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Oxygen-Tension Controlled Matrices for Enhanced Osteogenic Cell Survival and Performance

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Abstract

The success of a clinically-applicable bone tissue engineering construct for large area bone defects depends on its ability to allow for homogeneous bone regeneration throughout the construct. Insufficient vascularization, and consequently inadequate oxygen tension, throughout constructs has been largely cited as the most significant obstacle facing successful bone regeneration in large area defects. The development of constructs that support bone and vessel-forming cell growth and function throughout the scaffold structure are desired for large-area bone defect repair. Here, we developed oxygen tension-controlled matrices that support more homogenous oxygen levels throughout the constructs. Specifically, we examined polylactic co-glycolic acid (PLGA) scaffolds with optimized pore distribution and the percent pore volumes, and demonstrated significantly decreased oxygen and pH gradient from the exterior of the construct to the interior after long-term cell culture *in vitro*. We confirmed the ability of these optimized constructs to support the cellular survival via live/dead assay. In addition, we examined their ability to support the maintenance of two clinically relevant progenitor cell populations for bone tissue engineering and vascularization, namely mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs), and confirmed the expression of key bone and vascular markers via immunofluorescence.

Keywords

porosity; hypoxia; oxygen tension; pH; mesenchymal stem cells; bone tissue engineering; vascularization

INTRODUCTION

A well-recognized challenge significantly hindering the generation of clinically-applicable bone tissue engineering constructs involves heterogeneous and insufficient nutrient delivery throughout the construct.⁹ This phenomenon has been associated with the migration of cells

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towards areas of higher nutrient levels, resulting in significantly higher cell densities at the construct's periphery, and consequently, decreased oxygen tension and buildup of waste-products within the construct's interior regions.¹⁴ This diffusion limitation and oxygen gradient formation *in vitro* has been linked to decreased progenitor cell differentiation into the osteogenic cell lineage, decreased alkaline phosphatase levels and mineralization potential.^{3,4,6,7,21,27,29} In addition, lack of vascularization, increased inflammation and central necrosis has been observed upon implantation *in vivo*.^{3,24}

Various strategies have been attempted to enhance nutrient transport to the interior of bone tissue constructs, and improve cell survival. For instance, oxygen-generating biomaterials composed of oxygen-rich compounds (i.e., hydrogen peroxide, sodium percarbonate and calcium peroxide-based oxygen generating particles) have been designed, and incorporated into tissue engineered constructs in order to provide a sustained oxygen release over an extended period of time.^{5,19} However, issues concerning the possibility of residual reactive oxygen species and hydrogen peroxide, and excess salt byproducts, such as cations that can be harmful to the bio-environment require further examination. In addition, an optimal amount of oxygen delivery is critical to cell survival, and potentially uncontrolled varying oxygen dosages (i.e., hyperoxic or hypoxic) may be detrimental to cells and significantly decrease cell viability.^{1,15} Studies are required to examine the kinetics and factors that influence the release of oxygen, as well as long-term *in vivo* investigations.⁵ Finally, these approaches attempt to enhance cell survival by increasing the oxygen tension in the scaffold, but fail to address the issue of nutrient transfer and diffusion, which results in a build-up of metabolic waste-products in the interior of the constructs, and consequently central necrosis.

Alternative methods that have aimed to eliminate the development of hypoxic conditions include pre-vascularization of the bone tissue engineering construct with an arteriovenous (A-V) loop,¹¹ culture with bioreactors,²⁵ and incorporation of angiogenic growth factors.¹⁷ These methods have resulted in yet limited success due to either impractical clinical application, or insufficient acceleration of construct vascularization resulting in as the rate of construct vascularization result in limited construct size.

Here, we propose to design matrices with optimal pore size and pore volume in order to allow for a decreased oxygen gradient from the exterior surface of the construct to its interior.⁸ We specifically examined poly(lactide-co-glycolide) (PLGA) microsphere scaffolds with increased pore sizes with retained mechanical strength in the range of human cancellous bone. Through a systematic investigation, we identified optimal pore size range that allowed for significantly decreased oxygen and pH gradient from the exterior of the construct to the interior after long-term cell culture *in vitro*. In addition, we examined the ability of these optimized constructs to support the survival via a live/dead fluorescent assay, and performance of two well-recognized progenitor cell populations that are clinically relevant for bone tissue engineering and vascularization, namely mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs).

