
Formation of three-dimensional cell/polymer constructs for bone tissue engineering in a spinner flask and a rotating wall vessel bioreactor

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Abstract: The aim of this study is to investigate the effect of the cell culture conditions of three-dimensional polymer scaffolds seeded with rat marrow stromal cells (MSCs) cultured in different bioreactors concerning the ability of these cells to proliferate, differentiate towards the osteoblastic lineage, and generate mineralized extracellular matrix. MSCs harvested from male Sprague–Dawley rats were culture expanded, seeded on three-dimensional porous 75:25 poly(D,L-lactic-co-glycolic acid) biodegradable scaffolds, and cultured for 21 days under static conditions or in two model bioreactors (a spinner flask and a rotating wall vessel) that enhance mixing of the media and provide better nutrient transport to the seeded cells. The spinner flask culture demonstrated a 60% enhanced proliferation at the end of the first week when compared to static culture. On day 14, all cell/polymer constructs exhibited their maximum alkaline phosphatase activity (AP). Cell/polymer constructs cultured in the spinner flask had 2.4 times higher AP activity than constructs cultured under static conditions on day 14. The total osteocalcin (OC) secretion in the spinner flask culture was 3.5 times higher than the static culture, with a peak OC

secretion occurring on day 18. No considerable AP activity and OC secretion were detected in the rotating wall vessel culture throughout the 21-day culture period. The spinner flask culture had the highest calcium content at day 14. On day 21, the calcium deposition in the spinner flask culture was 6.6 times higher than the static cultured constructs and over 30 times higher than the rotating wall vessel culture. Histological sections showed concentration of cells and mineralization at the exterior of the foams at day 21. This phenomenon may arise from the potential existence of nutrient concentration gradients at the interior of the scaffolds. The better mixing provided in the spinner flask, external to the outer surface of the scaffolds, may explain the accelerated proliferation and differentiation of marrow stromal osteoblasts, and the localization of the enhanced mineralization on the external surface of the scaffolds. © 2002 Wiley Periodicals, Inc. *J Biomed Mater Res* 62: 136–148, 2002

Key words: bioreactor, scaffold, bone marrow stromal cells, osteoblasts, poly (D,L-lactic-co-glycolic acid), bone tissue engineering

INTRODUCTION

Large bone defects resulting from trauma, tumors, infections, or congenital abnormalities often require reconstructive surgery to restore function.¹ Current treatments include autograft or allograft transplantation and the use of synthetic materials such as metals and bone cements.² Autograft transplantation is the

preferred treatment but suffers from limited supply and donor site morbidity.³ Allografts, although more abundant, have been implicated in disease transmission.⁴ Metals or bone cements have been used for decades as permanent prostheses with proven utility but have often resulted in complications such as stress shielding-induced resorption of the surrounding bone and fatigue failure of the implant.⁵

During the last decade, new bone tissue-engineering strategies have been proposed that promise greater bone restoration without many of the limitations of the current therapies.⁶ These proposed strategies often require the use of degradable porous scaffolds of natural or synthetic biomaterials that can promote the migration of cells from the surrounding tissue or the growth of bone forming cells seeded within the porous network of the scaffold.^{7–9} Most of these bone tissue-engineering approaches envision

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seeding scaffolds with pluripotent marrow stromal cells (MSCs) obtained from the patient's bone marrow¹⁰ that have the potential to enhance new bone formation.¹¹ Although the autogenous origin of these cells prevents potential immune rejection, the amount of marrow available for transplantation is limited. Fortunately, techniques have been developed that allow osteoprogenitor cells from the bone marrow to be selected and expanded in culture over a billion fold, thus allowing the generation of a large transplantable cell population from a small biopsy.^{12–14} Scaffolds can be seeded with these MSCs and transplanted to the defect site or cultured *in vitro* for an additional period before transplantation.¹⁵ During this period, the cells will proliferate and also deposit bioactive extracellular matrix¹⁶ and growth factors that add osteoinductive potential to the scaffold.

The size of bone defects needing restoration is often large, requiring equally large three-dimensional scaffolds that can be fabricated into the desired shape to fill such defects. When synthetic porous poly(D,L-lactic-co-glycolic acid) (PLGA) disk-shaped scaffolds with a diameter of 7 mm and a thickness of 1.9 mm were seeded with rat MSCs and cultured under static conditions for 56 days, cell proliferation and mineralized tissue formation were restricted in a zone 120–250 μm from the scaffold surface, with only a small number of cells residing in the center of the polymer scaffold.¹⁶ The transport of low molecular weight metabolites, waste products, and other macromolecules within a cell-seeded matrix results primarily from diffusion generated by the existence of concentration gradients and convective forces from fluid flow.¹⁷ In the absence of fluid flow, the primary mechanism allowing transport of nutrients to the center of scaffolds cultured under static conditions is diffusion, which may not meet the significant metabolic requirements of cells seeded on three-dimensional scaffolds and cultured for extended time periods (more than 21 days).¹⁸ Some of the cells initially seeded at the interior of the porous scaffolds may migrate by chemotactic mechanisms towards the exterior surface where nutrient

concentration is higher. The remaining MSCs demonstrate decreased differentiation towards the osteoblastic lineage and lower expression of osteogenic phenotypic markers¹⁹ as a result of either the limited nutrient supply or the remaining low cell density, a condition known to have negative effects on the osteoprogenitor marrow stromal cell proliferation, differentiation, and mineralization.²⁰

Several bioreactor designs have been described for the culture of engineered tissues for *in vivo* implantation. Some of these bioreactors generate convective forces and can minimize nutrient transport limitations. One of the simplest bioreactor designs is the spinner flask (Fig. 1). Scaffolds are attached to needles hanging from the lid of the flask. Convective forces generated by a magnetic stirrer bar allow continuous mixing of the media surrounding the scaffolds. Spinner flasks have been used for the seeding of cells on scaffolds,²⁰ the culture of cell/polymer constructs for cartilage,^{21,22} and bone tissue regeneration.²³ Another bioreactor design that has been used with both microcarrier suspensions and scaffolds is the rotating wall vessel reactor (Fig. 1) originally designed to simulate a microgravity environment. This bioreactor is comprised of two concentric cylinders, whereas the microcarriers or the scaffolds are placed in the annular space.²⁴ Gas exchange occurs through the stationary inner cylinder whereas the outer cylinder is impermeable and rotates at a controlled rate. The free falling of the foams inside the bioreactor as a result of gravity can be balanced by the centrifugal forces due to the rotation of the outer-cylinder, thus establishing microgravity-like culturing conditions. Rotating wall vessels have been used in the culture of cells seeded on scaffolds or microcarriers in cartilage²² and bone^{25,26} tissue formation *in vitro*.

