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BONE MARROW CELL COLONIZATION OF, AND EXTRACELLULAR MATRIX EXPRESSION ON, BIODEGRADABLE POLYMERS

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Abstract

Poly(DL-lactide-co-glycolide)s (PLGAs) have been proposed as substrata for bone tissue engineering. In the experiments reported herein, we sought to identify the optimum lactide to glycolide ratio, from the series 85:15, 75:25, 50:50, or poly-(DL-lactide) (PLA), for the elaboration of bone matrix by cultured rat bone marrow cells (RBMC) on two-dimensional substrates. Having identified PLGA 75:25 as the optimum for bone matrix elaboration by RBMC, we produced three dimensional foams from this copolymer. For the two dimensional substrata, glass coverslips were spin-coated with one of the PLGAs, or PLA. Cultures were maintained for two weeks. We employed a new technique to label the elaborated bone matrix with the fluorescent antibiotic tetracycline. Bone matrix was present to a varying degree dependent on substrate composition: PLGA 75:25 = TCP > PLGA 85:15 >> PLA. No bone matrix was observed on PLGA 50:50 or on uncoated glass coverslips. Cell proliferation was similar on each surface except PLA on which they did not proliferate. Cell morphology was assessed by scanning electron microscopy. Based on these results, three dimensional devices were produced from PLGA 75:25. Our results demonstrate that the copolymer ratios that maximize cell proliferation are not identical to the that optimize bone matrix elaboration. Furthermore, despite the intended use of three dimensional matrices for connective tissue engineering applications, bone marrow-derived cells produced only a superficial matrix layer that did not invade the scaffold, whether produced by either the salt leaching or freeze-drying procedures employed.

Key Words: Poly-(DL-lactide) (PLA), poly(DL-lactide-co-glycolide) (PLGA), osteogenic cells, extracellular matrix, bone/substrate interface.

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Introduction

Bone defects are currently treated either with autogenous (Burchard, 1983; Friedländer, 1987), allogenous (DeBoer, 1988) or synthetic grafts (Saha and Pal, 1994; Matukas *et al.*, 1988). While autografts and allografts have been successful, both are limited, respectively, by donor tissue availability (Wakitani *et al.*, 1994) and risk of disease transmission/immune response (Buck and Malinin, 1989). Synthetic materials, such as bioceramics of calcium phosphates, meet some of the needs for bone replacement but are limited by their inherent stiffness, brittleness and low fatigue properties relative to bone.

Bone tissue engineering (the expansion of donor osteogenic cell populations on three dimensional matrices *in vitro* as a prelude to re-implantation) has generated widespread interest because it can be used to overcome the limitations of the foregoing techniques. For example, Caplan's group has described an innovative approach using calcium phosphate matrices (Ohgushi *et al.*, 1989; Goshima *et al.*, 1991); however, these implants remain *in vivo* for extended time periods. An alternative three-dimensional matrix, composed of biodegradable polymers, may be advantageous because the rate of polymer degradation can be controlled and this matrix may ultimately allow tissue remodeling.

Biodegradable polymers, such as poly(glycolide), poly(DL-lactide) and poly(DL-lactide-co-glycolide) have been used in numerous temporary therapeutic applications including sutures, implants and drug release systems (Vert *et al.*, 1984; Vert, 1989; Lewis, 1990; Zhang *et al.*, 1993). These α-hydroxy polyesters are biocompatible (Engleberg and Kohn, 1991) and degrade hydrolytically to glycolic acid and lactic acid, of which the latter is a metabolite in carbohydrate metabolism (Kulkarni *et al.*, 1966). The degradation of PLGA copolymers is a function of several variables, including polymer molecular weight, molecular weight distribution (von Recum *et al.*, 1996), polymer crystallinity and the lactide:glycolide ratio (Reed and Gilding, 1981): the higher the relative concentration of lactide, the slower the rate of degradation. The rate of polymer

degradation can be controlled such that bone formation is simultaneous with polymer scaffold degradation. PLGA has been investigated for bone cell interaction and growth and was shown to be osteoconductive (Hollinger, 1983; Hollinger *et al.*, 1986; Vert *et al.*, 1981). While previous *in vitro* studies have shown that the proliferation of bone marrow-derived cells is influenced by the lactide:glycolide copolymer composition, no direct evidence of bone matrix formation was provided (Ishaug *et al.*, 1994).

