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Tubular perfusion system culture of human mesenchymal stem cells on poly-L-lactic acid scaffolds produced using a supercritical carbon dioxide-assisted process

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Abstract

In vitro human mesenchymal stem cell (hMSC) proliferation and differentiation is dependent on scaffold design parameters and specific culture conditions. In this study, we investigate how scaffold microstructure influences hMSC behavior in a perfusion bioreactor system. Poly-L-lactic acid (PLLA) scaffolds are fabricated using supercritical carbon dioxide (SC-CO₂) gel drying. This production method results in scaffolds fabricated with nanostructure. To introduce a microporous structure, porogen leaching was used in addition to this technique to produce scaffolds of average pore size of 100, 250, and 500 μm. These scaffolds were then cultured in static culture in well plates or dynamic culture in the tubular perfusion system (TPS) bioreactor. Results indicated that hMSCs were able to attach and maintain viability on all scaffolds with higher proliferation in the 250 μm and 500 μm pore sizes of bioreactor cultured scaffolds and 100 μm pore size of statically cultured scaffolds. Osteoblastic differentiation was enhanced in TPS culture as compared to static culture with the highest alkaline phosphatase expression observed in the 250 μm pore size group. Bone morphogenetic protein-2 was also analyzed and expression levels were highest in the 250 μm and 500 μm pore size bioreactor cultured samples. These results demonstrate cellular response to pore size as well as the ability of dynamic culture to enhance these effects.

Keywords

supercritical fluids; scaffold; PLLA; human mesenchymal stem cells; tissue engineering; bioreactor

INTRODUCTION

Despite recent advances, commonly used techniques for the treatment of bone defects have significant disadvantages and cell-based tissue engineering (TE) represents a promising alternative treatment.^{1,2} An objective of TE is to create interactions between an artificial component and cellular components to ensure tissue regeneration. The artificial component

needs to support cellular growth and three dimensional organization, which requires the coexistence of micro and nanostructures, mimicking the extracellular matrix (ECM). In this study, microstructure is defined as scaffold features with a size scale between 1 and 1000 μm , and nanostructure is defined as scaffold features between 1 and 200 nm. The cells growing in a structural environment similar to their natural medium are driven to colonize the polymeric structure and to differentiate; thus, the porosity of scaffolds must have a specific size for the type of tissue to be replicated.³⁻⁵ In addition to the microstructure, the nanostructure is necessary to ensure the roughness of the pore walls that provide for cell adhesion, growth, migration, and differentiation.⁶

Several techniques have been proposed in the literature to obtain TE scaffolds,⁷ including solvent casting, particulate leaching,^{8,9} freeze drying,^{7,9} phase separation,¹⁰ rapid prototyping,^{5,11,12} foaming,^{11,13} sintering, or a combination of these techniques.

Solvent casting is relatively simple and it is possible to obtain controlled porosity and interconnection between pores using this method. However, post-treatments to eliminate the residual solvent and long processing times are necessary. Electrospinning and similar techniques can yield nanostructures, but are limited to primarily two-dimensional products and the scaffolds exhibit low mechanical strength. Gas foaming can be used to fabricate highly porous polymer foams without the use of organic solvents but the samples obtained lack a nanostructure.

As an alternative to overcome the limitations of these methods the adoption of supercritical carbon dioxide (SC-CO₂) based techniques has been proposed. These techniques take advantage of specific properties of gases at supercritical conditions including modifiable solvent power, high diffusivities, and solvent elimination.¹⁴⁻¹⁶ However, the general limitation of supercritical and non-supercritical techniques is the absence of nanostructure in scaffolds. Therefore, in this work a new supercritical CO₂ based process is used that, in contrast to other techniques, allows for the reproduction of micro and nanostructure. Termed supercritical gel drying it has no limitations in the size and shape of the structures that can be produced.¹⁷⁻¹⁹ Supercritical gel drying can be combined with porogen leaching in a process that consists of four steps:

1. Formation of a PLLA solution in an organic solvent, loaded with a solid, water soluble, leaching agent.
2. Formation of a gel by thermally induced phase separation.
3. Drying of the gel using SC-CO₂, forming a supercritical solution between the supercritical fluid and the organic solvent and flushing it away.
4. Porogen leaching in distilled water.

To the best of our knowledge, this work represents the first time scaffolds fabricated by this technique have been tested for a cellular response.

In addition to the key role of scaffold micro and nanostructure in hMSC proliferation and osteoblastic differentiation, the environment in which the cells are cultured also has a dramatic impact. hMSCs have been shown to respond to both mechanical stresses in the surrounding environment leading to an enhancement of osteoblastic differentiation.^{20,21} Thus, bioreactor culture is advantageous for three dimensional bone tissue engineering constructs. In this study, scaffolds are cultured in the tubular perfusion system (TPS) bioreactor. In this bioreactor system, scaffolds are loaded into a tubular growth chamber and media is perfused through the growth chamber using a pump. This system has previously been demonstrated to enhance hMSC osteoblastic differentiation using alginate scaffolds,²² but has not previously been used for synthetic microporous scaffold culture.

Therefore, this study aims to use the PLLA structures produced by supercritical gel drying with porogen leaching to culture human mesenchymal stem cells (hMSCs) and analyze cell response to this artificial environment. Different micropore size ranges were tested using the same nanostructure to select the most suitable range for hMSC culture in these scaffolds in the TPS bioreactor. To this end, the objectives of this study are first to evaluate hMSC response to PLLA scaffolds fabricated using supercritical gel drying with porogen leaching, second to demonstrate cell viability on these scaffolds cultured in the TPS bioreactor, and finally to evaluate the cellular response to dynamic culture and pore size.

