bioreactor model. Four scaffold architectures were contrasted, which varied in inter-layer lay-down angle and offset between layers, whilst maintaining a structural porosity of 60 ± 5%. We established that as layer angle was decreased (90° vs. 60°) and offset was introduced (0 vs. 0.5 between layers), structural stiffness, yield stress, strength, pore size and permeability decreased, whilst computational fluid dynamics-modeled wall shear stress was increased. Most significant effects were noted with layer offset. Seeding efficiencies in static culture were also dramatically increased due to offset (~45% to ~86%), with static culture exhibiting a much higher seeding efficiency than perfusion culture. Scaffold architecture had minimal effect on cell response in static culture. However, architecture influenced osteogenic differentiation in perfusion culture, likely by modifying the microfluidic environment.

Keywords
Tissue engineering, polycaprolactone, bone, scaffold, perfusion bioreactor
Introduction

Bone tissue engineering is a research area with notable potential in the realm of repair and regeneration of bone injuries. The tissue engineering approach involves the use of biodegradable scaffold systems to structurally support a target site whilst host cells and/or transplanted cell populations are induced by bioactive materials, growth factors, or other stimulatory systems to regenerate bone (Fujioka-Kobayashi et al., 2012; Kim et al., 2012; Lin et al., 2012; Wu et al., 2012; Wu et al., 2013). Thermoplastic polymer scaffolds have featured prominently within this field as the materials are relatively simple to process and are chosen on the basis of biodegradability and biocompatibility whilst providing desired mechanical characteristics (Rezwan et al., 2006; Neuendorf et al., 2008; Armentano et al., 2010). On one hand, a considerable amount of research has been focused on the characterization and optimization of the mechanical (Kharaziha and Fathi, 2010), inductive (Jones et al., 2006) and osteoconductive (Zhang et al., 2009) aspects of the scaffold constructs. On the other hand, a large proportion of work has focused on different cell and/or growth factor strategies to best achieve a desired differentiation and extracellular matrix (ECM) deposition at the target site (Holzwarth and Ma, 2011; Miranda et al., 2011; Gavenis et al., 2012; Zhao et al., 2013). Optimization of the microenvironment of biomaterial structures from a materials and engineering standpoint has been previously described as a pivotal aspect in the ultimate success of scaffold implants (Choi et al., 2010). A multi-faceted approach, wherein mechanical efficacy and target-site suitability are analyzed together with cell response to scaffold architecture, allows a more complete progression of knowledge in the applicability of scaffolds as tissue healing modalities.

Work on scaffold architecture to date primarily addresses global aspects such as differences in both porosity and lay-down orientation across test groups (Figure 1; (Zein et al., 2002)), material variations (Hoque et al., 2009), micro- or nanoarchitectures (Tuzlakoglu et al., 2005)
or material densities (Marcos-Campos et al., 2012). However, the aforementioned studies vary multiple parameters between groups, e.g., testing the influence of lay-down patterns without controlling porosity, which confounds the effects of each single parameter. In order to characterize the influence that certain aspects have on scaffold efficacy, adequate control over all scaffold parameters is necessary. In this study, we present a melt extruded (ME) thermoplastic polymer scaffold system within which we introduce architectural variations of inter-layer lay-down angle and offset whilst maintaining a constant porosity, scaffold material and extruded filament thickness. We propose that the architectural modifications will affect scaffold mechanical characteristics as well as cellular response in an in vitro culture model consisting of either static plate or custom-designed perfusion bioreactor culture methods.

Materials and Methods

Scaffold Design and Fabrication
MatLab (The MathWorks Inc., USA) was used to generate g-code, with filaments and layers spaced and rotated as specified, which controlled a melt extruder, the BioExtruder (designed by Domingos et al. (Domingos et al., 2009)) through Mach3 CNC Controller software (Newfangled Solutions LLC, USA). All scaffolds were fabricated from poly-ε-caprolactone (PCL; CAPA 6500, Perstorp, Sweden) to a porosity of 60±5 weight percentage (wt. %), with four experimental variants of lay-down angle and offset (OS; 0 or 0.5) between layers: A (90°), B (60°), C (90°OS), D (60°OS) (Figure 2 (a – d) rendered in SolidWorks 2009 (Dassault Systèmes S.A., France)). Scaffold diameter (12 mm) was based on the prior classification of ‘small’ defects (< 2 cm²) (Scopp and Mandelbaum, 2005).