In the present study, poly(DL-lactide) and poly(DL-lactide-co-glycolide) were used as supporting materials for primary bone marrow-derived cells. To determine a suitable biodegradable polymer for bone matrix formation, an established bone marrow cell culture system was used (Davies *et al.*, 1991; Davies, 1996) with 2-dimensional polymer-coated glass coverslips. The bone marrow-derived cell-polymer surface interaction was assessed using amorphous polymers of different ratios of lactide to glycolide to determine which polymer surface was most suitable for bone matrix formation. Having determined a biodegradable polymer that supports bone matrix formation in the two-dimensional system, the structural relationship between the elaborated bone matrix and an underlying three-dimensional polymer matrix was investigated. For tissue engineering applications, a three-dimensional substrate that promotes matrix formation is essential.

Materials and Methods

Polymer-coated glass coverslips

PLGA 85:15 (inherent viscosity = 0.66 dL/g), PLGA 75:25 (inherent viscosity = 0.67 dL/g), PLGA 50:50 (inherent viscosity = 0.59 dL/g), and PLA (inherent viscosity = 0.59 dL/g), (provided by Birmingham Polymer Inc., Birmingahm, AL) were each separately dissolved in chloroform (Caledon Laboratories Ltd., Georgetown, ON, Canada) at 2% (w/v). Glass coverslips (Bellco, Vineland, NJ) were sterilized at 200°C for 2 hours. One half milliliter of a 2% polymer solution was applied to sterile glass coverslips and spin-coated for 120 seconds at 5500 rpm using a photolithographic spinner (Headway Research Inc., Garland, TX). The coverslips were then air-dried, disinfected in 70% ethanol for 30 minutes and rinsed five times with α-minimal essential medium (α-MEM) prior to seeding with cells. Uncoated glass coverslips (control for spin-coating) were treated identically to polymer-coated coverslips and sterile tissue culture polystyrene dishes (TCP, control for cell culture) were used as received (Falcon, Div., Becton Dickinson & Co., Cockeysville, MD). Spin-coated glass coverslips were characterized by: (1) dynamic advancing and receding water contact angle measurements to assess the relative hydrophobicity of the polymer-coated glass coverslips; (2) X-ray photoelectron spectroscopy (XPS) to determine surface elemental composition; and (3) scanning electron microscopy (SEM) to determine surface topography. Advancing and receding water contact angles were obtained on a Ramé-Hart NRL telescopic goniometer (Naval Research Laboratories, Mountain Lakes, NJ). Values reported represent the average and standard deviation of five measurements per surface taken of three samples per surface. XPS data were collected on a Leybold (Cologne, Germany) LH Max 200 using a MgKα X-ray source at 15 kV and 20 mA emission current. An aperture size of 13 x 7 mm was used to collect data at take-off angles of 90° between sample and detector. Scanning electron micrographs were taken on a Hitachi (Tokyo, Japan) 2500 SEM operated at an acceleration voltage of 15 kV.

Preparation of polymer foams

Three-dimensional polymeric foams were prepared with PLGA 75:25 using the following techniques and characterized by SEM for average pore size.

(1) **The solvent-casting particulate leaching technique** (Mikos *et al.*, 1993): a 10% w/v solution of PLGA 75:25 in chloroform was prepared by dissolving 1 g of PLGA 75:25 in 10 ml of chloroform at room temperature (RT). 10 g of un-sieved sodium chloride crystals (Analar, BDH Inc., Toronto, ON, Canada) were added to the polymer solution which was thoroughly mixed by vortex and then immediately cast into 2.5 cm diameter Teflon molds. Cast polymer structures were left at RT for 48 hours during which time the chloroform evaporated. The remaining polymer-salt structure was then placed into water with continuous stirring at RT for 48 hours in an attempt to dissolve all salt and thereby leave a porous structure. During the first 8 hours of the 48 hour period, the aqueous phase was replaced after every 2 hours with fresh deionized water, obtained from a Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Millipore Corp, Bedford, MA) and used at 18 MΩ resistance. For the remaining 40 hours, the aqueous solution was replaced with fresh deionized water after every 8 hours period.

(2) **The freeze-dried emulsion technique** (Whang *et al.*, 1995): a 10% w/v solution of PLGA 75:25 in chloroform was prepared by dissolving 100 mg of PLGA 75:25 in 1 ml of chloroform at RT. The polymer solution was homogenized (Kinematica PCU PT10-35, speed setting 3; Brinkmann Instruments, Rexdale, ON, Canada) for 1 minute during which 4 ml of deionized water were slowly added, creating a creamy emulsion. This emulsion was poured into a cylindrical aluminum foil mold which was immediately immersed in liquid nitrogen and then freeze-dried for 48 hours at a pressure

of 30 mTorr.