The overall objective of this study was to answer the following questions. Did the mitigation of nutrient transport limitations external to three-dimensional cell/polymer constructs, influence the proliferation, differentiation, and expression of osteoblastic markers of rat MSCs seeded on porous PLGA scaffolds and

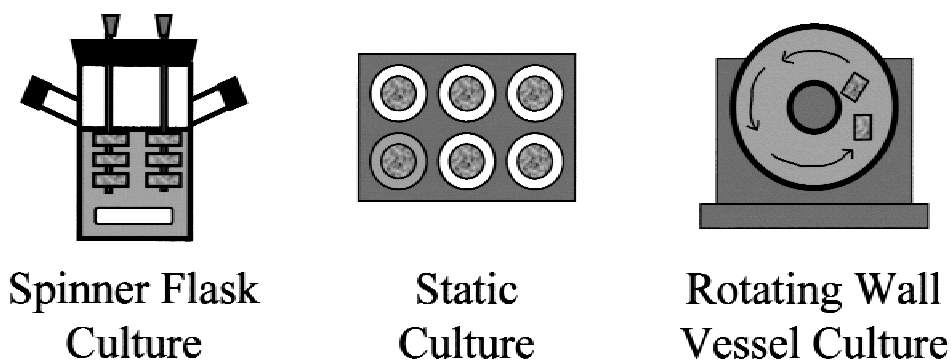


Figure 1. Drawings of the three bioreactors used in this study.

cultured in the presence of osteogenic media? Could the specific flow and mixing characteristics generated in a spinner flask and a rotating wall vessel bioreactor affect the growth and differentiation of MSCs seeded in porous PLGA scaffolds? Could the specific flow and mixing characteristics generated in these tissue-culture bioreactors affect the distribution of cells and mineralized matrix in the three-dimensional scaffolds after an extended culture period?

For this purpose, two model bioreactor designs, a spinner flask and a rotating wall vessel, were selected that generate convective forces in the media surrounding the cell/polymer constructs, thereby potentially decreasing the nutrient concentration gradients generated near the scaffold surface. These bioreactors have been investigated by Goldstein et al.,²³ but only for a short 14-day period, which allowed the monitoring of cell proliferation and distribution and early osteoblastic differentiation [alkaline phosphatase (AP) activity] but prohibited the measurement and distribution of deposited mineral and the secretion of osteocalcin, a marker of late osteoblastic differentiation. The proliferation, AP activity, and osteocalcin (OC) secretion of rat MSCs seeded on porous PLGA scaffolds and cultured in these bioreactors were monitored for a period of 21 days and compared with a static culture. Moreover, the distribution of cells and deposited mineralized matrix in the cell/polymer constructs after a culture period of 21 days was assessed by histology and tetracycline fluorescence, respectively.

MATERIALS AND METHODS

Preparation of the polymer scaffolds

To extend the findings in the work of Goldstein et al.²³ for a longer culture period that allowed full osteoblastic differentiation and the evaluation of mineralization, the scaffolds used in this study had similar preparation, dimensions, and pore morphology. 75:25 PLGA porous scaffolds were prepared by the solvent casting/particulate leaching method with NaCl as the leachable component.²⁷ NaCl crystals (Acros, Pittsburgh, PA), sieved into particles ranging in diameter 0.3–0.5 mm were combined with 75:25 PLGA (85 wt% NaCl and 15 wt% PLGA; Birmingham Polymers, lot D98036, weight average molecular weight of 81 kDa and polydispersity index of 3.7 as determined by gel permeation chromatography, Birmingham, AL) that was dissolved in methylene chloride (Fisher, Pittsburgh, PA). After solvent evaporation, the dry composite was broken manually in small pieces and compression molding was used to produce rods of 12.7 mm in diameter and 50 mm in length.²⁷ The rods were cut into pieces 6 mm in length using a diamond saw (Carver, Warsaw, IN). Sets of three foams were leached in 200 mL of distilled water for 3 days on a rotary table set at 120 rpm (distilled water was changed at least once every 12 h),

vacuum-dried for 24 h at 10 mtorr, and stored under vacuum until use. The prepared scaffolds had a porosity of 0.79 and a surface area of 0.25 m² per gram of polymer. Their physical properties have been characterized in detail previously.²⁸ Before seeding, the scaffolds were sterilized using ethylene oxide and then wetted with 200-proof ethanol.²⁷ The scaffolds were then placed in phosphate-buffered saline (PBS; Gibco BRL, Grand Island, NY) to remove the ethanol (PBS was changed at least twice for a period of 24 h). Approximately 2 h before cell seeding, 1 mL of a 50 µg/mL fibronectin solution (Sigma) in PBS was added drop-wise to the scaffolds to enhance cellular adhesion.²⁹ Finally, the scaffolds were washed drop-wise with PBS to remove residual fibronectin solution and they were then ready for seeding.

Rat MSCs isolation and primary culture

Rat MSCs were obtained from the marrow of femurs and tibias of 6-week old male Sprague–Dawley rats using well-established methods.^{14,30,31} After euthanasia by diethyl ether inhalation, femurs and tibias were aseptically excised, cleaned of soft tissue, and washed in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Grand Island, NY) containing 250 µg/mL gentamicin sulfate (GS; Sigma). The concentration of antibiotics was 5 times the normal amount used in cell culture as a precautionary measure to avoid contamination during the harvest. The metaphyseal ends were then cut off and the marrow was flushed from the midshaft with 5–10 mL of primary media [DMEM containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 50 µg/mL GS, 100 µg/mL ampicillin (Sigma), and 250 ng/mL fungizone (Sigma) using a syringe equipped with a 22-gauge needle and centrifuged at 400 g for 5 min. The resulting cell pellets from a tibia and femur pair were resuspended in 12-mL primary media and plated in a T-75 flask. After 3 days, haematopoietic and other unattached cells were removed. The cells were grown in a 5% CO₂, 95% relative humidity incubator at 37°C in the presence of primary media.