2. MATERIALS AND METHODS

2.1 PLGA Scaffold Fabrication

PLGA microspheres were prepared by an oil-in-water method as reported previously.^{2,12,18} PLGA microsphere scaffolds were fabricated as previously described.² Briefly, for control scaffolds, PLGA microspheres (diameter 425–600 μm) were placed into a steel mold, and thermally sintered at 100°C for 1 hour. For macro-porous PLGA microsphere scaffolds, porogen, NaCl crystals of diameter 200–300 μm , were mixed with PLGA microspheres at specific weight ratios (i.e., PLGA:NaCl ratios of 100:0, 90:10, 80:20, 70:30, 60:40), placed into a steel mold, thermally sintered at 100°C for 1 hour, and then the NaCl was leached out by soaking in water for 2 hours. We fabricated and used disc-shaped scaffolds (10 mm diameter, 2 mm height) for porosity analysis; we fabricated and used cylinder-shaped scaffolds (5 mm diameter, 10 mm height) for *in vitro* analysis of oxygen and pH levels, as well as cell viability analysis in the interior of the constructs.

2.2 Scaffold Porosity Evaluation

Scaffold porosity measurements ($n=3$ /scaffold group) were carried out using cone-beam micro-focus X-ray computed tomography (CT) analysis (mCT40; Scanco Medical AG) as previously described by Amini *et al.*² Two-dimensional density profile images were obtained at every 6 micron depth of the scaffold. These images were compiled to construct a three-dimensional reconstruction of the scaffold sample. Accessible Pore Volume script, proprietary software developed and supplied by SCANCO Medical, was utilized to evaluate 3D scan data and process the images for porosity measurements (i.e., volume fraction, size, connectivity, accessible internal pore volume as a function of pore dimension). This software allowed for advanced pore structure characterization, as it was developed to mimic mercury intrusion porosimetry in a non-destructive manner. Briefly, the software aimed to measure the scaffold pore volume that is accessible through pathways with at least a pre-determined diameter X . Specifically, the distance of every pore voxel to nearest object surface was determined, and thresholded to diameter X . Therefore, only areas with at least diameter X were included for measurement, and the volume of the resulting pore structure was determined and plotted versus the pore diameter. Therefore, interconnected porosity was evaluated qualitatively via visual evaluation for the homogeneity of the pore structure, and quantitatively via histogram analysis for porosity as a function of pore size dimension. For qualitative visualization of scaffold pore structure, images with color-coded pore sizes on a scale of 0 microns to 500 microns were analyzed. The highest pore sizes are color coded in red, and lowest in blue. Statistical significance of the accessible internal pore volume was evaluated using a two-way analysis of variance (ANOVA) test.

2.3 Cell Isolation. Mesenchymal Stem Cell Isolation

Bone marrow derived mesenchymal stem cells (MSCs) were isolated from New Zealand White rabbits. Mononuclear cells were isolated via layering over a Percoll density gradient, and centrifuging at 600 rpm for 20 min at room temperature. The mononuclear cell fraction was seeded and expanded in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% FBS and 1% Penicillin/Streptomycin (P/S, Gibco) at 37°C and 5% CO_2 . Passages 3–5 were used for experimentation. **Blood-derived Endothelial Progenitor**

Cell Isolation. Peripheral blood (50 mL) was collected via cardiac bleeding protocol (approved by the University of Connecticut Health Center Animal Care and Use Committee) from New Zealand White rabbits as previously reported⁴. Briefly, mononuclear cell fraction was isolated by layering the peripheral blood over a Percoll density gradient, and centrifuging at 600 rpm for 20 min at room temperature. The mononuclear cell fraction was re-suspended in endothelial cell growth medium (EGM2, Lonza; composed of endothelial cell basal medium-2 (EBM-2), 10% FBS, 1% P/S and EGM-2-SingleQuots growth factors and supplements), immediately seeded on dishes coated with 1 $\mu\text{g}/\text{cm}^2$ of rabbit type I collagen (C5608, Sigma), and cultured at 37°C and 5% CO₂. Non-adherent cells were removed after 4–7 days with gentle washes of PBS. Culture medium was changed every three days. Cells isolated 3–4 weeks post-isolation (passages 5–8) were used for experiments.