Cell Culture

Cell culture on polymer-coated glass coverslips. First passage primary bone marrow-derived cells were seeded on experimental and control (TCP and un-coated glass coverslip) surfaces using protocols and media described in detail elsewhere (Davies *et al.*, 1991). Briefly, bone marrow-derived cells were collected from both femora of young adult male Wistar rats (approximately 150 g) into a fully supplemented medium (FSM): α-MEM supplemented with 15% fetal bovine serum, 50 mg/ml ascorbic acid, 10 mM β-glycero-phosphate, 10^{-8} M dexamethasone (DEX) and antibiotics (0.1 mg/ml penicillin G, 0.05 mg/ml gentamicin and 0.3 mg/ml fungizone). Cells were maintained in culture for 6 days, and re-fed at day 2 and 5 with FSM. At day 6 , cells were trypsinized and seeded on the disinfected polymer surfaces at a concentration of 5 x 10^4 cells/ml. Polymer-coated coverslips, that were immersed in the tissue culture medium but not plated with cells, served as controls for polymer degradation. Tetracycline·HCl powder (Sigma, St. Louis, MO) was dissolved in α-MEM to prepare a stock solution of 90 mg/ml. A new tetracycline-containing fully supplemented medium (TFSM) was prepared of α-MEM containing 15% fetal bovine serum, 50 mg/mL ascorbic acid, 10 mM β-glycerophosphate, 10^{-8} M dexamethasone and tetracycline at 10% of the concentration described above. Cultures were re-fed at day 10, 12 and 15 with TFSM and observed during culture by inverted phase microscopy. Cultures were first fixed in Karnovsky's fixative (2.0% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer, pH 7.2-7.4). Following this, the cultures were dehydrated in series of graded alcohol solutions (70%, 100%), critical-point dried from carbon dioxide (Ladd Research Industries Inc., Burlington, VT), sputter-coated with gold (approximately 10 nm) (Polaron Instrument Inc., Doylestown, PA) and then examined first under ultra-violet (UV)-light as described below, then by SEM.

Imaging of bone matrix deposited during cell culture (i.e., tetracycline fluorescence). Tetracycline-labeled bone matrix was visualized under UV light (Lowenberg *et al.*, 1996; Parker *et al.*, 1997). The polymer-coated glass coverslips and control surfaces on which cells had proliferated for 21 days were photographed under UV-light using a Nikon camera (F- 601 equipped with AF Micro Nikkor 60 mm lens) using colour slide film (Ecktachrome, 400 ASA; Kodak, Rochester, NY). A custom-built box contained the UV-irradiation and limited light from external sources. The UV-source consisted of 4 lamps (365 nm wavelength; Microlites Scientific, Toronto, ON, Canada) positioned circumferentially inside the custom built box. A UV filter and a broad band interference filter (l = 550 nm, Melles Griot, Irvine, CA) was fitted to the lens in order to narrow the band of transmitted light to the 500-600 nm range. The emitted fluorescence of tetracycline is in the 530 nm range.

Cell proliferation study. First passage primary bone marrow-derived cells were used as above. Two different initial cell densities were used: a higher cell density of 5 x 10^4 cells/ml (or 1.5 x 10^5 cells/polymer surface) and a lower cell density of 2.7 x 10^4 cells/ml (or 8.1 x 10^5 cells/polymer surface). For each material tested a total of 36 polymer-coated coverslips were seeded with cells: 18 were seeded at the low cell density and 18 at the high cell density. For both high and low cell density experimental sets, cells were removed from three samples of each surface (polymer-coated and control groups) every day with trypsin (0.01% trypsin in citrate saline buffer, Gibco) and counted using a Coulter counter (Coulter Electronics Ltd., Luton, U.K.). The culture media was collected in a 7 ml polystyrene tube and analyzed with a pH meter (Accumet, Fisher Scientific, Nepean, ON, Canada).

pH measurements of PLGA 75:25 surfaces cultured with and without cells. Six PLGA 75:25 spin-coated glass coverslips were prepared (as described above), disinfected and placed in a 6-wells TCP dish. Polymer-coated glass coverslips were either seeded with first passage primary bone marrow-derived cells or immersed in 3 ml FSM alone. Similarly, plain TCP wells were either seeded with first passage primary bone marrow-derived cells or filled with 3 ml FSM alone. Media samples from the four sample types were collected daily and their pH was measured. The cultures and controls were maintained for 19 days (at 37°C and 5% CO_2) while media were replaced every 2-3 days. The experience was repeated twice, using a total number of four 6 well plates.