MATERIALS AND METHODS

Scaffold preparation

Scaffolds were prepared according to the following procedure. Poly(L-lactic acid) (PLLA) L210 (MW 210,000) with an inherent viscosity ranging between 2.6 and 3.2 dL/g (0.1% in chloroform, 25°C) was purchased from Boehringer Ingelheim (Ingelheim, Germany). A solution of PLLA 15% w/w in dioxane (Sigma Aldrich, St. Louis, MO) was prepared and 99.8% pure ethanol (Sigma Aldrich) as the non-solvent was added at dioxane/ethanol ratio of 1.7. The solution was stirred and heated at 60°C until it became homogeneous. Scaffolds with different average microporosity were produced through the addition of D-fructose (m.p. 119–122°C) (Sigma Aldrich) particles with average diameters of 100, 250, and 500 µm.

The solution was enriched with the leaching agent (fructose), homogenized, and poured into steel cylindrical containers with the diameter of 4 mm and the height of 3.5 cm. The solution was then compressed to 10 bar to obtain uniform contact between the leaching agent particles and the polymer and produce interconnected pores. The solution was then incubated at –18°C for 1 h to obtain a gel that was subsequently dried using SC-CO₂ (99% purity) (SON, Società Ossigeno, Napoli, Italy).

The drying vessel was filled from the bottom with SC-CO₂ up to the desired pressure using a high pressure pump (Milton Roy-Milroyal B, Pont-Saint-Pierre, France). Optimized supercritical CO₂ extraction conditions of the solvent from the polymeric gel (200 bar and 35°C) were selected and extraction was completed in 4 h.¹⁹ A depressurization time of 10 min was used to bring the system to atmospheric pressure.

The dried samples were cut to obtain plug scaffolds of 4 mm in diameter and 5 mm height. To avoid the shrinkage of the nanostructure of the surface, the samples were cut using a blade previously immersed in liquid nitrogen. The samples were then soaked in distilled water to remove porogen, sterilized in 70% ethanol and rinsed with PBS.

Mechanical tests

Compressive mechanical properties of the scaffolds were measured using an INSTRON 4301 (Instron Int. Ltd, High Wycombe, UK). The compressive modulus is defined as the initial linear modulus on the stress-strain curves. Cylindrical samples with a diameter of 4 mm and a thickness of 5 mm were compressed at a crosshead speed of 1 mm/min. Seven specimens were tested for each sample ($n = 7$).

Solvent residue analysis

Dioxane residue was measured by a headspace (HS) sampler (model 7694E, Hewlett Packard, Palo Alto, CA) coupled to a gas chromatograph (GC) interfaced with a flame ionization detector (GC-FID, model 6890 GC-SYSTEM, Hewlett Packard). Dioxane was separated using two fused-silica capillary columns connected in series by press-fit: the first column (model Carbowax EASYSEP, Stepbios, Italy) connected to the detector, 30 m

length, 0.53 mm i.d., 1 μ m film thickness and the second (model Cp Sil 5CB CHROMPACK, Stepbios, Italy) connected to the injector; 25 m length, 0.53 mm i.d., 5 μ m film thickness. GC conditions were the one described in the USP 467 Pharmacopoeia with some minor modifications (oven temperature from 45 to 210°C for 15 min). The injector was maintained at 135°C (split mode, ratio 4:1), and Helium was used as the carrier gas (5 mL/min). Head space conditions were: equilibration time, 30 min at 95°C; pressurization time, 0.15 min; and loop fill time, 0.15 min. Head space samples were prepared in 20 mL vials, filled with internal standard DMI (3 mL) and 500 mg of NaCL and water (0.75 mL) in which samples of PLLA scaffold were suspended.

Scaffold porosity

The porosity (ϵ) represents the “void space” of the scaffold and was calculated from the

density of the scaffold $\left(\rho_s = \frac{\text{Scaffold Weight}}{\text{Scaffold Volume}}\right)$ and the density of untreated PLLA $\left(\rho_p = 1.24 \frac{\text{g}}{\text{cm}^3}\right)$:

$$\epsilon = 1 - \frac{\rho_s}{\rho_p}$$

The scaffold density was determined by measuring its dry volume and weight.

EXPERIMENTAL SETUP

Following scaffold fabrication and characterization, cellular studies were completed. The first short term study aimed to evaluate proliferation and osteoblastic differentiation of hMSCs cultured on the scaffolds in a dynamic culture environment. Six experimental groups were used including scaffolds with pore sizes of 100, 250, and 500 μ m cultured in both static (six well plates) and dynamic (TPS bioreactor) conditions. In addition two monolayer control groups were completed, a control cultured in control media and a control cultured in osteogenic media. Timepoints were taken at days 1, 4, 8, and 12 and samples analyzed for alkaline phosphatase (ALP) protein and deoxyribonucleic acid (DNA) content. Following this, a long term study was completed. The same six experimental groups were used and monolayer control groups were not used. Timepoints were taken at days 1, 8, 16, and 24 and samples were analyzed for ALP and bone morphogenic protein-2 (BMP-2) gene expression. In addition, to visualize cells, histological staining was completed and SEM images were taken.