2.4 Local oxygen tension and pH measurements

Needle-type fiber optic oxygen microsensors (501656, World Precision, Saratoga, FL) and pH microsensors (502123, World Precision, Saratoga, FL) were utilized to analyze oxygen levels and pH levels in the interior of MSC-seeded control and macro-porous scaffolds.^{2,28} Briefly, 100,000 MSCs were seeded on each scaffold type (i.e., 0% NaCl, 10% NaCl, 20% NaCl, 30% NaCl, 40% NaCl), and cultured for 21 days in osteogenic media (DMEM, 10% FBS, 10 nM dexamethasone, 50 $\mu\text{g}/\text{ml}$ ascorbic acid, 5 mM β -glycerophosphate) 37°C and 5% CO₂. At this point, a 25 gauge needle was utilized to pre-form a 2.5 mm deep channel on the side (mid-length) of the scaffold for which the probe would then be inserted (305127, Becton Dickinson). Prior to sample measurements, the oxygen microsensor was calibrated following a conventional two-point calibration protocol described by the manufacturer. Briefly, oxygen-free water and water-vapor saturated air were used as calibration standards. The oxygen-free water standard was prepared by dissolving one gram of sodium sulfite (S430, Fisher Scientific) in 100 milliliters of water in a sealed vessel, and the water-vapor saturated air was prepared by placing a wet piece of cotton in a sealed vessel. Prior pH measurements, the pH microsensors were calibrated with buffer solutions of pH 5.0, 6.0, 7.0 and 8.0. Oxygen tension and pH measurements in the medium were carried out by inserting a probe in the medium next to all experimental scaffold groups. Oxygen tension and pH measurements of the interior of the cell-seeded scaffolds was carried out by placing the probe tip in the center of the scaffold by way of the pre-formed channel made in the constructs. Oxygen tension and pH measurements are expressed as the mean of three samples per scaffold group $\pm$ standard deviation.

2.5 Cell Viability in Interior of Cell-Seeded Construct

Live-dead cell viability assay (Invitrogen, Carlsbad, CA) was used to analyze cell survival in the interior of cell-seed constructs. We seeded and cultured 100,000 MSCs on scaffolds (0% NaCl/100% PLGA, and 20% NaCl/80% PLGA) in osteogenic media for 21 days 37°C and 5% CO₂. Samples were bisected lengthwise from each scaffold group (n=3) to allow for the examination of cell viability in the sample's interior. Live-dead cell viability assay was performed according to the manufacturer's protocol to label live cells green with calcein AM, and dead cells red with ethidium homodimer-1 probes. Confocal microscopy (Zeiss LSM ConfoCor2, 20 $\times$ magnification) was utilized to image cells interior of the scaffolds.