Cell culture on polymeric foams. Polymer foams were placed into 6 well Falcon dishes and disinfected with 70% ethanol for 30 minutes. The foams were seeded with 3 ml of first passage primary bone marrow-derived cells at a concentration of 5 x 10^4 cells/ml (or 1.5 x 10^5 cells/foam). Cultures were maintained for 28 days, re-fed every 2-3 days with FSM and from day 9 to day 28, with TFSM. Cultures on foams were then fixed in Karnovsky's fixative, dehydrated in graded alcohols and freeze-dried.

Results

Polymer-coated glass coverslips

Polymer-coated glass coverslips were characterized

Table 1. XPS elemental composition and advancing (θ_A) and receding (θ_R) water contact angles (n = 15, mean ± standard deviation) of polymer-coated glass coverslips.

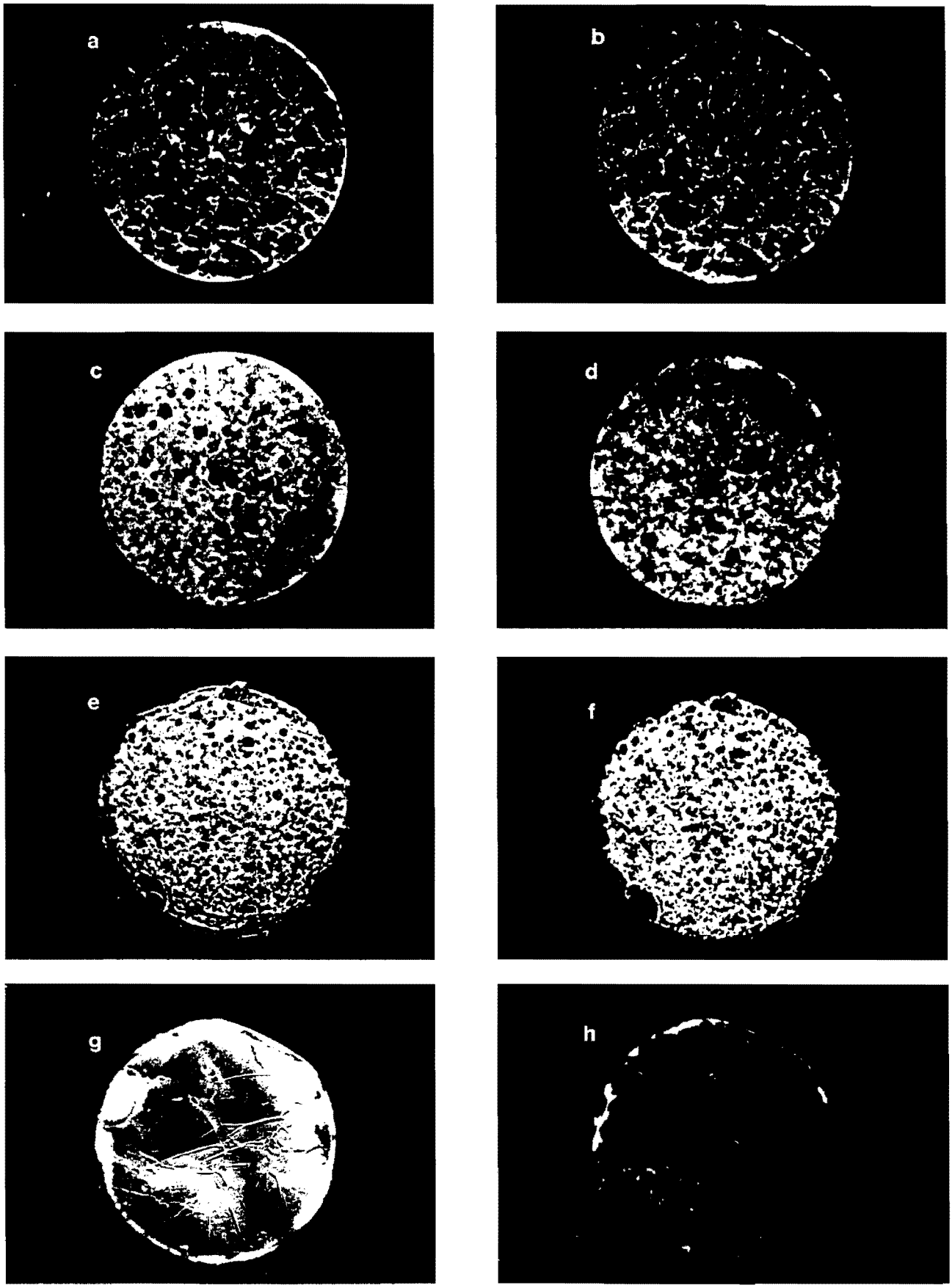
Polymer-coated glass coverslip	XPS elemental composition (90° takeoff angle)			Contact Angle (θ_A/ θ_R) (n = 15)
	Si	C	O	
PLA	0.5	62.4	37.2	77° ± 1° / 55° ± 1°
PLGA 85:15	0.6	60.7	38.6	70° ± 2° / 48° ± 2°
PLGA 75:25	0.8	61.1	38.0	71° ± 2° / 49° ± 2°
PLGA 50:50	0.7	59.0	40.3	68° ± 2° / 45° ± 1°
Uncoated Glass	24.4	18.3	57.3	60° ± 1° / 22° ± 2°