Cell seeding on polymer scaffolds

The cultures were enzymatically lifted from the T-75 flasks with 4 mL of trypsin-EDTA solution (0.05% trypsin; Gibco BRL) when they reached 70–80% confluency, at 7 days after harvest. The cells were counted and added in a cell suspension drop-wise to the polymer scaffolds. The volume of the cell suspension added to each scaffold was 1 mL, having a cell density of 10⁶ cells/mL. The cells were allowed to adhere to the scaffolds for 2 h and then the cell/polymer constructs were placed in 6-well plates, covered with 10 mL of media, and left overnight in the incubator before being placed in the bioreactors. The seeding method used here has been shown to generate a ho-

homogeneous cell distribution in the porous network of these polymer scaffolds.²³

Culture of cell/polymer constructs in bioreactors

In the static culture, each cell/polymer construct was maintained in one well of a six-well plate with 10 mL of osteogenic culture media (primary media supplemented with 100 nM dexamethasone; 10 mM β -glycerophosphate (Sigma); and 50 μ g/mL ascorbic acid-2-phosphate, all from Sigma).¹³ In the rotating wall vessel culture, 6 cell/polymer constructs were placed in the annular space between two concentric cylinders of a rotating wall vessel (Synthecon, Houston, TX) and the vessel was filled with 55 mL of osteogenic media (the constructs occupied approximately 5 mL of the 60 mL total bioreactor volume). The outer and inner cylinders had 57 mm and 20.6 mm diameters, respectively, resulting in an annular thickness of 18.2 mm. The length of the rotating wall vessel was 27 mm. The diameter and the length of the cylindrical cell/polymer constructs were 12.7 mm and 6 mm, respectively. The rotation speed of the outer cylinder must be optimized to generate the appropriate centrifugal forces that balance the free-falling of the foams inside the bioreactor resulting from the gravitational forces, thus establishing microgravity-like culturing conditions. A rotation speed of 30 rpm provided the optimum balance of the centrifugal and gravitational forces. The spinner flask system consisted of a dual side arm cylindrical flask with a diameter of 58 mm (Belco, Vineland, NJ) and a No. 12 rubber stopper, which served as a cover. Three pairs of cell/polymer constructs were placed on three 22-gauge needles (two scaffolds per needle, 10 mm apart) that pierced the rubber stopper. The three lower constructs were 20 mm from the bottom of the flask, and the three upper constructs were placed 36 mm from the bottom of the flask. The needles were arranged in a circle 12 mm from the center of the flask. A volume of 120 mL of osteogenic media was added to completely cover the scaffolds reaching 45.5 mm from the bottom of the flask. A magnetic stirrer was placed at the bottom of the flask and rotated freely at 30 rpm. Media in all cultures were changed every 2–3 days.

DNA measurement

The cellularity of the cell/polymer constructs was determined using a fluorometric DNA assay.³² Briefly, foams were removed from the bioreactors at day 7, 14, and 21, washed with ddH₂O, and homogenized in 1.4 mL of cold 10 mM ethylenediaminetetraacetic acid (EDTA) solution (pH 12.3) in a polypropylene centrifuge tube with a tissue homogenizer. The homogenates were then sonicated for 10 min in an ice bath and incubated at 37°C for 20 min before being returned to ice. A volume of 200 μ L of 1M KH₂PO₄ solution was added to neutralize the pH. DNA standards were prepared from a stock DNA solution that contained highly polymerized calf thymus DNA (type I, Sigma) at a concentration of 50 μ g/mL. A volume of 200 μ L of the stan-

dards or the homogenized samples was mixed with 1.3 mL of a 200 ng/mL Hoechst 33258-dye (Polysciences, Warrington, PA) in a 100 mM NaCl and 10 mM Tris buffer solution. The fluorescence emission at 455 nm was read with an excitation wavelength at 350 nm on a fluorescence spectrophotometer (VersaFluor Fluorometer, Bio-Rad). Cell standards were used to convert the total amount of DNA into number of cells.

AP activity measurement

AP activity was measured using a commercially available kit (ALP-10, Sigma).¹⁶ Briefly, foams were placed in a polypropylene centrifuge tube containing 1 mL of a 1M Tris solution at neutral pH and homogenized in a tissue homogenizer. The homogenates were then sonicated for 10 min in an ice bath. A volume of 20 μ L of each sample was added to 1 mL of reconstituted reagent with deionized water provided by the kit at 30°C. The absorbance was measured every minute for 4 min at 405 nm using a HP 8452A Diode Array Spectrophotometer (Walldronn, Germany). The slope of the absorbance versus time was used to calculate the AP activity.

OC measurement

OC secreted in the culture media was measured using a commercially available sandwich immunoassay (BT-490), specific for rat osteocalcin only, from BTI (Stoughton, MA).³³ The immunoassay is based on two goat OC antibodies, recognizing both the carboxylated and the decarboxylated rat OC, each directed toward an end of the (C- or N- terminal) protein. The N-terminal monoclonal antibody is bound to the wells and reacts with rat OC standard or sample whereas the C-terminal rat OC antibody completes the sandwich. Detection is achieved using a horseradish peroxidase conjugate of a donkey anti-goat IgG and subsequent addition of a peroxidase substrate (3,3',5,5'-tetramethyl benzidine) and hydrogen peroxide. The OC concentration was proportional to the absorbance measured at 450 nm in an ELISA plate reader. The BTI rat OC EIA kit provided a detailed description of the assay, standards for rat OC that allowed the generation of a dose response curve, and all the required reagents.

Calcium deposition measurement

Calcium deposition in the scaffolds was measured by the ortho-cresolphthalein complexone method (Sigma Diagnostics, Procedure No. 587, St. Louis, MO). Briefly, foams were removed from the bioreactors at day 14 and 21, washed with ddH₂O, and placed on an orbital shaker to incubate overnight in the presence of 2 mL of 0.5N acetic acid. Equal volumes of the calcium binding reagent (0.024% ortho-cresolphthalein complexone and 0.25% 8-hydroxyquinoline)

and the calcium buffer (500 mmol/L 2-amino-2-methyl-1,3 propanediol and other non-reactive stabilizers) provided in the assay kit were mixed to generate the assay working solution. A volume of 300 μL of working solution was added to 10 μL of sample in a 96-well plate. To generate a standard curve, serial dilutions of CaCl_2 were prepared (1–250 $\mu\text{g}/\text{mL}$). The plate was incubated at room temperature for 10 min and then read at 575 nm. The calcium deposition in each scaffold is reported as mg Ca^{2+} equivalents.

Histology and tetracycline fluorescence microscopy

Scaffolds were cryo-sectioned into 20- μm sections in a Microm 505 cryotome and stained with toluidine blue for visualization of cells. Mineral deposition was visualized by adding tetracycline-HCl (Sigma) in the culture media at a final concentration of 10 $\mu\text{g}/\text{mL}$. Tetracycline is a well-known fluorochrome-labeling agent for bone tissues.³⁴ Tetracycline accumulates at bone forming sites and fluoresces brightly when activated with fluorescent light. In this study,

the tetracycline incorporated into the mineral matrix was visualized using a Nikon E600 fluorescence microscope equipped with a Sony DXC-950P CCD camera.

Statistics

Multiple samples were collected in each measurement ($n = 3\text{--}5$), and the results were expressed as means $\pm$ standard deviation. Multiple pairwise comparisons have been performed with the Tukey-Kramer procedure³⁵ at a significance level of 95%.