2.6 Evaluation of cell-scaffold construct performance *in vitro*

To assess the ability of the oxygen tension controlled matrices to support osteogenic and vasculogenic stem cell growth, MSCs and EPCs were seeded on the scaffolds and cultured for 2 days *in vitro*. Specifically, oxygen tension controlled matrices (20% NaCl/80% PLGA scaffolds) were seeded with a total of 250,000 cells per scaffold (i.e., MSCs for the osteogenic experimental group, and EPCs for the vasculogenic experimental group), and cultured in 1-to-1 mix of endothelial and osteogenic media for 2 days 37°C and 5% CO₂. Prior to culture on scaffolds, MSCs were cultured in osteogenic media (DMEM, 10% FBS, 10 nM dexamethasone, 50 µg/ml ascorbic acid, 5 mM β-glycerophosphate) on tissue culture plate (TCP) for 7 days. MSC expression of osteogenic markers, RunX2 and Collagen Type I, and EPC expression of endothelial markers, CD31 and von Willabrand Factor (vWF) was analyzed by immuofluorescence. After 2 days *in vitro*, cell-seeded constructs were fixed in 10% formalin for 1 hour at room temperature, rinsed with PBS, and permeabilized with 0.25% Triton X-100 for 10 minutes. Constructs were rinsed with PBS, blocked in 10% normal goat serum for 1 hour, and then incubated with the following primary antibodies for 1 hour: anti-RunX2 antibody (Abcam, ab76956, 1:50), anti-Collagen I antibody (Abcam, ab34710, 1:100), anti-CD31 antibody (Abcam, ab28364, 1:50), anti-von Willebrand Factor antibody (Abcam, ab6994, 1:1000), Anti-β-Tubulin (Millipore 05-661, 1:200). Samples were then washed and labeled with the corresponding secondary antibody: anti-mouse IgG secondary antibody-FITC (sc-2010, Santa Cruz) or anti-rabbit IgG secondary antibody-FITC (sc-2012, Santa Cruz) diluted 1:100 in 1% BSA/PBS for 40 min at room temperature. Finally, cell nuclei were counterstained using propidium iodide (81845, Sigma, St. Louis, MO). Stained constructs were examined via confocal microscopy (Zeiss LSM ConfoCor2, 20× magnification).

3. RESULTS

3.1. Scaffold Porosity

The scaffold fabrication method, porogen leaching combined with microsphere sintering, as previously described by Amini *et al.* allowed for a significant and tunable increase in scaffold porosity.² MicroCT analysis was utilized to reconstruct three-dimensional images of all scaffold groups (PLGA scaffolds with sequentially increasing porogen weight percentage) with specific pore size dimension ranges. As seen in Figure 1, we first reconstructed the accessible pore volume of the scaffolds in the pore size dimension range of 100 to 500 microns. We define the accessible pore volume of the scaffolds to be the pore volume that is accessible from the external surface for cell infiltration. Generally, as percentage of porogen used increased, we observed an increase in accessible pore volume. For example, control scaffolds (i.e., no porogen used) displayed pore sizes in the lowest range.

3.2 Pore-size Distribution: Micro-CT imaging and analysis

By varying the porogen content during the scaffold fabrication process, we were able to tune the scaffold's pore size distribution. As seen in Figure 2, in a histogram plot comparing pore percentage and pore size range, PLGA microsphere scaffolds fabricated with at least 20% porogen displayed bi-modal pattern in relation to their percent of pores, peaking at 150 to

200 μm and then again in the range of 300 to 350 μm , whereas control scaffolds displayed a uni-modal pattern peaking at approximately 25% of pores are in the range of 150 to 200 μm . Porogen content increase from 10 to 20wt% displayed single to bi-modal transition in pore size distribution. In addition, scaffolds fabricated with at least 20% porogen displayed significantly greater pore sizes in the range of 200 to 400 μm (Figure 2).

PLGA microsphere scaffolds fabricated with 20% porogen displayed significantly increased percent of pores in the range of 200 to 400 μm . In addition, we have previously demonstrated that these scaffolds fabricated with 20% porogen retain mechanical strength in the range of human cancellous bone (i.e., compressive modulus ~ 250 MPa, and compressive strength ~ 4 MPa).² Also, to note, control PLGA microsphere scaffolds (i.e., scaffolds that did not have added porogen) display an average of 39 mm^3 void volume, and 39% porosity (i.e., void volume divided by total volume), whereas PLGA microsphere scaffolds fabricated with 20% NaCl display an average of 49 mm^3 void volume, and 45% porosity.