Scaffold Characterization and Mechanical Testing
Scaffold porosity was determined gravimetrically. Scaffolds were scanned using micro-Computed Tomography (μCT; Scanco Medical μCT 40; Figure 2 (e – h)) to verify porosity was accurate (i) and establish mean pore size (j) for samples. Axial permeability of scaffolds was quantified by measuring flow of water through the structures within a confined tube, using
an Instron MicroTester (Model 5848; Instron®, USA), then calculating permeability using Darcy’s law, as applied by Melchels et al. (Melchels et al., 2010). Axial mechanical compression tests to failure were conducted on 4.5 mm scaffolds (n = 6 per type) using the Instron MicroTester in dry conditions and in Dulbecco’s Phosphate Buffered Saline (PBS; Invitrogen, USA) at 37°C, with a displacement rate of 0.5 mm/sec up to 66% strain.

Surface Modification and Sterilization
All scaffolds were incubated in 5M NaOH (5 hours at 37 °C) then rinsed in Milli-Q water (Merck Millipore, USA) until pH 7. Samples were sterilized with 60 minute 70% EtOH immersion, evaporation and subsequent 60 minute UV-C irradiation. Axial compressive tests were conducted to assess degradation.

Perfusion Bioreactor Construction
A perfusion system was designed to allow continuous bidirectional media flow axially through scaffolds. This consisted of front-end reservoirs (50 mL syringe; Terumo) attached by silicone tubing to 6-port connectors (Value Plastics, USA) that split to scaffold chambers (5 mL syringe; Terumo) and terminated at back-end reservoirs (50 mL syringe; Terumo) (Figure 3). Aervent Disposable 0.2 μm Filters (Merck Millipore) were attached to both ends of the system. A programmable syringe pump (AL-6000, World Precision Instruments, USA) external to the incubator was used to perfuse media by displacing air from two 50 mL syringes. Four-way stopcocks (Value Plastics) were used to allow for media change during culture. Sections of the silicone tubing were used to separate scaffolds within chambers.

Computational Fluid Dynamics
Computational fluid dynamic (CFD) analysis was conducted using Ansys Fluent (Ansys, Inc. Build Oct 12 2010, USA) with a flow rate of 1 mLhr⁻¹. Analysis was done for wall shear stress; a limit of 3 Pa was used (Weinbaum et al., 1994).

Cell Culture
MC3T3-E1 cells (ATCC, USA) were cultured in α-minimum essential medium (MEM) (Invitrogen) with 10 vol% fetal bovine serum (FBS; Invitrogen) and 1 vol. % penicillinstreptomycin (10000 U/mL- 10000 µg/mL; Invitrogen); henceforth ‘culture media’. Culture conditions of 37°C, 98-100% humidity and 5% CO₂ were maintained. Osteogenically inductive media (henceforth ‘osteogenic media’) was made using the same procedure, with the addition of ascorbic acid (0.1%, 100 mM; Sigma-Aldrich, USA), dexamethasone (0.1%, 1 mM; Sigma) and β-glycerophosphate (1%, 1 M; Sigma).

**Static Culture**
Scaffolds of 10 mm height (henceforth ‘seeding experiment’) were seeded with 1.25 million cells within 500 µL of culture media, pipetted axially. Plates were incubated for 2 hours, whereafter 4 mL/scaffold of culture media was added. Media (4 mL/scaffold) was changed on days 1, 3 and 7 of culture. Scaffolds of 4.5 mm height (henceforth ‘differentiation experiment’) were cultured in well plates, with 625,000 cells within 225 µL of culture media pipette-seeded into the top of each scaffold. Plates were incubated for 2 hours, whereafter culture media was added (2 mL/scaffold). Media (2 mL/scaffold) was changed on days 2, 5 and 7. Scaffolds were then transferred into new plates and split into control and osteogenic media, and 2 and 4 week time points (n = 6 of each type/condition/time point). Media was changed on day 1, 3 and 5 each week.