Figure 1. Scanning electron micrograph of PLGA 75:25 -coated glass coverslip after 14 days in culture medium without cells (37°C, 5% CO_2) demonstrates cracks on the surface. (White spots are due to salt deposition.) (Field width = 339.6 μm).

for surface elemental composition by XPS and for surface hydrophobicity by dynamic advancing (θ_A) and receding (θ_R) contact angles. The XPS and contact angle data are summarized in Table 1 where the mean and standard deviations are reported (for contact angle data = 5 measurements/sample x 3 samples each, total 15 samples). As expected, the homopolymer, PLA has a slightly higher ratio of carbon to oxygen than any of the copolymers of PLGA. In addition, PLA is more hydrophobic than the copolymers which show similar surface wetting behaviour.

Polymer-coated glass coverslips were further characterized by SEM to determine the surface morphology of the coating. Polymer coatings were compared before and after 2 weeks of incubation in cell culture medium alone to asses the homogeneous nature of the surface coating prior to, and during, cell culture. All polymer-coated glass coverslips appeared to be planar and smooth at 250X magnification prior to cell-seeding (SEM not shown). Figure 1 represents typical scanning electron micrographs of polymer-coated glass coverslips after 14 days of incubation (at 37°C and 5% CO_2) in the cell culture medium alone (i.e., without cells). The cracks

Figure 2 (on the facing page 227). Paired photographs obtained by normal and UV illumination of the specimens. Tetracycline labeling of the matrix produced by bone marrow-derived cells on various polymer surfaces. (Diameter of polymer surfaces: 2.5 cm). The fluorescence observed after fixation under UV light reveals the presence of the mineralized matrix produced. (a) PLA shows tissue bundles which in (b) are shown to correspond with the fluorescent striae. (c) PLGA 85:15 under normal illumination and (d) PLGA 85:15 under UV light illumination. (e) PLGA 75:25 under normal illumination and (f) PLGA 75:25 under UV light illumination. (g) PLGA 50:50 under normal illumination and (h) PLGA 50:50 under UV light illumination where no fluorescence was observed.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observed on these polymer coatings may result from polymer degradation or SEM sample preparation.

Bone marrow cultures

Light photomicrography. Polymer-coated glass coverslips were seeded with bone marrow-derived cells and examined every second day by phase microscopy for changes in cell morphology. Cells on PLA surfaces had a rounded morphology, adopting a "stand-off" position, as reported on some charged polymer surfaces (Davies *et al.*, 1986) and did not spread. Cells on all other surfaces had a flattened morphology and seemed to adhere to the surfaces. The proliferation of bone marrow-derived cells seemed to be affected by polymer composition, with proliferation increasing with increased glycolic acid composition in the copolymer. After 14 days in culture, the samples were fixed and examined under UV illumination for tetracycline labeling. Figure 2 provides representative images viewed by both normal and UV light of each polymer-coated glass coverslip. Figures 2a and 2b summarize the light and UV-illuminated micrographs of PLA samples: tetracycline-labeled striae were observed towards the margins of the coverslips consistent with the detachment of cell bundles and matrices from the surface. The centers of the PLA-coated coverslips were devoid of fluorescent labels. Figures 2c and 2d provide normal and UV light micrographs of PLGA 85:15 samples: discrete foci are tetracycline-labeled, demonstrating the increased matrix formed on PLGA 85:15 with respect to PLA. Figures 2e and 2f summarize light and UV-illuminated micrographs for PLGA 75:25; in contrast to PLA and PLGA 85:15 samples, a more continuous and homogenous fluorescent label was observed on PLGA 75:25 samples indicating a further increase in the matrix formed. The tetracycline labeling observed on PLGA 75:25 was comparable with that observed on TCP controls (figures not shown). Figures 2g and 2h summarize normal and UV light micrographs observed of PLGA 50:50. The lack of fluorescence indicates that no labeling was observed on PLGA 50:50.