RESULTS

The cellularity of the cell/polymer constructs was determined by a DNA assay at the 7, 14, and 21 days of culture (Fig. 2). During the first and second week,

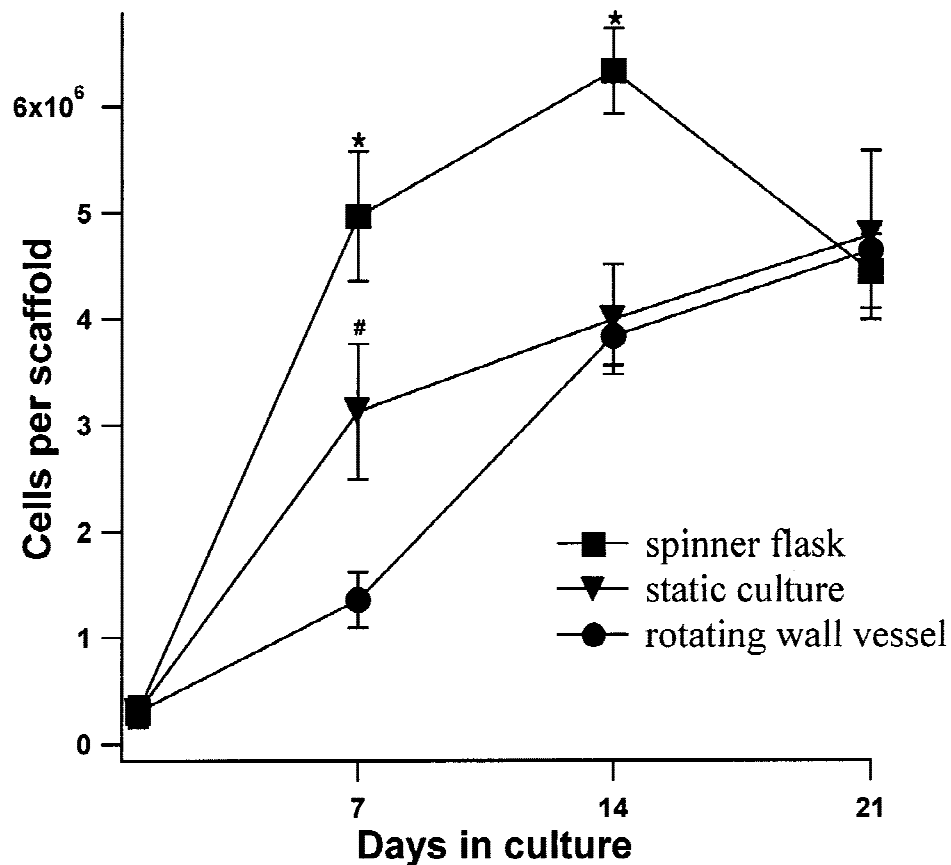


Figure 2. Cellularity of cell/polymer constructs during the 21-day culture period in the three bioreactors. The cellularity of all cell/polymer constructs cultured in the spinner flask, static culture, and rotating wall vessel, is denoted with squares, triangles, and circles, respectively. Pairwise comparisons were performed using the Tuckey-Kramer procedure with a significance level of 99%. When cell/polymer constructs from different bioreactors are compared for the same time point and the cellularity of cell/polymer constructs cultured in one bioreactor is significantly higher from the other two bioreactors then the bioreactor that generated the highest cellularity for this specific time point is denoted with a star (*). For the same time point, if the cellularities between the cell/polymer constructs cultured in the other two bioreactors are significantly different, then the higher of the two is denoted with the number sign (#).

the cell number increased in all three bioreactors, but the highest increase was observed in scaffolds cultured in the spinner flask. Scaffolds cultured in the spinner flask for 7 days had a 60% higher cellularity when compared to scaffolds cultured under static conditions and 4 times higher cellularity when compared to scaffolds cultured in the rotating wall vessel. It seems that the spinner flask accelerated the proliferation of MSCs seeded on polymer scaffolds. On day 14, cells seeded on scaffolds cultured in the spinner flask continued to proliferate and had 60% higher cellularity when compared to the other two culture configurations. During the third week, the cell number on the scaffolds cultured in the static and the rotating wall vessel cultures continued to increase whereas in the spinner flask it decreased. By day 21, the scaffolds cultured in all three bioreactors had approximately the same number of cells.

The AP activity of pre-osteoblastic cells is an indi-

cator of their commitment towards the osteoblastic lineage. The AP activity was determined for the 7th, 14th, and 21st day of culture, and normalized for the total cell number per scaffold for each time point. In Figure 3, the normalized AP activity for different time points is presented as the percentage of the maximum AP activity per cell measured in this experiment. All three cultures had a peak in AP activity on day 14. The highest AP activity was observed in the spinner flask culture and it was 2.4 times higher than AP activity of cell/polymer constructs cultured under static conditions. By day 21, the AP activity dropped considerably, and the spinner flask culture had the highest measurable AP activity (1.75 times higher than the static culture). The only detectable AP activity in the cell/polymer constructs cultured in the rotating wall vessel was observed on day 14, but it was two orders of magnitude lower than the AP activity in the spinner flask culture on the same day.