Human mesenchymal stem cell culture

hMSCs were purchased from Lonza (Walkersville, MD) and were expanded on tissue culture polystyrene flasks in control media composed of high glucose DMEM (Gibco, Carlsbad, CA) with 4 mM L-glutamine (Gibco), 0.1 mM nonessential amino acids (Gibco), 1.0% penicillin/streptomycin (v/v) (Gibco), and 10% mesenchymal stem cells qualified FBS (Gibco) as described previously.^{8,22–24} Media was changed every 3–4 days, according to manufacture specifications. Cells were stored in a cell culture incubator at 37°C and 5% CO₂ and passaged into a new flask every 7 days ($p < 6$) using trypsin/EDTA (Lonza). To obtain the osteogenic media, 100 nM β -dexamethasone (Sigma-Aldrich), 10 mM β -glycerophosphate (Sigma-Aldrich), and 50 mg/L ascorbic acid (Sigma-Aldrich) were added to the control media.^{8,22–24}

hMSCs seeding on PLLA scaffolds

Sterilized and rinsed scaffolds were soaked in DMEM supplemented with 10% fetal bovine serum for 4 h. hMSCs were removed from tissue culture and pelleted. The cell pellet was then resuspended at a density of 1.2×10^7 cells/mL to prepare the solution for seeding. Scaffolds were removed from DMEM and seeded with 10 μ L of the solution (1.2×10^5 cells/scaffold) via pipetting directly on the scaffold surface. The scaffolds were then put in the incubator for 4 h without media to allow cell attachment on the scaffolds surface. To measure attachment efficiency, cells which were not attached to scaffold were removed after 4 h and mixed with trypan blue (Sigma-Aldrich) and counted on a standard hemocytometer. Four counts were made for each sample ($n = 4$). Attachment efficiency was then calculated by the following formula: Efficiency = (Unattached Cells/Total Cells Added Scaffold). On study day -1 (cell seeding day), all cell seeded scaffolds were cultured in static into six-well plates using control media for 24 h, to facilitate the cell adhesion. On study day 0 dynamically cultured scaffolds were loaded into the TPS growth chambers while six static scaffolds were placed in osteogenic media in each well of six well plates. In addition, two control groups were performed: monolayer hMSCs grown on tissue culture polystyrene six well plates in control media and monolayer of hMSCs grown on tissue culture polystyrene six well plates in osteogenic media. Control group cells, used to demonstrate the osteoblastic differentiation of seeded samples, were cultured in 5 mL of media, for the duration of the study with media changes every 3 days for all groups.

Bioreactor for dynamic culture

The bioreactor system consists of a tubular growth chamber and media reservoir connected via a tubing circuit as described previously.^{22,24} Media flow was driven by an L/S Multichannel Pump System (Cole Parmer, Vernon Hills, IL) at 0.3 mL/min for all studies. The tubing circuit was sterilized via autoclave and consisted of platinum cured silicon tubing (Cole Parmer) for all areas except the one that passes through the pump which was composed of Pharmed BPT tubing (Cole Parmer) chosen for its high mechanical durability. The growth chamber was packed with cell seeded PLLA scaffolds using a sterile spatula. The TPS bioreactor was then kept in the incubator for the duration of the study with media changes every 3 days.

Scanning electron microscopy

PLLA scaffolds were analyzed using two scanning electron microscopes. In the first part of the work, scaffolds were cryofractured using liquid nitrogen and then were sputter coated with gold (Agar Auto Sputter Coater mod. 108 A, Stansted, UK) at 30 mA for 180 s [scanning electron microscopy (SEM) mod. LEO 420, Assing, Italy] to analyze cell and pore size and the overall scaffold structure.

Following cell culture, the scaffolds were analyzed to evaluate cell adhesion, diffusion, and proliferation inside the PLLA structures. Scaffolds were soaked 12 h in 4% paraformaldehyde to fix the biological material on the polymeric surface, then were dried for 24 h at room temperature. The samples were then sputter coated with carbon at 30 mA for 180 s and then analyzed by SEM (SU-70 Hitachi).

Protein assays

Protein was extracted using the M-PER (Pierce, Rockford, IL) mammalian protein extraction reagent, following standard protocols.^{8,25} A p-nitrophenyl phosphate liquid substrate system (pNPP) (Sigma-Aldrich) was used to analyze intracellular ALP concentrations from the extracted protein. The extracted protein sample was suspended in PBS and added to 100 μ L of pNPP and incubated at room temperature for 30 min in the

dark. The absorbance was read using a M5 SpectraMax plate reader (Molecular Devices, Sunnyvale, CA) at 405 nm by the PicoGreen assay. Data were normalized to scaffold weight and DNA. All samples were analyzed in triplicate ($n = 3$).

DNA quantification

DNA was isolated from samples to normalize the ALP assay and to relate to cell proliferation.^{8,25} Cell pellets or scaffolds were resuspended in 200 μ L of PBS. Scaffolds were mechanically agitated following resuspension and the supernatant was retrieved. DNA was isolated using a DNeasy Tissue Kit (Qiagen, Valencia CA) following the kit standard protocols into 400 μ L of eluate. DNA was then quantified by mixing 100 μ L of DNA eluate with 100 μ L of diluted Quant-iT Pico-Green dsDNA reagent (Molecular Probes, Carlsbad, CA), incubating for 5 min in the dark and measuring fluorescence using an M5 SpectraMax plate reader with excitation/emission of 480/520 nm. All samples were analyzed in triplicate ($n = 3$).

Gene expression

RNA was isolated using trizol (Invitrogen, Carlsbad, CA) and mechanical agitation and purified using an RNeasy mini plus Kit (Qiagen, Valencia, CA) following standard protocols.^{8,25} Then isolated RNA was reverse transcribed to cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The expression of BMP-2 (Taqman Assay ID: Hs00154192_m1) and ALP (Hs00758162_m1) was analyzed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs00960641_m1) as an endogenous control gene for all samples. Gene expression assays (Applied Biosystems) were combined with the cDNA to be analyzed and Taqman PCR master mix (Applied Biosystems). The reaction was performed on a 7900HT real time PCR System (Applied Biosystems) using conditions of 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The relative gene expression level of each target gene was, then, normalized to the mean of the GAPDH in each group. Fold change was calculated using the $\Delta\Delta$ CT relative comparative method. Samples were analyzed in triplicate and standard deviations were reported ($n = 3$).