3.3. Matrix Oxygen Tension and pH

In order to examine the effect of increasing porogen concentration in scaffolds (i.e., porosity and accessible pore volume) and their ability to better support cell survival, we measured oxygen tension and pH levels throughout *in vitro* cultured constructs with optic microsensors (Presens). Specifically, MSCs were seeded uniformly along the length of the scaffold and cultured for 21 days *in vitro*. The oxygen and pH levels were measured at the periphery and interior of control constructs, as well as constructs fabricated with increasing percentage of porogen (i.e., 10%, 20%, 30% and 40%) with optic microsensors (Figure 4). We observed a direct relationship with increasing porogen and levels of oxygen and pH at the interior of the constructs (Figure 5). Oxygen tension levels in the interior of the control scaffold fell to approximately 0.5%, which are hypoxic conditions (Figure 5A). Compared to control scaffolds, matrices fabricated with at least 20% porogen displayed significantly increased oxygen levels in the interior of the constructs, and for this reason, we refer to these matrices as oxygen tension controlled matrices. Specifically, oxygen tension controlled matrices displayed oxygen tension of 3.42% after 21 days of *in vitro* culture with MSCs. There was not a significant difference in oxygen levels in the interior of constructs fabricated with 20%, 30% or 40% porogen.

Similarly, pH levels were measured at the interior of the scaffolds as a method to evaluate whether increasing construct pore sizes may decrease the pH gradient from the exterior of the constructs to the interior (Figure 5B). Again, we observed the greatest pH gradient and most significant drop in pH in the interior of control constructs to a pH level of approximately 6.84. Constructs fabricated with at least 20% porogen displayed significantly higher pH than control scaffolds, and they were not significantly lower than that measured at the periphery of the construct. Oxygen tension controlled matrices seeded with MSCs displayed a pH of approximately 7.32 in the interior of the constructs after 21 days *in vitro*.

3.3. Evaluation of viability and performance of osteogenic and endothelial progenitor cells on oxygen tension controlled matrices *in vitro*

In addition to assessing oxygen and pH levels in the interior of constructs cultured with MSCs, we also examined cell viability. After seeding and culturing MSCs on control and oxygen tension controlled matrices for pre-determined time points, the constructs were bisected along the length of the construct and stained green with calcein AM and red with ethidium bromide, to assess cell survival and death, respectively. Confocal microscopy was utilized to specifically examine cell viability and activity at the center interior of the constructs, as well as the exterior of the constructs.

As seen in Figure 6, there was not a significant difference in cell distribution and viability from the exterior to the interior of the constructs when comparing MSCs cultured on control and oxygen tension controlled matrices in a short-term 7 day time point. Live cells stained with calcein AM were observed throughout the exterior and interior of both control and oxygen tension controlled matrices. However, long-term 21 day *in vitro* culture of mesenchymal stem cells (MSCs) resulted in enhanced and more homogeneous cell survival throughout the oxygen tension controlled matrices, compared to that of control constructs. By 21 days *in vitro*, minimal cell survival was observed on the interior of control constructs as compared to that of the exterior.

Further, we examined the maintenance of both osteogenic and vasculogenic cell expression on our oxygen tension controlled matrixes. Specifically, oxygen tension controlled matrices were seeded with a total of 250,000 either pre-differentiated MSC-derived osteoblasts or EPCs, and were cultured in a mix of 1:1 ratio osteogenic and endothelial differentiation medium for 2 day *in vitro* (pre-implantation stage for future *in vivo* studies). As seen in Figure 7, MSC-derived osteoblasts maintained their differentiation as demonstrated by the positive immunostaining of osteogenic markers collagen type 1 (Col1) and RunX2. Furthermore, EPCs maintained the endothelial cell phenotype as demonstrated by the positive immunostaining of vascular endothelial markers CD31 and von Willabrand Factor (vWF).

4. DISCUSSION

The lack of oxygen and nutrient diffusion in tissue engineering constructs ultimately lead to cell and tissue necrosis, and is the limiting factor for developing functional tissues for clinical applications. In this study, we have examined these critical factors and evaluated approaches to overcome them for enhanced bone tissue engineering. Specifically, we report a fabrication method for the development of constructs that allow for the controlled oxygen tension levels throughout after long-term *in vitro* culture. We performed an in-depth MicroCT analysis to identify critical mean pore size and interconnected pore volume of these constructs. Lastly, we measured precise local oxygen and pH levels in the interior of the constructs, and confirmed more homogenous levels of both throughout our biodegradable, oxygen tension-controlled constructs in long term *in vitro* culture.