**Perfusion Culture**
Seeding experiment scaffolds were seeded with a cell suspension of 1.25 million cells/6.5 mL of culture media per scaffold, infused into bioreactor reservoirs. The pump was programmed to loop infinitely at a rate of 1 mL/hr per chamber (0.61 µm/s, 15 mL volume, bidirectional). Media was changed to cell-free on day 1. Chambers were terminated on days 1 & 7. Differentiation experiment scaffolds were pre-cultured as in static culture methods. The scaffolds were then transferred to bioreactor chambers. The perfusion system in this case was used exclusively for osteogenic culture. Media was slowly introduced into the system (< 1
mL/hr), so as to not disturb or detach pre-cultured cells. Perfusion consisted of 0.5 mL/hr (31 µm/s, 12 mL volume) flow per chamber; media was changed weekly.

**Cell-Construct Characterization**

Assessment of cell seeding efficiency, homogeneity of attachment, viability, proliferation, differentiation and cell/scaffold morphology was conducted.

**Cell Attachment and Morphology**

Cell seeding homogeneity, spreading, attachment, viability and morphology were assessed with fluorescence microscopy (Zeiss Axio Imager M2), confocal scanning laser microscopy (CLSM; Leica SP5 Confocal Microscope) and scanning electron microscopy (SEM; Quanta 200 SEM). Live/dead staining was conducted (seeding experiment day 7), with 5 µg/mL fluorescein diacetate (FDA; Invitrogen) and 0.5 µg/mL propidium iodide (PI; Invitrogen) in PBS. Samples were washed in PBS and cut to produce a thin (2 mm) centro-axial section. These were transferred to the staining solution and incubated at 37°C for 5 minutes. Samples were imaged using fluorescence (excitation: 488 nm (FDA) and 568 nm (PI)). CLSM assessment (seeding experiment day 7) samples were sectioned using the same method, after which sections were rinsed in PBS (Ca²⁺ Mg²⁺) then fixed in 4% paraformaldehyde (PFA; Sigma) prior to a final PBS rinse. Sections were transferred to a PBS solution containing 5 µg/mL of 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen) and 0.8 U/mL concentration of rhodamine-conjugated phalloidin (Invitrogen) and kept at 4°C in darkness for 24 hours. Excitation wavelengths of 358 nm (DAPI) and 480 nm (rhodamine-conjugated phalloidin) were used to visualize nuclei and actin. SEM assessment (seeding experiment day 7) samples were bisected axially, fixed with 3% glutaraldehyde and stored at 4°C. On the day of treatment, 3 washes of 0.1 M cacodylate buffer (Sigma) were done, followed by a soak in 1% osmium tetroxide (Sigma) in cacodylate buffer. Samples were then dehydrated using incremental ethanol soaks, and chemical drying using 100% hexamethyldisilazane (HMDS) (Sigma) was
performed thereafter. Samples were sputter coated with gold in a 10⁻² bar argon atmosphere (EMS150R S; Electron Microscopy Sciences, USA) prior to imaging.

**Seeding Efficiency, Proliferation and Differentiation**

Cell seeding efficiency and proliferation were assessed using the Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen). Seeding experiment scaffolds were removed at day 1, whilst differentiation experiment scaffolds were removed at days 14 and 28. Samples were stored at -80°C in 0.2% Triton™ X-100 (Sigma) in PBS to induce cell lysis (Brown and Audet, 2008). Upon thawing, scaffold sections were placed in micro-centrifuge tubes (Quantum Scientific; LabAdvantage) along with lysate, prior to processing with 0.1 mm diameter glass beads (Bullet Blender Beads GB01, Next Advance Inc., USA) using the Bullet Blender® Storm (BBY24M-CE, Next Advance Inc., USA). The PicoGreen assay was conducted as per manufacturer’s instructions (Invitrogen), prior to microplate reading with the POLARstar Omega (BMG LABTECH GmbH, Germany). An excitation wavelength of 480 nm and emission of 520 nm were used; samples were analyzed in triplicate. Results for seeding (day 1) were calculated using cell number based on dsDNA content (one cell containing 7.7 pg DNA (Kim et al., 1988)), and the seeding efficiency was taken as the percentage of cells remaining from the initial seeded amount. Proliferation was quantified by dsDNA content over the analyzed points. Differentiation was quantified at days 14 and 28 by levels of alkaline phosphatase (ALP), measured using the pNPP ALP assay, as per manufacturer’s instructions (Sigma), and contrasted using ALP (µg) per DNA (µg) content for each sample.