Histological analysis

PLLA scaffolds were collected and fixed in 4% paraformaldehyde (Sigma) and 0.1M sodium cacodylate (Sigma) buffer, containing 10 mM CaCl₂ (Sigma) at pH 7.4 at 4°C for 4 h. After fixing, the scaffolds were placed in cassettes and washed with 0.1M sodium cacodylate buffer and 10 mM CaCl₂ at pH 7.4 at room temperature, for 24 h. The scaffolds were then dehydrated for histological processing washing with ethanol, followed by two Citrisolv (Fisher Scientific) washes. The samples were then embedded in paraffin (Fisher Scientific) and sectioned to 5 μ m thickness sections and placed on glass slides. Sections were oven dried at 64°C for 2 h, deparaffinized in Citrisolv and rehydrated in ethanol. Hematoxylin and Eosin (H&E) staining was performed to visualize the cells using standard protocols.

Statistical analysis

All samples were performed in replicates ($n = 3-7$). Data were analyzed first using ANOVA single factor analysis and then using Tukey multiple comparison test to demonstrate differences between groups assuming a normal data distribution with a confidence of 95% ($p < 0.05$). Mean values of triplicates and standard deviation error bars are reported on each figure as well as relevant statistical relationships.

RESULTS

Scaffold fabrication

PLLA scaffolds were readily and reliably fabricated with micro and nanostructure in a short time (8 h) to set specifications. Following porosity analysis of PLLA samples, all samples had porosity values higher than 90% as reported in Table I. Porosity values were $95.5 \pm 0.1\%$, $96.0 \pm 0.2\%$, and $96.3 \pm 0.1\%$ for the 100, 250, and 500 μm pore size scaffolds, respectively. Micropores were successfully produced using porogens of 100, 250, and 500 μm in average diameter. The size difference in these pores can be observed in SEM images (Fig. 1). In addition to this microporous structure, a nanostructure is formed by a continuous network of nanofilaments originating during the gelation step. Nanofilaments located on the wall of a micropore with a diameter of approximately 200 nm are observed in Figure 2.

The compressive modulus of these samples was then determined to demonstrate sufficient mechanical strength for bone tissue engineering. The compressive modulus was found to range between 100 ± 1 and 120 ± 1 kPa for the scaffolds tested (Table I). Specifically, scaffolds with 100 μm pore size had a higher modulus of 120 ± 1 whereas the 500 μm pore size scaffolds had a lower modulus of 100 ± 1 . The 250 μm pore size modulus had a compressive modulus more similar to that of the 100 μm pore size at 117 ± 0 kPa.

A solvent residue analysis was then performed, to verify the successful elimination of the solvent from the scaffolds. For all samples tested, a residue solvent value was found, which approached the detection limit of the instrument of 5 ppm.

hMSC growth and osteoblastic differentiation

After a 4 h incubation, approximately 70% of the total cells seeded were attached to the scaffolds based on cell counts, indicating hMSCs readily adhere to the PLLA scaffolds. SEM images indicated that by day 8 cells had spread and infiltrated scaffolds (Fig. 3). hMSCs can be observed covering pores demonstrating continued cell adhesion to the samples. This observation can also be made in histological sections in which the cells are stained using Hematoxylin and Eosin (Fig. 4). In all groups cells appear both lining and infiltrating the pores of the scaffolds by day 8.

Following an investigation of cell adhesion, DNA quantification was completed to determine the proliferation of seeded cells (Fig. 5). To account for any seeding difference all values were normalized to the respective day 1 sample. Through 12 days both the monolayer control groups proliferated at a steady rate with a day 12 increase of 5.56 ± 1.5 $\mu\text{g}/\mu\text{g}$ and 14.28 ± 7.30 $\mu\text{g}/\mu\text{g}$ fold for the control media and osteogenic media groups, respectively. Cell growth varied between static and dynamic culture and pore size; however, all day 12 samples maintained at least 70% of the cell number from day 1. The lowest day 12 relative cell numbers were observed in the 250 μm statically cultured group with a DNA decrease of 0.70 ± 0.24 $\mu\text{g}/\mu\text{g}$ fold compared to day 1. Slightly higher proliferation was observed in the 500 μm static groups with a day 12 increase of 1.14 ± 0.45 $\mu\text{g}/\mu\text{g}$ fold. The highest day 12 proliferation of the static groups was the 100 μm pore size with an increase of 2.94 ± 0.67 $\mu\text{g}/\mu\text{g}$ fold compared to day 1. Interestingly the 100 μm had the lowest day 12 relative cell number among the TPS cultured group with a decrease of 0.75 ± 0.21 $\mu\text{g}/\mu\text{g}$ fold compared to day 1. The 500 μm TPS cultured group had a nearly twofold higher proliferation with an increase of 1.49 ± 0.35 $\mu\text{g}/\mu\text{g}$ fold. The highest day 12 scaffold proliferation was observed in the 250 μm bioreactor groups with an increase of 3.69 ± 2.84 $\mu\text{g}/\mu\text{g}$ fold.