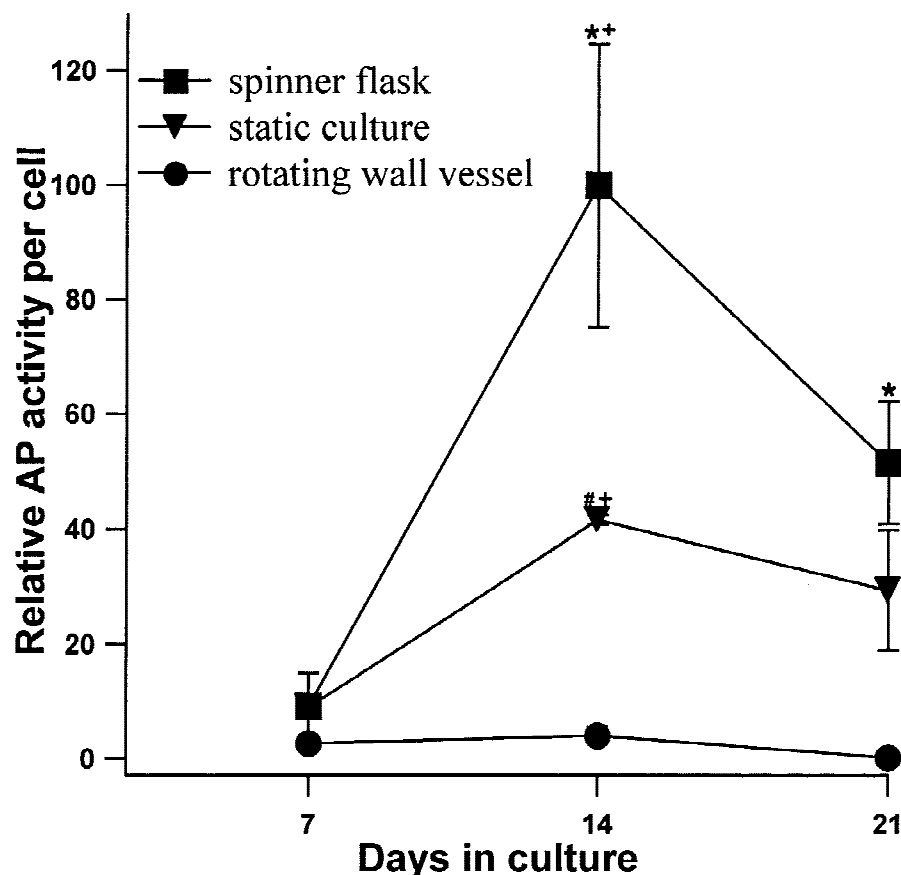


Figure 3. AP activity of cell/polymer constructs during the 21-day culture period in the three bioreactors. The AP activity of all cell/polymer constructs cultured in the spinner flask, static culture, and rotating wall vessel, is denoted with squares, triangles, and circles, respectively. Pairwise comparisons were performed using the Tuckey-Kramer procedure with a significance level of 99%. When cell/polymer constructs from different bioreactors are compared for the same time point and the AP activity of cell/polymer constructs cultured in one bioreactor is significantly higher from the other two bioreactors then the bioreactor that generated the highest AP activity for this specific time point is denoted with a star (*). For the same time point, if the AP activities between the cell/polymer constructs cultured in the other two bioreactors are significantly different, then the higher of the two is denoted with the number sign (#). When the AP activities between different time points on the same bioreactor are compared and for a certain time point it is significantly higher from all the other ones, the plus symbol (+) appears at that point.

OC, also known as Gla protein, is the most abundant non-collagenous protein in mature bone and is synthesized by mature osteoblasts and incorporated in the mineralized matrix.³⁶ A portion of secreted OC is not incorporated in the bone nodules but is released into the surrounding media. OC is a widely used marker for bone formation *in vivo* and a number of immunoassays have been developed for the detection of human serum OC.³⁷ The OC secretion in the media surrounding the scaffolds was measured during every media change and is presented for the three different bioreactor cultures in Figure 4. Because OC accumulates in the media, the reported values were normalized for the media volume and the number of days between each media change. OC was detected in the media of the spinner flask and the static culture as soon as day 11. As expected from the AP activity results, the spinner flask culture consistently had the highest OC secretion with the exception of day 14, where the difference in the concentration of OC in the spinner flask and the static culture media was not sig-

nificant. The OC secretion in the spinner flask media had a pronounced peak on day 18 corresponding to 0.1 pg/cell/day. The total OC release for the 21 days of culture in the media of the spinner flask was approximately 0.44 pg/cell and was 3.5 times higher than the total OC release in the static culture. No OC secretion was detected in the rotating wall vessel culture throughout the 21 days culture period.

The calcium deposition in three-dimensional scaffolds is an indication of the level of mineral deposition and a marker of the full maturation of osteoblastic cells. As indicated by the alkaline phosphatase activity and the osteocalcin secretion results, the spinner flask culture had the highest calcium deposition on day 21 (Fig. 5). The calcium deposition in cell/polymer constructs cultured in the spinner flask bioreactor was 6.6 times higher than the calcium deposition in the static controls and 33 times higher than the calcium deposition in cell/polymer constructs cultured in the rotating wall vessel. Calcium deposition was evident in the cell/polymer constructs cultured in the spinner flask,

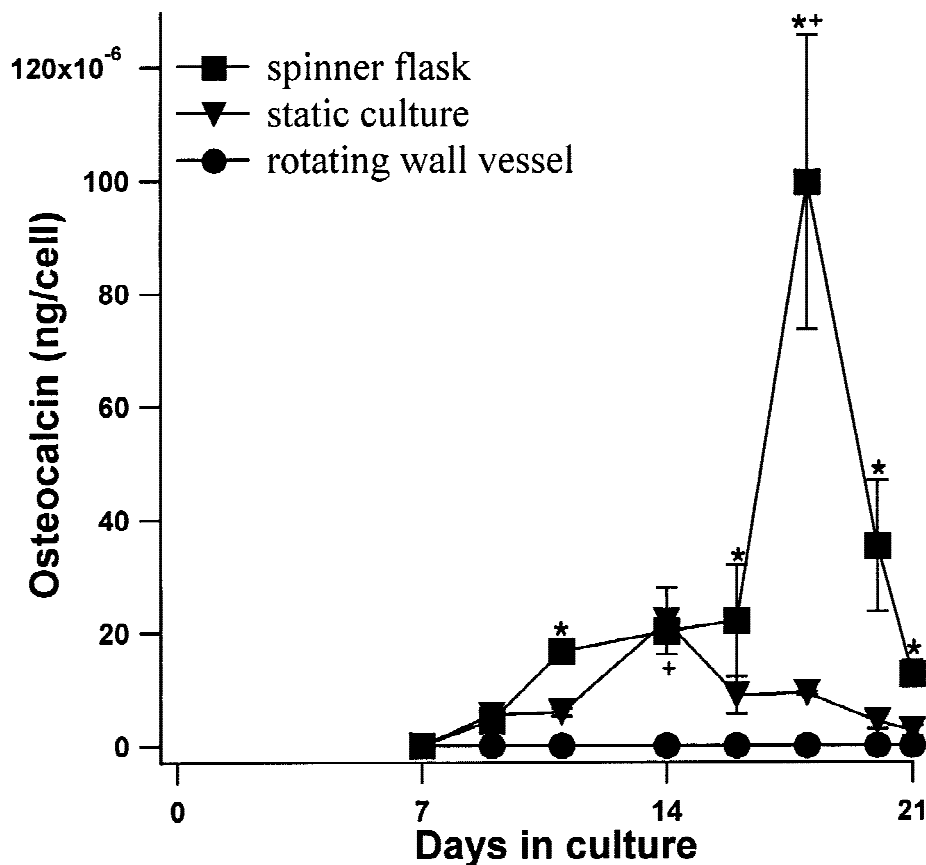


Figure 4. OC secretion from cell/polymer constructs during the 21-day culture period in the three bioreactors. The OC secretion from all cell/polymer constructs cultured in the spinner flask, static culture, and rotating wall vessel, is denoted with squares, triangles, and circles, respectively. Pairwise comparisons were performed using the Tuckey-Kramer procedure with a significance level of 99%. When cell/polymer constructs from different bioreactors are compared for the same time point and the OC secretion from cell/polymer constructs cultured in one bioreactor is significantly higher than the other two bioreactors then the bioreactor that generated the highest OC secretion for this specific time point is denoted with a star (*). When the OC secretion between different time points on the same bioreactor is compared and for a certain time point it is significantly higher from all the other ones, the plus symbol (+) appears at that point.