**Statistical Analyses**

Analysis of Variance (ANOVA) tests were used to assess differences, with Tukey’s post-hoc tests used to determine inter-type relationships. Tests were conducted using SPSS 21.0 with p-values < 0.05 taken as significant. Figure bars show mean ± standard deviation.

**Results**
**Scaffold Characterization**

*Structural Quality, Computational Fluid Dynamics and Permeability*

Four scaffold types were produced: A, B, C and D, as shown in Figure 2 (a - d) schematically and by μCT (e – h). Analysis of μCT data showed porosity was clearly controlled across the four scaffold types whilst a decreasing pore size was imposed (n = 6; Figure 2 (i, j)). CFD of the four scaffold types yielded an overall distribution of wall shear stress across the axis (Figure 4 (a – d), data in (f)). The majority of data points in the box plot fit below a shear stress of 1 Pa, with outliers imposing shear stress maximums across the groups of 0.837 Pa (A), 1.103 Pa (B), 1.979 Pa (C) and 2.527 Pa (D). Significance was found between the four types (p < 0.05) with structural offset imposing the strongest changes in the data. Scaffold permeability data corroborated predictions, with types featuring higher permeability undergoing lower shear stress magnitude (Figure 4 (e)).

**Mechanical Testing**

PCL is a highly hydrophobic polymer and hence cell attachment potential as well as fluid ingress through dense structures is limited in the native material. Surface treatment of the polymer was used to decrease hydrophobicity and enhance biological applicability. Treatment was found to reduce mean scaffold stiffness by 10% (not significant; data not presented). Scaffold compressive modulus, yield stress and strength (Figure 5 (a – c)) as well as strain at yield and at strength (e, f) were calculated from the stress-strain relationship demonstrated in (g) and tangent modulus (h). The tangent modulus was used to expose relevant peaks to allow determination of the structural yield and strength. The relevant inflection points are indicated (h), with the same strain values used to mark where these values would fall on the stress-strain curve (g). The recovered height of scaffolds after testing increased more in scaffolds tested after PBS immersion (d). Scaffold architecture has a clear influence on the mechanical values of the scaffolds, with a decrease in stiffness, yield stress and strength seen across the scaffold types.
Cell Culture

Cell Viability and Morphology

Cell penetration through the full scaffold depth was seen in all FDA/PI imaged specimens, with a higher proportion of dead cells visible in C and D type scaffolds in perfusion culture. As scaffolds were sectioned with a scalpel blade, the cells that were cut and thus suffered membrane rupture appear as areas of red around the filament cross-section (Figure 6 (a – d) – static, Figure 7 (a – d) – perfusion). Slight green autofluorescence of PCL was observed. CLSM images indicate filament coverage and bridging between filaments for all scaffold types, most notably under static culture conditions (Figure 6 (e – h)), with markedly lower cell number under perfusion (Figure 7 (e – h)). SEM again indicated static culture scaffolds having higher cell coverage, including visible bridging, compared with perfusion samples that had a contrasting lower number of cells (Figure 6 (i – l) – static, Figure 7 (i – l) – perfusion).

Seeding Efficiency, Cell Proliferation and Alkaline Phosphatase Expression

Seeding efficiency was assessed by DNA assays (Figure 8). There was an increase in seeding efficiency with decreasing scaffold permeability in static culture, whereas scaffolds in perfusion culture showed the opposite trend. Cell number in the bioreactor decreased from day 14 to day 28, whilst cell numbers increased under static conditions (Figure 9 (a)). No significant differences were found between types at any singular time point apart from perfusion at day 28 (Figure 9 (a)). ALP per DNA values increased with time in perfusion culture, whilst peaking on day 14 in static culture and dropping by day 28 (Figure 9 (b)).

Discussion

As predicted, the variance in lay-down angle and introduction of offset into designs strongly influenced pore size at a constant scaffold pore density of 60±5%. A clear, significant decrease was observed between types A – C (p < 0.05), however the pore size reduction imposed by introduction of interlayer offset was negligible for the 0-60° lay down pattern. (C and D, n.s.). This is a result of the algorithm used by the µCT software to determine pore sizes by fitting
maximally-large spheres in pore spaces. For the 0-90° pattern the pores are relatively large in all directions but are strongly reduced in size in the z-direction upon the introduction of offset, whereas for the 0-60° pattern the pore size in the xy-plane is much smaller, and hence the overall pore size is less effected when offset is introduced.