Following quantification of proliferation, intracellular ALP protein was measured as a marker of early osteoblastic differentiation (Fig. 6). On day 4, statically cultured groups and the monolayer osteogenic control showed slightly elevated levels as compared to day 1

levels. The 100, 250, and 500 μm statically cultured groups exhibited ALP values of 0.11 ± 0.05 , 0.20 ± 0.16 , and 0.22 ± 0.20 μM 4-nitrophenol/ μg DNA, respectively. These values represent an approximate twofold increase from day 1 numbers for the 100 and 500 μm scaffolds and approximately a fourfold increase for the 250 μm scaffolds. The osteogenic control also increased approximately threefold, while the bioreactor cultured groups and control media group maintained ALP levels close to day 1 values. By day 8, the statically cultured scaffolds decreased back to approximately day 1 levels while the bioreactor cultured scaffolds and the osteogenic control increased significantly. The 100, 250, and 500 μm had ALP values of 0.50 ± 0.26 , 1.67 ± 0.87 , and 1.00 ± 0.57 μM 4-nitrophenol/ μg DNA, respectively. The osteogenic control also exhibited an increase to 1.17 ± 0.54 . This represented an approximate 13-fold increase compared to day 1 numbers. Bioreactor groups also increased from day 1 numbers with fold changes of approximately 4, 11, and 6 for the 100, 250, and 500 μm groups, respectively. The control group remained at baseline level as expected. By day 12, dynamically cultured scaffold groups returned to a lower ALP level while the osteogenic control decreased, but to a level higher than other groups.

ALP was also analyzed in the long term study at the mRNA level [Fig. 7(a)]. All groups underwent a peak at day 8 but no significant differences were detected between the groups at this day. This could be due to an earlier peak of ALP mRNA in these groups. In addition to ALP, osteogenic signaling molecule BMP-2 [Fig. 7(b)] was analyzed at days 1, 8, 16, and 24. BMP-2 expression levels on day 1 were elevated in the bioreactor groups as compared to the statically cultured scaffolds. This trend continued on day 8, where the 500 μm and 250 μm bioreactor-cultured scaffolds exhibited statistically significant increased fold changes of 13.65 ± 1.67 and 6.93 ± 0.96 , respectively. On day 16, BMP-2 expression levels increased with increasing pore size and with dynamic culture. Static cultured samples exhibited fold changes of 0.29 ± 0.02 , 0.62 ± 0.07 , and 0.72 ± 1.2 , respectively, for the 100, 250, and 500 μm groups. TPS-cultured scaffolds exhibited higher BMP-2 expression levels with fold changes of 1.40 ± 0.28 , 3.73 ± 1.01 , and 6.04 ± 0.28 for the 100, 250, and 500 μm pore sizes, respectively. On day 24, the significantly higher expression levels were observed in the 250 and 500 μm bioreactor cultured groups. Lower BMP-2 expression levels continued to be observed in the 100 μm bioreactor group and the statically cultured groups including no BMP-2 being detected in the 500 μm statically cultured group.

DISCUSSION

The objectives of this study were to evaluate hMSC response to PLLA scaffolds fabricated using supercritical gel drying, to demonstrate cell viability on these scaffolds cultured in the TPS bioreactor, and to evaluate the cellular response to dynamic culture and pore size. To accomplish these objectives, PLLA scaffolds were first fabricated using supercritical gel drying with porogen leaching; thus producing gels characterized by high porosity and distinct nanostructure.¹⁹ The nanostructure was formed by a continuous network of nanofilaments formed during the gelation step in microstructure derived from porogen leaching. This nanostructure is fundamental to guide cell adhesion, proliferation, and migration.²⁶ Nanostructure of 50 and 24 nm was shown to reduce the adhesion of hMSCs compared to 200 or 1500 nm.²⁷ In addition to affecting proliferation and adhesion surface topography can affect hMSC differentiation and matrix production.^{28,29} Comparing titanium scaffolds without surface texture to those that do hMSCs produced significantly more mineralization and collagen on the scaffolds with surface topography.²⁸ Thus surface nanostructure is important to support hMSC attachment and proliferation as well as differentiation. Because of the well documented effects of scaffold nanostructure, this study focuses primarily on the effect of bioreactor culture and microporosity with this nanostructure present.

Solvent residue analysis was performed on PLLA scaffolds to verify the successful elimination of the solvents from the polymeric gels; for all PLLA scaffolds, a residue solvent value lower than 5 ppm was found, lower than the limits of USP 467 Pharmacopeia (380 ppm for Dioxane). Mechanical testing indicated these scaffolds to have compressive moduli from 100 to 120 kPa.

Following fabrication and characterization of scaffolds, hMSC attachment to scaffolds and long term viability was tested. Efficient seeding was accomplished with a seeding efficiency of over 70%. SEM and histological images indicated cells were readily able to infiltrate the scaffolds and attach to the nanostructure lining the pores. DNA quantification indicated the majority of the hMSC population remained viable throughout the 12 day study. In statically cultured scaffolds, the highest proliferation was observed on the 100 μm pore size scaffolds. It is hypothesized that this scaffold provided for closer cell interactions leading to increased cell proliferation. Cell density is a potent regulator of hMSC proliferation rate with low cell densities leading to reduced proliferation due to poor cell communication and high cell densities leading to reduced proliferation due to contact inhibition.^{30,31} Smaller pores permit for closer cell interactions,³² thus it is hypothesized in larger pore scaffolds cell–cell distance may have been too great for optimal proliferation, thus leading to the modest proliferation in the 500 μm static culture group and the slight decrease in cell number in the 250 μm group. Another potent regulator of cell proliferation is nutrient transport and waste removal. Nutrient transfer and waste removal are limited to hundreds of microns, thus culturing three dimensional scaffolds in a static environment can lead to nutrient gradients and non-homogenous cell distributions.^{20,33} In the bioreactor, the 250 and 500 μm pore size groups exhibited the higher proliferation levels compared to static cultures of the same pore sizes. It is hypothesized that in dynamic culture these pore sizes group allowed for greater infiltration of media flow into the scaffold, resulting in higher proliferation from increased nutrient transport. The highest proliferation was observed in the monolayer control groups, however, monolayer culture of hMSCs lacks a scaffold to support the cells upon *in vivo* implantation. Furthermore, upon reaching confluence in monolayer proliferation would no longer continue.