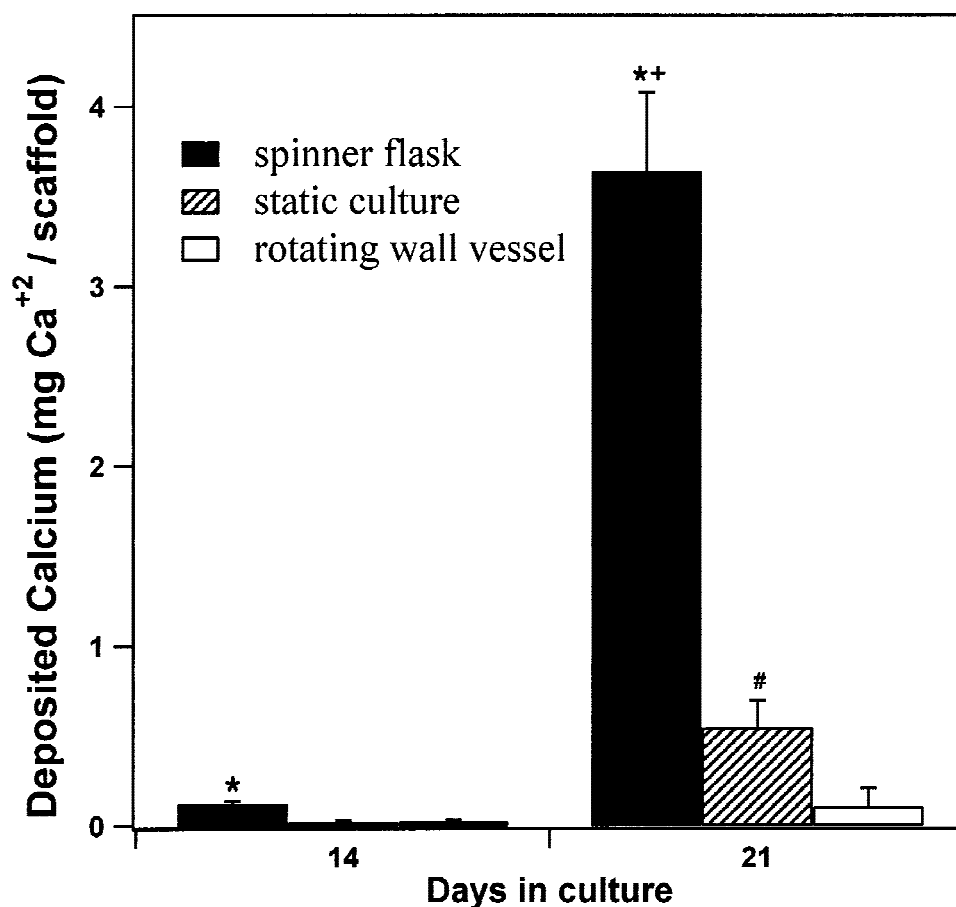


Figure 5. Calcium deposition in cell/polymer constructs during the 21-day culture period in the three bioreactors represented as equivalents of Ca^{2+} based on CaCl_2 standards used for the construction of the dose response curve. Pairwise comparisons were performed using the Tuckey-Kramer procedure with a significance level of 99%. When cell/polymer constructs from different bioreactors are compared for the same time point and the calcium deposition in cell/polymer constructs cultured in one bioreactor is significantly higher than the other two bioreactors then the bioreactor that generated the highest calcium deposition for this specific time point is denoted with a star (*). For the same time point, if the calcium deposition in the cell/polymer constructs cultured in the other two bioreactors is significantly different, then the highest of the two is denoted with the number sign (#). When the calcium deposition between different time points on the same bioreactor is compared and for a certain time point it is significantly higher from all the other ones, the plus symbol (+) appears at that point.

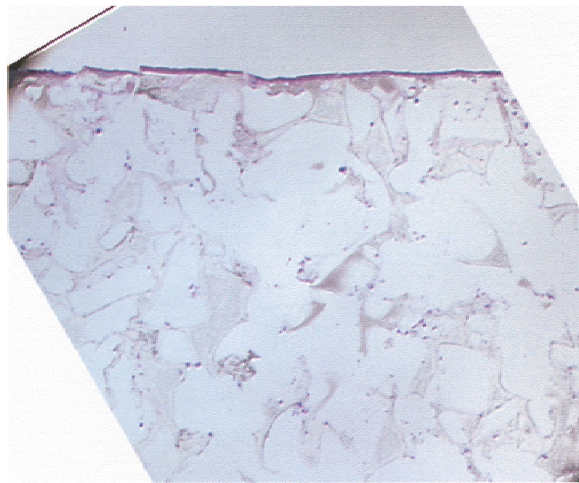
the rotating wall vessel, and the static controls from day 14. On that day, the highest calcium deposition was measured in cell/polymer constructs cultured in the spinner flask bioreactor, although the amount of calcium deposited until this time point was 30 times lower than the amount deposited on day 21 in scaffolds cultured in the same bioreactor. On day 14, the calcium deposition in cell/polymer constructs cultured in the rotating wall vessel and under static conditions was approximately 4 times lower than the amount deposited on the same day in constructs cultured in the spinner flask.

The histological sections of the scaffolds demonstrated that in all three cultures there was a sparse distribution of cells, with their majority forming a thick layer near the scaffold's surface. Representative histological sections from the cell/polymer constructs cultured in the spinner flask for 21 days are shown in

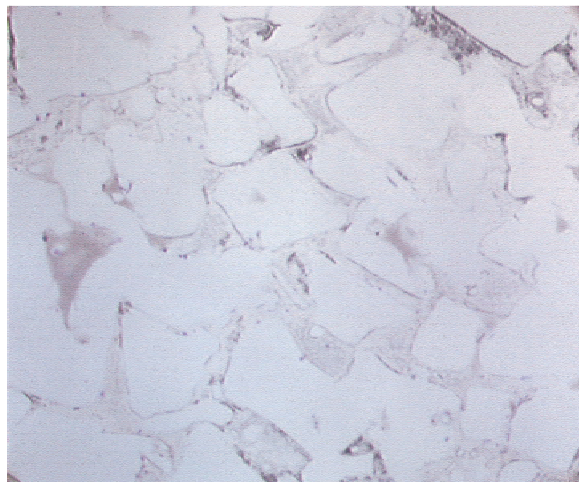
Figure 6. As expected, the mineralization was very strong on the surface of the scaffolds in the vicinity of the highest cell density (Fig. 7). The thickness of the mineralized layer was clearly higher in the cell/polymer constructs cultured in the spinner flask for 21 days [Fig. 7(a)] compared to the static culture for the same culture period [Fig. 7(b)] in accordance with the calcium deposition data. No tetracycline fluorescence appeared in cell/polymer constructs cultured for only 1 day under static conditions [Fig. 7(c)].