Scanning electron microscopy

SEM was used to further examine the cell-material interface. Figure 3 summarizes the results observed which corroborate those obtained by light microscopy. Figure 3a shows that the few cells that interacted with PLA were isolated and displayed a rounded morphology. As shown in Figures 3b and 3c, although no matrix was observed in contact with the PLA surface, detached bundles of cells formed fine filaments which stretched across the surface. These filaments were tetracycline-positive as determined by fluorescent microscopy and thus contained a mineralized extracellular matrix. On PLGA 85:15 (not shown), PLGA 75:25 (Figure 3d) and PLGA 50:50 (Figure 3e), cells overlapped, forming a dense layer on top of the coated surface. Collagen-type filaments were observed on all surfaces.

Cell proliferation study

Bone marrow-derived cells were seeded on polymer-coated glass coverslips and control surfaces at two initial

Figure 3. (*a and b on the previous page, c, d and e on this page*) Morphology of osteoblasts cultured on various polymers as viewed by SEM: (a) on PLA, cells display a small rounded morphology and no matrix is produced (Field width = 44 μm); (b) on PLA, cells produce matrix on cell bundles (Field width = 65.3 μm); (c) on PLA, cell bundles are bridged from one cell cluster to another (Field width = 399 μm); (d) on PLGA 75:25, cells are flattened and matrix is produced (Field width = 64.8 μm); (e) on PLGA 50:50, cells are flattened and present a similar morphology to those observed on PLGA 75:25 (Field width = 65.4 μm).

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cell densities and counted periodically over a 7 day period. In our laboratory, bone marrow-derived cells are routinely seeded at 5 x 10^4 cells/ml (or 3 x 10^4 cells/cm^2). However, at this concentration the cells were embedded in their secreted matrix at day 6 and were not easily removed by trypsinization, cell counting was therefore hindered. Cells were seeded at 2.7 x 10^4 cells/ml (or 1.6 x 10^4 cells/cm^2) to facilitate cell counting, as the cell multilayering and matrix elaboration was delayed. Figure 4 summarizes the results obtained from the cell proliferation study for cells seeded at 5 x 10^4 cells/ml (Figure 4a) and those seeded at 2.7 x 10^4 cells/ml (Figure 4b). Figure 4a shows that cells proliferated at approximately the same rate on PLGA 85:15, PLGA 75:25 and PLGA 50:50. In contrast, cells on TCP proliferated at a 3-fold rate and those on PLA showed minimal proliferation. The results in Figure 4b mirror those in Figure 4a.

pH measurements of PLGA 75:25 coated glass coverslips

The pH values of four different media were measured and compared: PLGA 75:25 with and without bone marrow-derived cells in culture and TCP with and without bone marrow-derived cells in culture (results not shown). Slight fluctuations in pH were observed between pH 7.3 and 8.3 for all surfaces. The lowest pH values were measured on TCP and PLGA 75:25 cell-seeded surfaces, both showing similar pHs. It seems, therefore, that cell metabolism mostly affected the pH in