In addition to cell proliferation, osteoblastic differentiation was evaluated. ALP was used as an early osteoblastic marker and BMP-2 was evaluated as an important osteogenic signalling molecule. Statically cultured samples exhibited elevated ALP levels on day 4 indicating these samples may be undergoing differentiation more rapidly than bioreactor- cultured samples. Only small differences were observed for different pore sizes of statically cultured groups with slightly higher day 4 levels observed in the 250 and 500 μm groups. Though bioreactor-cultured samples did not exhibit a peak in ALP expression until day 8, the peak had a higher magnitude than the statically cultured samples. It was also much more dependent on pore size as no significant differences were observed between pore sizes of statically cultured constructs but with higher ALP levels again in the 250 and 500 μm groups in dynamically cultured constructs. It is hypothesized that dynamic culture magnifies the cellular response to pore size by stimulating the cells via fluid shear stress. Fluid shear stress has been widely demonstrated to enhance osteoblastic differentiation,^{20,34–38} and it is hypothesized that a greater percent of cells in larger pore size scaffolds are directly exposed to fluid shear stresses. ALP mRNA levels peaked at day 8, but did not show significant differences between groups. It is hypothesized this is because ALP mRNA peaked previous to day 8 in order to stimulate downstream protein production. BMP-2 expression levels follow a similar trend as ALP protein levels with higher levels in the bioreactor samples as compared to static controls. In addition, BMP-2 expression was shown to be dependent on pore size in dynamic culture with larger pore sizes corresponding to higher expression levels after day one. This further demonstrates the ability of the bioreactor culture to enhance osteogenic signal expression. Pore size and porosity have previously been shown to be

powerful mediators of hMSC osteoblastic differentiation through facilitation of autocrine and paracrine signalling pathways.⁸ Previous studies have analyzed the effect of pore size *in vitro* including findings that pore sizes greater than 500 μm increased osteogenic signal expression as compared to 180–300 μm pore sizes.¹² Larger pore size (500 μm compared to 200 μm) has also been demonstrated to increase cell proliferation.³⁹ It is hypothesized that this effect is due to increased nutrient transport throughout the scaffolds with larger pore sizes.^{5,12,39} Though the effect of pore size has been fairly widely investigated in static culture, relatively few studies have investigated the effect in dynamic culture. Focusing on porosity rather than pore size, it has previously been demonstrated that osteoblastic differentiation of rat BMSCs was influenced by scaffold geometry in a perfusion system.⁴⁰ A more recent study found that though scaffold pore size influenced osteoblastic differentiation in static culture, bioreactor culture was detrimental to both proliferation and differentiation of hMSCs.⁴¹ This was not found to be true in this study as proliferation and differentiation tended to increase during bioreactor culture. The difference could be due to the difference in bioreactor design. In the study demonstrating decreased hMSC proliferation, the flow perfusion system used was a direct perfusion system forcing media through the pores of the scaffold. Depending on the pore size, overall porosity and interconnectivity of a scaffold, this method can result in high shear stresses on cells lining the pores of the scaffold. In the tubular perfusion system, media is perfused in and around the scaffolds, thus the magnitude of shear stresses placed on cells is much less than forced perfusion systems of the same flow rate. Despite media not being directly perfused through the scaffolds tubular perfusion system culture still was able to increase both proliferation and osteoblastic differentiation. Based on these results, it can be concluded that scaffold microstructure greatly influences hMSC proliferation and differentiation in perfusion culture and scaffold geometry and flow rates must be tailored in tandem to optimize culture conditions.

CONCLUSIONS

This work demonstrated that 3D PLLA scaffolds could be produced by supercritical gel drying with porogen leaching and possessed mechanical strength for use as bone tissue engineering scaffolds. hMSCs were able to adhere, proliferate, and differentiate into the scaffold structure in both static and dynamic culture. Effects of the architecture of the scaffold were magnified in dynamic culture leading to increased proliferation and osteoblastic differentiation. Thus, we conclude that PLLA scaffolds produced by supercritical gel drying with porogen leaching are effective scaffolds for bone tissue engineering using hMSCs and can be cultured in the tubular perfusion system to enhance hMSC proliferation and differentiation.