For the advancements in bone tissue engineering, it is necessary to evaluate the scaffold architecture parameters as it facilitates the improvement of the scaffold design process.

Several of the scaffold parameters that should be examined include porosity, pore size, and interconnectivity. Although various techniques may be used to evaluate scaffold architecture (i.e., scanning electron microscopy (SEM) analysis, flow and mercury porosimetry, gas pycnometry and adsorption), many present with shortcomings, including being destructive or toxic, time consuming and not resulting in highly accurate measurements.²⁶ The methods used in this study with MicroCT serves as the most attractive qualitative and quantitative technique as it is nondestructive, and capable of providing a comprehensive set of data. The accuracy of this method was validated and confirmed using mercury porosimetry in a study conducted by Petrie Aronin *et al.*²⁰ Although past studies has used similar MicroCT methods to measure scaffold pore volume, we were able to study the changes in pore size and interconnected pore volume as we increased the porogen used during the scaffold fabrication process.

In this study, we have utilized a previously described fabrication method of porogen leaching and microsphere sintering to develop poly(lactic-co-glycolic acid) (PLGA) microsphere scaffolds that exhibit tunable oxygen tension and pH levels in the scaffold's interior regions after long term *in vitro* cell culture. Specifically, by adding porogen to a mixture of PLGA microspheres, and leaching out the porogen via soaking in water after thermal sintering the molded mixture, we developed biodegradable scaffolds with increased pore sizes and accessible pore volume, while retaining mechanical strength in the range of human cancellous bone.² We demonstrated that scaffolds with increasing accessible pore volume corresponding specifically to pore sizes in the range of 200 to 400 μm (i.e., the critical pore size range for neovascularization of engineered bone constructs), resulted in a less significant drop in oxygen tension and pH from the exterior to interior regions of the MSC-seeded constructs after 21 days *in vitro*. For instance, oxygen tension controlled matrices fabricated with 80% PLGA microspheres and 20% porogen (by weight) demonstrate 30.6% of its accessible pore volume to pore sizes of 200 μm , 12.5% of its accessible pore volume to pore sizes of 300 μm , and 6.0% of its accessible pore volume to pore sizes of 400 μm , in contrast to 12.3%, 2.0% and 0.9% of that in scaffolds that did not undergo porogen leaching (i.e., control scaffolds). In all, oxygen tension controlled matrices had an increased interconnected pore volume of approximately 50%, and approximately 30% of the pores had a diameter of at least 200 μm . However, it is important to note that our oxygen tension controlled matrix constructs do demonstrate a sacrificed lower mechanical strength (data previously published).²

We further investigated the effects of increasing accessible pore volume in the range of pore sizes critical for vascularization on oxygen tension levels, as well as pH levels on the interior of constructs cultured with MSC-derived osteoblasts after 21 day *in vitro*. We found that the observed increasing cell survival trend can be attributed to the increased oxygen tension levels, as well as more normal pH levels in the interior of the scaffolds, as compared to that of control scaffolds after 21 day *in vitro*. The increasing accessible pore volume corresponded to less hypoxic oxygen levels, and less acidic pH levels, allowing for enhanced MSC-derived osteoblasts survival throughout the construct after long-term 21 day culture.

Previous studies have demonstrated that changes in oxygen tension and pH levels *in vitro* significantly affect the biologic activity of pre-osteoblastic stem cells. Specifically, a

reduction in oxygen level has been shown to inhibit various activities of pre-osteoblastic stem cells, specifically their rate of proliferation and osteogenic differentiation.^{6,7,13,16,21–23} Previous studies have examined markers of the osteoblastic phenotype, including ALP and calcium, and observed enhanced levels with increased oxygen tension levels. In addition, Kohn *et al.* demonstrated that small shifts in extracellular pH *in vitro* resulted in significant changes in the ability of bone marrow stromal cells to express key markers of osteoblast phenotypes. For instance, ALP activity and collagen synthesis decreased two-to-three fold as pH decreased from 7.5 to 6.6.¹⁰ This pH shift is in similar range that was observed in our study, and may help reason the decreased cell proliferation on the interior of our control constructs that displayed a decreased pH in the range of 6.8, compared to a pH of 7.4 in our optimized constructs.