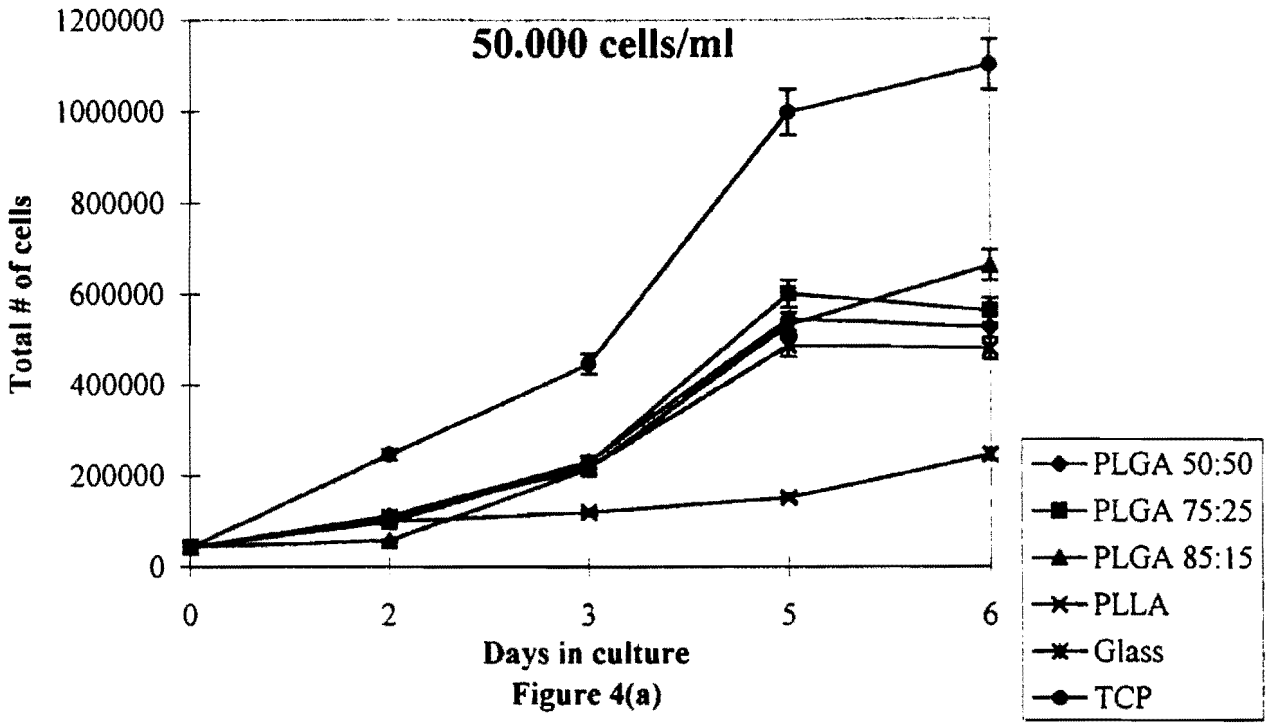

Figure 4(a)