DISCUSSION

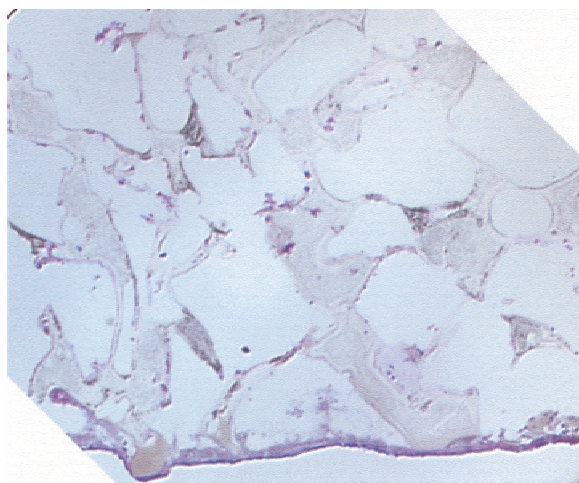
This study demonstrated that the mitigation of nutrient transport limitations, external to three-dimensional cell/polymer constructs, influences the



(a)

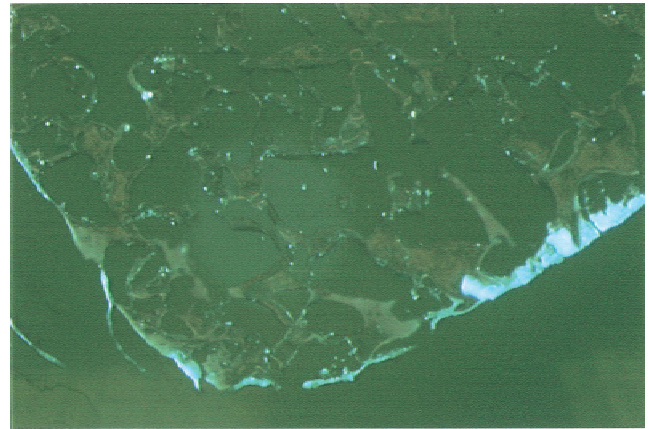


(b)

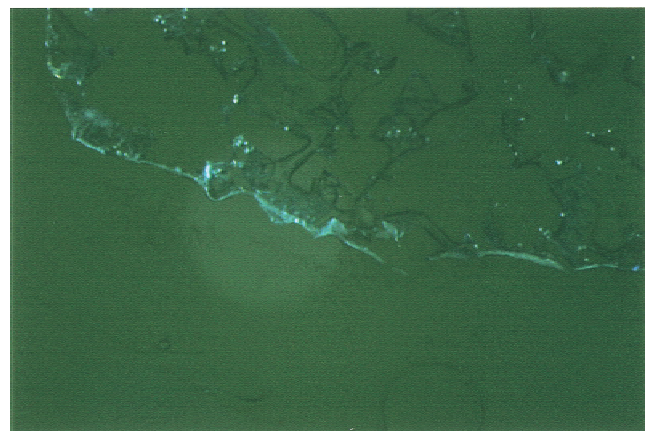


(c)

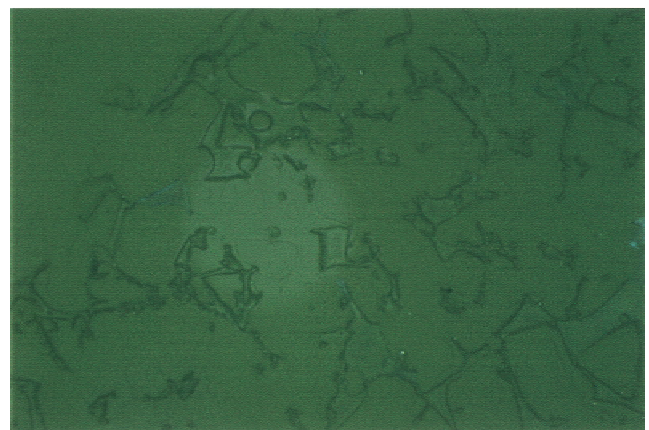
Figure 6. Representative histological sections from the (a) top, (b) middle, and (c) bottom part of cell/polymer constructs cultured in the spinner flask for 21 days and stained with toluidine blue.



(a)



(b)



(c)

Figure 7. Tetracycline labeling from representative sections of cell/polymer constructs cultured for (a) 21 days in the spinner flask bioreactor, (b) 21 days under static conditions, and (c) 1 day under static conditions. Tetracycline accumulates at bone forming sites and fluoresces when activated with fluorescent light.

proliferation, differentiation, and expression of osteoblastic markers of MSCs seeded on three-dimensional scaffolds and cultured in the presence of osteogenic media. Porous PLGA polymer scaffolds were seeded

with rat MSCs, and the cell/polymer constructs were cultured for an extended period of 21 days in two model tissue-culture bioreactors (a spinner flask and a rotating wall vessel) and in conventional static culture. The MSCs seeded in polymer scaffolds and cultured in the spinner flask bioreactor demonstrated: 1) accelerated proliferation during the first 2 weeks of culture; 2) the strongest AP activity with a maximum on the 14th day of culture; 3) the highest OC secretion in the media with a peak on the 18th day of culture; and 4) the highest calcium deposition at the end of the culture period. The temporal expression of cell growth and expression of the osteoblastic phenotype has three distinct periods: 1) a period of strong proliferation accompanied with the formation of extracellular collagenous matrix which lasts 10–12 days; 2) a matrix maturation phase that is accompanied by a down-regulation of proliferation and up-regulation of AP expression, a protein specific to the bone cell phenotype, which occurs between the 12th and the 18th day; and 3) a heavy mineralization phase that is marked by the further decrease of proliferation, a decline of the AP activity, and the induction of osteocalcin expression, which occurs between the 16th and the 20th day.^{38,39} The trends observed in the cell/polymer constructs cultured in the spinner flask bioreactor and the static culture are in agreement with the temporal expression and cell growth of osteoblastic cells described. Similar cellular behavior was seen by Goldstein et al.²³ for the early osteoblastic differentiation. The trends in cell number and alkaline phosphatase activity of the cultured cells were consistent between the two studies. The decreased cellularity observed at the end of the third week on the cell/polymer constructs cultured in the spinner flask is in accordance with the apoptotic behavior of mature osteoblasts residing in mineralized nodules.⁴⁰ Cell/polymer constructs cultured in the rotating wall vessel demonstrated a slower proliferation which may have delayed or dampened the normal differentiation pattern of osteoblastic cells observed in the other two bioreactors. Moreover, the enhanced proliferation, AP activity, OC secretion, and calcium deposition observed in the spinner flask culture indicate a positive influence of the potential mitigation of external mass transport limitations on the growth and differentiation of MSCs towards the osteoblastic phenotype.