In this study, we successfully and clearly demonstrate the direct relationship between increasing pore size and accessible pore volume, and increasing oxygen tension levels in the interior of our constructs. We used oxygen microsensors to locally quantify oxygen tension levels at the exterior and interior of the constructs that were fabricated with varying levels of porogen. We confirmed our ability to tune and control the oxygen tension in the interior of the scaffolds by the amount of porogen used during the fabrication process of the constructs. Furthermore, the significant difference in oxygen tension levels can in fact be clearly attributed to the pore size and accessible pore volume, and not a difference in cell number since cell density in all constructs were not statically different in long term culture.²

Our oxygen tension controlled matrices supported the survival and activity of MSCs and endothelial progenitor cells, critical progenitor cell populations for vascularized bone regeneration. The fundamental implications of this study are critical for establishing success of clinically relevant bone tissue engineering constructs, and will lay the groundwork for future bone tissue engineering studies. Our future studies will utilize this approach for the repair and regeneration of a critical-sized load-bearing bone defect in rabbits. In addition, in-depth studies will establish oxygen tension profiles and the corresponding cell behavior, in order to determine universal parameters such as scaffold pore distribution and the specific percent pore volumes that promote enhanced bone tissue engineering.

5. CONCLUSIONS

Oxygen tension is a key factor that influences the regeneration of bone and vascular tissue at a bone defect site. In this study, we used a unique method to measure both oxygen tension levels and pH locally at the interior of the constructs after long-term *in vitro* culture, and demonstrated significantly higher oxygen and pH levels in the interior of constructs that were fabricated with at least 20% porogen by weight compared to control constructs. We confirmed the ability of our oxygen tension controlled constructs to support the cellular survival, as well as their ability to support the maintenance of two clinically relevant progenitor cell populations for bone tissue engineering and vascularization, namely mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs). Therefore, we have developed oxygen tension-controlled matrices that allow for enhanced osteogenic and vasculogenic cell viability, and subsequently, may allow for increased success of vascularized bone regeneration upon implantation.

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Figure 1.

MicroCT reconstructed images of accessible pore volume of oxygen tension controlled matrices with specific pore size dimension ranges. Accessible pore volume images by imposing specific pore diameter parameters of (A) 100–500 microns, (B) 100–200 microns, (B) 200–300 microns, (B) 300–400 microns, and (E) 400–500 microns on 20% NaCl/80% PLGA scaffolds from a top-view.

Figure 2. Histogram comparing the pore size distribution in NaCl-leached PLGA microsphere scaffolds. Area under the curve equals 100% of pores.

Figure 3.
Percent of accessible pore volume in the range of 200 – 400 μm diameter for NaCl-leached PLGA microsphere scaffolds.

Figure 4.

(A) Photograph of oxygen and pH microsensor set-up. Insert shows the tip of the microsensor. (B) Microsensors were utilized to measure the local oxygen and pH levels in the center of cell seeded scaffolds. Insert figure demonstrates the dimensions of the scaffold and location where microsensors were inserted to measure the oxygen and pH levels.

Figure 5.
(A) Percent oxygen tension and (B) pH levels in the interior region of constructs cultured with MSC-derived osteoblasts after 21 days *in vitro*.

Figure 6. Effect of increasing porosity on cell viability in the interior of the PLGA microsphere scaffolds at 7 and 21 days (scale = 200 μm).

Figure 7. Immunofluorescence staining of osteogenic markers (RunX2 and Collagen type I) on MSC-seeded, and endothelial markers (CD 31 and von Willebrand factor) on endothelial progenitor cell seeded constructs. Tubulin staining is also shown for both MSCs and EPCs, and the staining was recorded after the scaffolds were cultured for 9 days *in vitro*. Green staining indicates FITC staining of CD31, von Willabrand factor and tubulin. Red staining represents nuclear (propidium iodide) staining.