REFERENCES

1. Gitelis S, Saiz P. What's new in orthopaedic surgery. *J Am Coll Surg*. 2002; 194:788–791. [PubMed: 12081070]
2. Mistry AS, Mikos AG. Tissue engineering strategies for bone regeneration. *Adv Biochem Eng Biotechnol*. 2005; 94:1–22. [PubMed: 15915866]
3. Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. *Biomaterials*. 2005; 26:5474–5491. [PubMed: 15860204]
4. Murphy CM, Haugh MG, O'Brien FJ. The effect of pore size on osteoblast activity in collagen-glycosaminoglycan scaffolds. *Tissue Eng A*. 2008; 14:914–914.
5. Kim K, Yeatts A, Dean D, Fisher JP. Stereolithographic bone scaffold design parameters: Osteogenic differentiation and signal expression. *Tissue Eng Part B Rev*. 2010; 16:523–539. [PubMed: 20504065]

6. Yoshimoto H, Shin YM, Terai H, Vacanti JP. A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering. *Biomaterials*. 2003; 24:2077–2082. [PubMed: 12628828]
7. Stamatialis DF, Papenburg BJ, Bettahalli MS, Girones M, de Boer J, van Blitterswijk C, Wessling M. Membranes for bioartificial organs and tissue engineering. *Tissue Eng A*. 2008; 14:755–755.
8. Betz MW, Yeatts AB, Richbourg WJ, Caccamese JF, Coletti DP, Falco EE, Fisher JP. Macroporous hydrogels upregulate osteogenic signal expression and promote bone regeneration. *Biomacromolecules*. 2010; 11:1160–1168. [PubMed: 20345129]
9. Zhang JC, Zhang H, Wu LB, Ding JD. Fabrication of three dimensional polymeric scaffolds with spherical pores. *J Mater Sci*. 2006; 41:1725–1731.
10. He LM, Liu B, Guan XP, Xie GY, Liao SS, Quan DP, Cai DZ, Lu J, Ramakrishna S. Microstructure and properties of nano-fibrous Pcl-B-Plla scaffolds for cartilage tissue engineering. *Eur Cells Mater*. 2009; 18:63–74.
11. Liu XH, Won YJ, Ma PX. Porogen-induced surface modification of nano-fibrous poly(l-lactic acid) scaffolds for tissue engineering. *Biomaterials*. 2006; 27:3980–3987. [PubMed: 16580063]
12. Kim K, Dean D, Wallace J, Breithaupt R, Mikos AG, Fisher JP. The influence of stereolithographic scaffold architecture and composition on osteogenic signal expression with rat bone marrow stromal cells. *Biomaterials*. 2011; 32:3750–3763. [PubMed: 21396709]
13. Kim TK, Yoon JJ, Lee DS, Park TG. Gas foamed open porous biodegradable polymeric microspheres. *Biomaterials*. 2006; 27:152–159. [PubMed: 16023197]
14. Mooney DJ, Baldwin DF, Suh NP, Vacanti LP, Langer R. Novel approach to fabricate porous sponges of poly(d,l-lactic-co-glycolic acid) without the use of organic solvents. *Biomaterials*. 1996; 17:1417–1422. [PubMed: 8830969]
15. Tsvintzelis I, Pavlidou E, Panayiotou C. Porous scaffolds prepared by phase inversion using supercritical CO₂ as antisolvent. I. Poly(l-lactic acid). *J Supercrit Fluids*. 2007; 40:317–322.
16. Duarte ARC, Mano JF, Reis RL. Preparation of starch-based scaffolds for tissue engineering by supercritical immersion precipitation. *J Supercrit Fluids*. 2009; 49:279–285.
17. Reverchon E, Cardea S, Rapuano C. A new supercritical fluid based process to produce scaffolds for tissue replacement. *J Supercrit Fluids*. 2008; 45:365–373.
18. Cardea S, Pisanti P, Reverchon E. Generation of chitosan nanoporous structures for tissue engineering applications using a supercritical fluid assisted process. *J Supercrit Fluids*. 2010; 54:290–295.
19. Reverchon E, Pisanti P, Cardea S. Nanostructured PLLA-hydroxyapatite scaffolds produced by a supercritical assisted technique. *Ind Eng Chem Res*. 2009; 48:5310–5316.
20. Yeatts AB, Fisher JP. Bone tissue engineering bioreactors: Dynamic culture and the influence of shear stress. *Bone*. 2011; 48:171–181. [PubMed: 20932947]
21. Rodrigues CA, Fernandes TG, Diogo MM, da Silva CL, Cabral JM. Stem cell cultivation in bioreactors. *Biotechnol Adv*. 2011; 29:815–829. [PubMed: 21726624]
22. Yeatts AB, Fisher JP. Tubular perfusion system for the long-term dynamic culture of human mesenchymal stem cells. *Tissue Eng C Methods*. 2011; 17:337–348.
23. Chen CW, Betz MW, Fisher JP, Paek A, Chen Y. Macroporous hydrogel scaffolds and their characterization by optical coherence tomography. *Tissue Eng C Methods*. 2010; 17:101–112.
24. Yeatts AB, Gordon CN, Fisher JP. Formation of an aggregated alginate construct in a tubular perfusion system. *Tissue Eng C Methods*. 2011; 17:1171–1178.
25. Kim K, Dean D, Lu A, Mikos AG, Fisher JP. Early osteogenic signal expression of rat bone marrow stromal cells is influenced by both hydroxyapatite nanoparticle content and initial cell seeding density in biodegradable nanocomposite scaffolds. *Acta Biomater*. 2011; 7:1249–1264. [PubMed: 21074640]
26. Ma PX. Scaffolds for tissue engineering. *Mater Today*. 2004; 7:30–40.
27. Dulgar-Tulloch AJ, Bizios R, Siegel RW. Human mesenchymal stem cell adhesion and proliferation in response to ceramic chemistry and nanoscale topography. *J Biomed Mater Res A*. 2009; 90:586–594. [PubMed: 18563822]