The trend observed in wall shear stress, modeled with CFD, mirrored the trend in pore size, however whilst the large sample size (~100,000 data points) in the CFD model caused a considerable number of outliers, unlike in pore size, significance remained between C and D type scaffolds. Again as in pore size, the experimentally determined permeability differed most between the lay-down patterns in the absence of an offset (A to B), however significant difference was also detectable for the offset-groups (C to D). The trends indicated by the computational and experimental systems were in agreement, which supported reliance on the CFD model for determination of maximum shear value thresholds of the bioreactor system. In reference to shear stress values, García et al. (García et al., 1997) tested shear stress required to detach osteoblasts from fibronectin (Fn) coated or no-Fn (control group, bovine serum albumin (BSA) only) glass slides, using a spinning disc device. It was found that under control conditions a 2 Pa shear stress was sufficient to detach 50% of cells, whilst Fn coating raised the required shear to 5 Pa or more. In contrast, the reference value used in our modeling was derived from Weinbaum et al. (Weinbaum et al., 1994) who describe that a shear stress range of 0.8 – 3.0 Pa is exhibited on osteoblasts in vivo. With both the factor of safety imposed on the flow rate in the model (0.5 mL/hr in bioreactor, 1 mL/hr modeled), and the vector plot indicating only few peripheral filaments underwent high shear magnitudes, it was concluded that a bioreactor flow rate of 0.5 mL/hr would enact lower shear than this threshold value and thus be within the 0.8 – 3.0 Pa physiological range.

Throughout the literature, different cell types have been tested with imposed fluid shear in vitro to assess response to the stresses. Notably, a shear stress of 1.6 Pa was found to positively
influence chondrocyte glycosaminoglycan levels and morphology (Smith et al., 1995), whilst contrastingly a shear stress above 1.0 Pa caused apoptosis and cell detachment in endothelial cells (Macario et al., 2008). It is clear that cell response to shear stress is both cell- and substrate-dependent and hence despite stress predictions and ceilings being imposed on the system, deviation from expectations may result.

The scaffold designs were chosen based on the predicted mechanical properties of each architectural variant. Whilst a 90° lay-down gives the most tiled filaments axially (along the long (Z) axis; every 2 layers the cycle is tiled) and hence affords the strongest axial arrangement possible within such a structure, a 60° lay-down (3 layer tiling), with triangular cross-sectional pores, offers stronger transverse rigidity. Introduction of an offset into types further increases tiling (4 for 90°, 6 for 60°), along with increasing these listed strengths. Offset designs were also chosen to increase axial surface area, reduce permeability and offer a further modality through which pore size could be modified whilst maintaining scaffold density. It should be noted that whilst the primary physiological loading modality of osteochondral bone-phase scaffolds would be axial, various transverse and shear loads may be present in vivo. The design of the scaffolds imposed logistical constraints in transverse mechanical testing as a circumferential variability would exist. As the scaffolds would serve as the bone component of osteochondral scaffolds, shear loading would be primarily experienced in the cartilage phase. Any material failures caused by excess shear would thus occur at the top phase and transfer only transiently to bone scaffolds. Mechanical tests were hence only performed on the scaffolds in an axial orientation. Testing was performed both in ambient air and in simulated physiological solution (37°C PBS), and consisted of high strain (66%) compression past failure. As predicted, testing scaffolds in physiological solution caused a reduction in stiffness and strength. Scaffolds tested in these conditions were not surface treated or previously subject to UV or EtOH sterilization, hence it was concluded that swelling or any other infiltration of
solution into the scaffold did not occur, with differences resulting from temperature variability. As the melting point of PCL is ~60°C, an increase in temperature from ~24°C to 37°C would increase the chain mobility in the amorphous phase of the polymer and lower structural stiffness. Compressive modulus values of the scaffolds decreased across the four types, with significant difference (p < 0.05) noted between all apart from C & D. Stiffness decreased in PBS, however no change was significant. Yield stress and strength values of the samples were calculated using the tangent modulus (Figure 5 (g, h)). A global decrease in mechanical properties was observed across the four types, however significant difference was not notable between any condition. The strength of scaffolds indicated a stronger similarity to wall shear stress values, with offset providing a much more prominent impact on the maximum stress the structures could withstand. Significance was noted in offset (p < 0.05), however there was no effect of lay-down angle in standard or offset samples. Immersion in PBS clearly impacted strength values, with significance present between dry and PBS in types C and D (p < 0.05). Interestingly, physiological fluid also led to significantly greater elastic recovery in all groups (p < 0.05). Height recovery under dry conditions appeared offset-dependent (p < 0.05) whilst after immersion no such trend was indicated. Strain values at yield were similar between all test groups. Conversely, strain values at yield indicated a decrease after PBS immersion, corroborating the previously described decrease in strength under the same conditions. The elevated temperature may have increased polymer chain mobility, resulting in earlier failure as well as improved height recovery in the PBS groups.