The cell/polymer constructs in the spinner flask culture experienced the convective forces generated by the rotation of the magnetic stirrer in the bottom of the flask. The type of fluid flow in spinner flasks can be approximated by isotropic turbulence.^{21,41} Although the media volume is 120 mL, the cells experienced in their direct vicinity a small fraction of the media. If the rate of nutrient consumption from the immediate vicinity of the cells is faster than the replacement rate of that nutrients then a nutrient concentration gradient is

generated on the scaffold's surface and the seeded cells may experience malnutrition or decreased concentrations of growth factors vital for their growth and differentiation. In a static culture the only transfer mechanism of nutrients is molecular diffusion, while in the spinner flask the generation of convective forces enhances nutrient transport thereby decreasing the existing nutrient concentration gradients. The PLGA scaffolds were seeded with a cell density of 10^6 cells per scaffold and during the culture period they cell population increased more than 10-fold. When PLGA porous scaffolds seeded with MSCs were transplanted in the rat mesentery, bone-like tissue formation was limited to an external layer with a thickness less than 400 μm after 49 days *in vivo*.¹⁵ The bone-like tissue formed nodules surrounded by fibrovascular tissue that provided the necessary nutrients for the cellular metabolism. The inability of cells to survive in the absence of vascularization implied the inability of molecular diffusion to transfer nutrients even at moderate distances. It has been proposed that the largest volume of a transplantable tissue is 3 μL .⁴² The mitigation of the transport limitations on the external surface of cell/polymer constructs provides a convincing mechanistic explanation for the ability of the spinner flask to stimulate the growth and differentiation of marrow stromal osteoblasts seeded on polymer scaffolds.

The existence of convective forces in the spinner flask exposed the cells residing on the external surface of the PLGA scaffolds to shear forces. The sensitivity of osteoblasts to fluid shear stress is well established for cell cultures within flow chambers.^{43–47} The proposed mechanisms for the stimulation of osteoblasts by shear forces include the activation of growth factor signaling pathways,⁴⁸ cytoskeleton-integrin interactions,⁴⁹ and the formation of stress fibers and their attachment to focal adhesion points.^{50,51} The spinner flask bioreactor exposed the cell/polymer constructs to shear forces that may have contributed to the enhanced differentiation of MSCs towards the osteoblastic phenotype.

The rotating wall vessel is designed specifically to allow the culture of shear-sensitive mammalian cells in a microgravity-like environment. The rotating wall vessel bioreactor generates laminar rotational flow fields.²² The generation of nutrient transport limitations should have been mitigated in the cell/polymer constructs cultured in the rotating wall vessel bioreactor. The cell/polymer constructs grown in the rotating wall vessel displayed minimal differentiation towards the osteoblastic phenotype. There was an extremely low AP activity peak, compared to the static control, an absence of any detectable OC in the collected media throughout the 21 days culture period, as well as a low calcium deposition. This somewhat counter-intuitive result contradicts earlier reports on

the culture of osteoblastic cells in rotating wall vessels. Osteoblast-like cells seeded on PLGA lighter-than-water scaffolds and cultured in a rotating wall vessel exhibited increased AP activity and mineral deposition compared to static controls.²⁶ Rat MSCs cultured in a rotating wall vessel bioreactor in the presence of hydroxyapatite-coated hollow ceramic microspheres resulted in the formation of three-dimensional cell/carrier aggregates that generated extracellular matrix and mineral nodules.²⁵ The common theme in both of these successful attempts to culture osteoblastic cells in a rotating wall vessel was the small size of the carriers. Successful attempts of culturing large cell/polymer constructs have been reported for tissue-engineered cartilage.²² The main assumption in the operation of the rotating wall vessel bioreactor is the ability to generate microgravity like conditions, with the scaffolds been dynamically suspended in the flow field. When multiple, large scaffolds are placed in the rotating wall vessel, although free settling could be achieved, collision of the scaffolds with the reactor's walls could not be avoided. As stated previously, each cylindrical scaffold had a diameter of 12.7 mm and a length of 6 mm whereas the rotating wall vessel had an annular thickness of 18.2 mm and a length of 27 mm. These collisions disturb momentarily the desirable free settling, and they may traumatize the cells that reside on the surface of the scaffolds and come in direct contact with the surface of the wall. For collision-free culture of three-dimensional MSC/polymer constructs, the use of a much larger rotating wall vessel would be needed.

Although the mitigation of the external nutrient transport limitations influenced the MSC's proliferation and differentiation towards the osteoblastic phenotype, it did not influence the distribution of the cells and the deposited mineral matrix in the scaffolds. In all three culture systems, the histology sections revealed a dense cell layer on the surface of the scaffolds and a considerably lower cell distribution in the scaffold's interior. The inhomogeneous cell distribution in the scaffolds was in agreement with the distribution of the deposited mineral matrix in the static culture and the spinner flask (although the mineral matrix was heavier in the spinner flask culture). The transport of nutrients in the interior of the scaffolds in all three cultures was limited to diffusion. The generation of convective forces in the spinner flask did not extend to the interior of the scaffolds. The potential existence of internal nutrient transport limitations may be responsible for the inhomogeneous distribution of cells and mineral deposits in the three-dimensional scaffolds. The observed inhomogeneous cell distribution contradicts recent reports where: 1) MSCs seeded in porous PLGA scaffolds generated homogeneously distributed mineralized matrix after six weeks of culture under static conditions⁵² and 2) MSCs seeded in three-

dimensional PLGA meshes were found to be distributed homogeneously 4 weeks post-seeding when the cell/polymer constructs were cultured in orbitally mixed 24-well petri dishes.⁵³

Internal mass transport limitations can be minimized if the culture media are perfused through the porous network of the scaffolds. Murine osteoblasts seeded on collagen sponges and culture in a perfusion bioreactor were viable and homogeneously distributed in three-dimensional sponges contrary to the static controls where the cells appeared more dense near the sponge surface.¹⁹ Improved cell uniformity was observed in marrow stromal cells seeded on porous PLGA scaffolds and cultured in a perfusion bioreactor, compared to a spinner flask, a rotating wall vessel, and a static culture.²³ Perfusion of the media through three-dimensional bone tissue-engineering constructs will allow for the continuous replenishment of nutrients throughout the volume of the construct and will expose all the seeded cells to shear forces that may further stimulate them.

CONCLUSION

We have examined three different culturing conditions: 1) spinner flask culture, 2) static culture, and 3) rotating wall vessel culture for their ability to promote the proliferation and differentiation of marrow stromal osteoblasts seeded on PLGA porous scaffolds. The potential mitigation of external mass transport limitations in the spinner flask culture can have beneficial effects on the proliferation and differentiation of marrow stromal osteoblasts seeded on PLGA porous scaffolds. Cell growth and mineralization for all culturing conditions were limited to the outside of the scaffolds because the internal nutrient transport limitations were not eliminated. Improved culture conditions are needed to permit cellular growth throughout cell-polymer constructs.

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