28. Mendonca DB, Miguez PA, Mendonca G, Yamauchi M, Aragao FJ, Cooper LF. Titanium surface topography affects collagen biosynthesis of adherent cells. *Bone*. 2011; 49:463–472. [PubMed: 21549232]
29. Shanmugasundaram S, Chaudhry H, Arinze TL. Microscale versus nanoscale scaffold architecture for mesenchymal stem cell chondrogenesis. *Tissue Eng A*. 2011; 17:831–840.
30. Bitar M, Brown RA, Salih V, Kidane AG, Knowles JC, Nazhat SN. Effect of cell density on osteoblastic differentiation and matrix degradation of biomimetic dense collagen scaffolds. *Biomacromolecules*. 2008; 9:129–135. [PubMed: 18095652]
31. Kim K, Dean D, Mikos AG, Fisher JP. Effect of initial cell seeding density on early osteogenic signal expression of rat bone marrow stromal cells cultured on cross-linked poly(propylene fumarate) disks. *Biomacromolecules*. 2009; 10:1810–1817. [PubMed: 19469498]
32. Oh SH, Park IK, Kim JM, Lee JH. In vitro and in vivo characteristics of PCL scaffolds with pore size gradient fabricated by a centrifugation method. *Biomaterials*. 2007; 28:1664–1671. [PubMed: 17196648]
33. Volkmer E, Drosse I, Otto S, Stangelmayer A, Stengele M, Kallukalam BC, Mutschler W, Schieker M. Hypoxia in static and dynamic 3D culture systems for tissue engineering of bone. *Tissue Eng A*. 2008; 14:1331–1340.
34. Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, Mikos AG. Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. *Proc Natl Acad Sci USA*. 2002; 99:12600–12605. [PubMed: 12242339]
35. Grellier M, Bareille R, Bourget C, Amedee J. Responsiveness of human bone marrow stromal cells to shear stress. *J Tissue Eng Regen Med*. 2009; 3:302–309. [PubMed: 19283726]
36. Li D, Tang T, Lu J, Dai K. Effects of flow shear stress and mass transport on the construction of a large-scale tissue-engineered bone in a perfusion bioreactor. *Tissue Eng A*. 2009; 15:2773–2783.
37. McCoy RJ, O'Brien FJ. Influence of shear stress in perfusion bioreactor cultures for the development of three-dimensional bone tissue constructs: A review. *Tissue Eng B Rev*. 2010; 16:587–601.
38. Stolberg S, McCloskey KE. Can shear stress direct stem cell fate? *Biotechnol Prog*. 2009; 25:10–19. [PubMed: 19197983]
39. Mygind T, Stiehler M, Baatrup A, Li H, Zoua X, Flyvbjerg A, Kassem M, Bunger C. Mesenchymal stem cell ingrowth and differentiation on coralline hydroxyapatite scaffolds. *Biomaterials*. 2007; 28:1036–1047. [PubMed: 17081601]
40. Gomes ME, Holtorf HL, Reis RL, Mikos AG. Influence of the porosity of starch-based fiber mesh scaffolds on the proliferation and osteogenic differentiation of bone marrow stromal cells cultured in a flow perfusion bioreactor. *Tissue Eng*. 2006; 12:801–809. [PubMed: 16674293]
41. Bjerre L, Bunger C, Baatrup A, Kassem M, Mygind T. Flow perfusion culture of human mesenchymal stem cells on coralline hydroxyapatite scaffolds with various pore sizes. *J Biomed Mater Res A*. 2011; 97:251–263. [PubMed: 21442726]

FIGURE 1.
3D PLLA scaffolds structures obtained with pore sizes of (a) 100 μm , (b) 250 μm , and (c) 500 μm .

FIGURE 2.
Nanofilaments on the walls of a micropore in a PLLA scaffold.

FIGURE 3.

SEM images of hMSCs growing on PLLA scaffolds following 8 days of static culture. Cell aggregates can be observed growing homogeneously on (a) 100 μm , (b) 250 μm , and (c) 500 μm synthetic scaffolds.

FIGURE 4.

Hematoxylin and Eosin staining of 100 μm (a,d), 250 μm (b, e), and 500 μm (c, f) PLLA scaffolds after 8 days of static (a–c) and dynamic culture (d–f). Cells have infiltrated pores of scaffolds in all groups by day 8. Scale bar represents 100 μm . [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 5.

Fold change of DNA content normalized to day one based on DNA quantification from PicoGreen. The symbol (*) denotes statistical significance within a timepoint ($p < 0.05$).

FIGURE 6.

Intracellular alkaline phosphatase protein normalized to DNA. Note increased day 8 ALP amounts in bioreactor groups and osteogenic control. Day 8, 250 µm pore size bioreactor group is statistically different from all static scaffold groups and monolayer control group. All other day 8 groups are statistically similar. The symbols (*, #) denote statistical significance within a timepoint ($p < 0.05$).

FIGURE 7.

Quantitative reverse transcriptase polymerase chain reaction analysis on days 1, 8, 16, and 24 of (a) alkaline phosphatase and (b) bone morphogenetic protein-2. All groups are normalized to day 1 100 μ m static. In ALP data note no relevant significant difference between groups. In BMP-2 data note statistically significant increases of expression levels in 250 μ m and 500 μ m bioreactor groups on days 8, 16, 24. The symbols (*, #) indicate statistical significance from all other groups within a timepoint ($p < 0.05$). The abbreviation ND refers to no gene detected within 40 cycles.

TABLE I

Effect of Leaching Agent Size on Porosity and Compressive Modulus of Gel-Dried PLLA Scaffolds

Average Fructose Particles Size Range (μm)	Polymer Concentration (%)	Compressive Modulus (kPa)	Porosity (%)
100	15	120 ± 1	95.5 ± 0.2
250	15	117 ± 0	96.0 ± 0.2
500	15	100 ± 1	96.3 ± 0.1

Means $\pm$ standard deviation presented ($n = 9$).