A high level of cell viability was observed across all groups (static culture Figure 6 (a – d) and perfusion culture Figure 7 (a – d)). Higher viability was observed within the static samples, although both culture methods showed a similar trend of cell spreading around strut circumferences and most prominently at the interface between struts. As the FDA/PI stains
were performed on scaffolds from the initial culture, which had no pre-culture and were seeded within the perfusion system, a dramatic difference in seeding efficiency was noted. As the cells appeared to clump at the joints throughout the entirety of the structures, it may be inferred that if sufficient cellular infiltration was initially provided, scaffolds with lower permeability should sustain more cells due to a higher number of these joint-regions. On the other hand, as all the scaffolds are equal in density, sharing filament thickness and hence total filament length per scaffold, with surface area variance only due to differing inter-filament contact area, the maximum sustainable number of cells should not significantly differ between samples. Architecture may aid the spreading and bridging of cells in initial culture stages, but such relevance would diminish as constructs would mature in vitro or in vivo.

CLSM and SEM images (Figure 6, Figure 7) clearly demonstrated that polymer filament coverage was much more comprehensive, with sheath development and bridging apparent at day 7, in static culture than in perfusion culture. For a 7 day culture, scaffold types A and B had significant cellular development, with discrete bridging observable at inter-layer joints. Types C and D developed similar levels of cellularization. The images presented qualitatively herein are from the initial seeding experiment culture; the consequences of the low seeding efficiency of the perfusion system are observable across all images of perfused samples, with low-minimal amounts of cell attachment visible. The differentiation experiment culture featured a pre-seed and all quantitative data (bar seeding efficiency) is from this method.

It is clear from our results that static seeding efficiency can be improved by modifying scaffold architecture to decrease permeability and increase axial surface area. This is most clearly demonstrated when contrasting the standard and offset scaffold architectures (types A&B vs. C&D). Whilst static samples with zero offset achieved seeding efficiency commonly seen with macroporous PCL scaffolds (Chen et al., 2011; Sobral et al., 2011; Hong et al., 2012; Kim and Kim, 2012), the offset groups significantly surpassed the values, achieving seeding efficiencies
seen in PCL scaffolds featuring nanometer-sized foam (Chen et al., 2011), or scaffolds featuring pore size gradients (Sobral et al., 2011). Although the effects on axial surface area are similar between a pore size gradient and a scaffold layer-offset, it should be noted that using larger filament spacing (0.1 vs. 1 mm axially), we achieved improved seeding efficiencies (~70% vs. ~86%). As the scaffolds used were made of PCL etched with NaOH and featured macroscopic pores, the efficacy of static seeding when performed on an offset design is noteworthy. It should be mentioned that unlike in tortuous foams, where high cell seeding density may be observed but cells may not penetrate through structures homogenously, or ‘conventional’ structures such as the non-offset scaffold presented herein, the offset architectures capture both homogeneous cell distribution and high seeding efficiency. Direct comparisons of these scaffold designs have been made in the literature (Melchels et al., 2010). Seeding within the perfusion system exhibited the opposite trend to static, with decreases in permeability (and/or offset) resulting in a decreased cell adherence. It may be postulated that this is a result of increased wall shear stress that was imposed on the lower permeability structures; seeing as PCL remains somewhat hydrophobic despite NaOH etching, lower shear than predicted may be sufficient to cause cell detachment. The problem with our system may have resulted from simply an excess flow rate during attachment and not during sustained culture, however it has been found (Alvarez-barreto et al., 2007) that in scaffolds with pore sizes between 300 – 450 µm, a perfusive flow of 9 mL/hr (12.43 µm/s vs. our 0.31 µm/s) for 2 hours was sufficient to improve seeding efficiency over a static condition. It may be debated, however, that as Alvarez-barreto et al tested polystyrene and poly(l-lactic acid) (PLLA) with much lower filament thicknesses, cell-surface interactions on a microscopic scale may have been modulated. Design features of the bioreactor may have also influenced the outcomes of the culture. For instance, Wendt, et al., demonstrated increase in seeding efficiency when using a perfusion bioreactor (57±5% to 75±6%), on scaffolds made of Polyactive, a copolymer of
poly(ethylene glycol) and poly(butylene terephthalate) (Wendt et al., 2003). Whilst again material variability should be accounted for, Wendt et al. also used FEP tubes and minimized contact area and dead corners in his set up. Overall, the trend exhibited by all aforementioned studies demonstrates an increase in cell adherence under perfusion.

When the perfusion seeding method was replaced with a static seed and pre-culture period to standardize the initial cell number, cell numbers were consistent across all scaffold types at the 14 day time point (although lower in perfusion than static). The type A scaffolds showed significantly higher cell proliferation than all other types under perfusion by day 28, comparable to that observed in the static culture groups. Static control scaffolds showed significant increase in cell number from day 14 to day 28 (p < 0.05), reaching levels exhibited in static osteogenic samples. ALP/DNA was higher on day 14 than day 28 in static osteogenic samples (p < 0.05), conversely higher on day 28 vs. day 14 in perfusion samples (p < 0.05) and higher in perfusion day 28 than static day 14 or 28 (p < 0.05). A low DNA content was present in all perfused samples but type A on day 28, however ALP/DNA values were comparable across this time point. As the scaffolds had a large surface area to volume ratio, cell lysis was difficult to accomplish through traditional freeze-thaw cycles and hence the Bullet Blender® provided means through which a comprehensive lysis could be achieved. This theory was corroborated by testing the lysate contents of a scaffold group for DNA prior to spinning; no detectable DNA levels were found in the untreated samples. Additionally, a different flow rate such as non-continuous perfusion may be more beneficial for deposition of mineralized tissue, as purportedly a desensitization occurs after prolonged mechanostimulation (Jaasma and O'Brien, 2008; Partap et al., 2010; Plunkett et al., 2010).

PCL is a highly inert material; architectural changes affect culture environment within the scaffold yet do not manifest as cell-surface stimuli at a cellular scale. Modifying scaffold surfaces prior to contrasting architectural influence would expose effects on a microscopic
scale, by introducing a higher degree of interaction between cells and the substrate. The effect of such structures on cells would then probably vary between pore sizes of different scaffold types. However, the use of an inert material allows the institution of a highly controlled environment wherein macro- and microscopic fluid flow, the resultant shear stresses and the mechanical characteristics of the scaffold may be assessed. Controlling scaffold density whilst modifying architectural factors directly affects these parameters and thus may expose subtle variation in cell response. Overall, minimal direct effects were present in static culture, with architecture predominantly affecting seeding efficiency. Conversely, whilst perfusion culture offered additional challenges and produced variable results, the tailorability of the system possible through manipulations in flow, pore size and permeability showed that distinct behaviors may be encouraged through parameter optimization.

**Conclusion**

Architecture had a clear effect on scaffold mechanical properties, including modulus and permeability, and also on cell seeding efficiency. Most significant differences were evident when a layer offset was used, with structural characteristics such as pore size, strength and plastic deformation exhibiting a larger change than when contrasting differences imposed by lay-down angle. Notably, the seeding efficiencies were much higher in static culture than in perfusion culture, with offset providing a dramatic increase in efficiency. Overall, architecture had minimal effects on cell response in static culture when using an inert thermoplastic, PCL, and future work may benefit from using more bioactive materials. Nonetheless, cellular response does appear to be dependent on mechanical stimulation by perfusion culture, which can be altered by modifying scaffold permeability, pore size and shape of microstructures.

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References


**List of Figures**

Figure 1. Schematic of lay-down and offset in 3d-printed scaffolds. A single scaffold layer consisting of parallel filaments, viewed from the top (a). A 90° lay-down, consisting of a second layer of parallel filaments globally rotated by 90° about the z-axis (b). An inter-layer offset of a factor of 0.5 filament-to-filament distance introduced in the 90° design (c). First two layers of a 60° lay-down, illustrating a second layer of parallel filaments globally rotated by 60° about the z-axis (d). The third layer in the 60° sequence, completing the layer-tiling sequence for this angle (e). A 0.5 inter-layer offset introduced into a 60° lay-down design (f).

Figure 2. The four scaffold architectural variants sectioned at 3 mm to reveal internal geometry; A: 90°, B: 60°, C: 90°OS, D: 60°OS (a - d). Micro-computed tomography (μCT) scan renders of sections of the four scaffold types (e – h). Porosity distribution (i), pore size (j) and filament thickness (k) between the four scaffold types. Differing Roman numerals indicate significant difference between tested groups (p < 0.05).

Figure 3. Custom-built perfusion bioreactor schematic. A 6-bay syringe pump was used to push air forward and backward, displacing media during culture (illustrated in reservoirs and scaffold chamber). Each scaffold type had a separate inlet reservoir, chamber and outlet reservoir.
Figure 4. Computational Fluid Dynamics (CFD) vector plots indicating wall shear stresses within the four tested scaffold types at a flow rate of 1 mLhr⁻¹ (a – d). Scaffold permeability, $\kappa$, calculated from scaffold resistance to axial water flow (e). Wall shear stress across the four scaffold types as calculated by CFD (f). Differing Roman numerals indicate significant difference between tested groups ($p < 0.05$). Histogram of wall surface shear stress (Pa); expressed as a percentage of elements within a stress range for scaffold types A – D, as indicated on plots.

Figure 5. Mechanical characterization of PCL scaffolds with four architectural types. Axial compression was performed dry and after soaking in 37° PBS for 30 mins. Compressive modulus taken over 4-6% strain of the resultant non-linear curve (a); yield stress (b); stress maximum at failure (strength) (c); height recovery after testing (original height 4.5 mm; compressed to 1.5 mm); strain at the yield point (e); strain at the strength point (f); stress strain curve example of a tested scaffold sample (g); tangent modulus of the same sample (h). Peaks determined from the tangent modulus (slope of stress) were used to calculate yield stress (broken circle) and strength (full circle) and are indicated in both (g) and (h) at the same strain levels. Differing Roman numerals indicate significant difference between tested groups ($p < 0.05$); upper case and lower case are tested independently to each other. Stars indicate significant difference between condition pairs as indicated by bars ($p < 0.05$).

Figure 6. Static culture: FDA (live, green) / PI (dead, red) fluorescent stain on day 7 - Z-stack of between 40 and 60 images (a – d); DAPI (blue) / phalloidin (rhodamine, red); nuclei/actin stain on day 7 – Maximum projection images (e – h); SEM image of cells on scaffold filament junctions on day 7 using 20 kV and 200x magnification (i – l).

Figure 7. Perfusion culture: FDA (live, green) / PI (dead, red) fluorescent stain on dDay 7 - Z-Stack of between 40 and 60 images (a – d); DAPI (blue) / phalloidin (rhodamine, red);
nuclei/actin stain on day 7 – Maximum projection images (e – h); SEM image of cells on scaffold filament junctions on day 7 using 20 kV and 200x magnification (i – l).

Figure 8. Mean seeding efficiency of scaffolds seeded in static and perfusion bioreactor systems, expressed as a percentage of DNA quantities on day 1 post-seeding compared to seeded population. Significantly different groups are indicated with different Roman numerals whilst similar groups share symbols; p < 0.05.

Figure 9. DNA content per scaffold – data for 14 and 28 day cultures, with static control (SC), static osteogenic (SO) and perfusion osteogenic (PO) groups described (a); ALP measured using pNPP assay with 30 minute incubation, normalized to DNA content per scaffold (b), same groups as in graph (a). Significantly different groups are indicated with different Roman numerals whilst similar groups share symbols; p < 0.05.
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6
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Figure 7
Figure 8
Figure 9

(a) DNA (μg) / Scaffold

(b) ALP / DNA (x 10^3 ng pNPP / ng)

Culture Condition

14 Day  28 Day