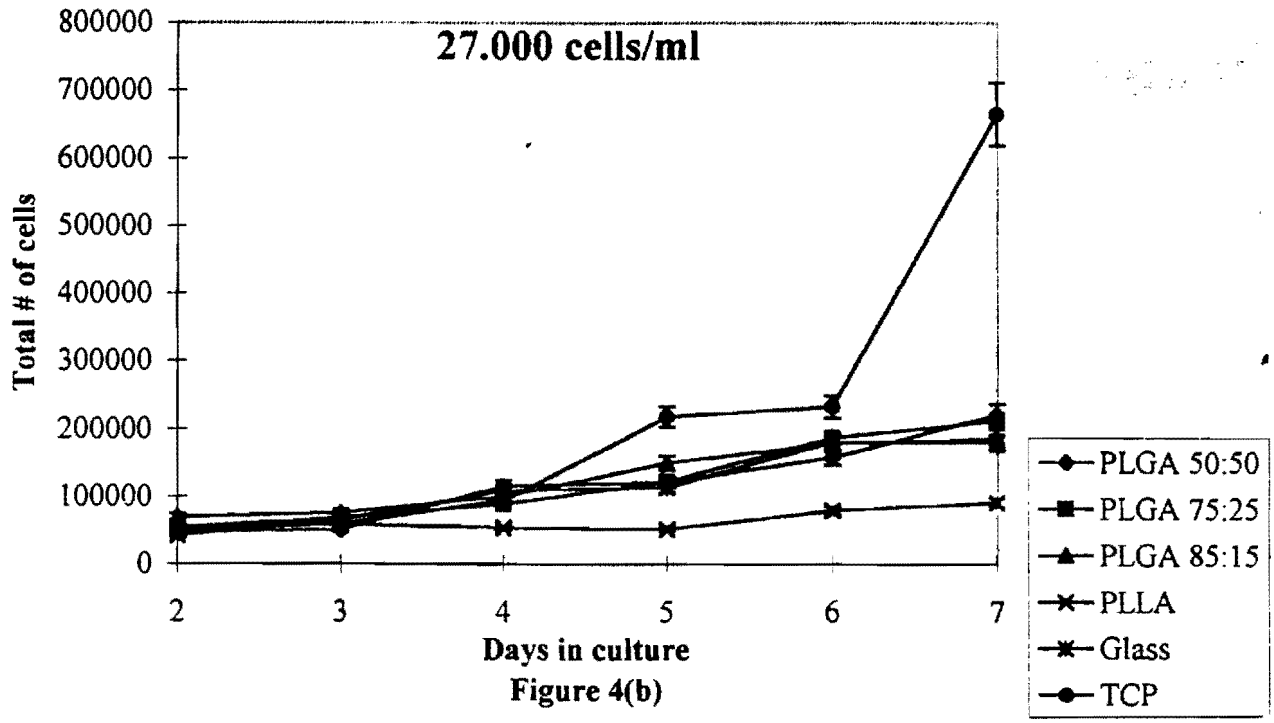

Figure 4(b)

Figure 4. Analysis of cell proliferation on different polymer surfaces: (a) Cells were seeded on the different surfaces at a starting concentration of 5 x 10^4 cells/ml. Saturation on the TCP control surface was reached after 5 days (n=3). (b) Cells were seeded on the different surfaces at a starting concentration of 2.7 x 10^4 cells/ml. PLA is the only polymeric surface where cells do not proliferate after 7 days.

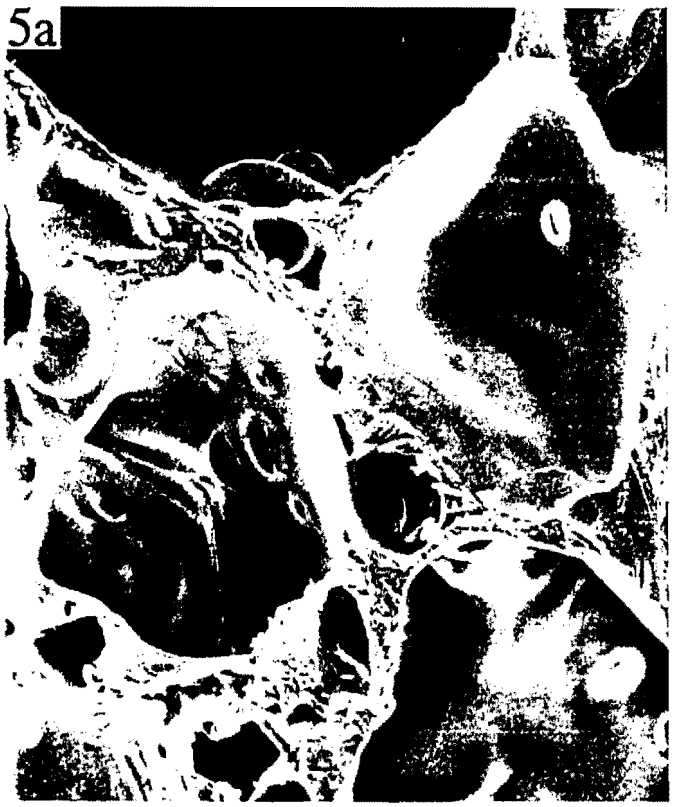

Figure 5. (*at left*) (a) Scanning electron micrograph of the surface of a PLGA 75:25 three-dimensional device obtained by the solvent-casting particulate-leaching technique. The matrix is highly porous with a size pore of 100-300 μm. (Field width = 283 μm). (b) Scanning electron micrograph of the surface of a PLGA 75:25 three-dimensional device obtained by the freeze-drying emulsion technique. The pore size of the matrix ranges between 10-50 μm, Field width = 152 μm.

the culture medium, polymer degradation, if any, did not lower the pH.

3-Dimensional Polymer foams of PLGA 75:25

The morphology of the 3-dimensional structures created by the salt leaching and the freeze-drying techniques had a foam-like appearance as determined by SEM, shown in Figures 5a and 5b, respectively. The salt leaching technique produced a polymeric foam with pore sizes ranging between 100 and 300 μm whereas the freeze-drying technique produced a polymeric foam with pore sizes ranging between 10 and 50 μm. The foam morphologies obtained by these methods were different. The salt leaching technique produced a foam with pores shapes similar to the shape of the particles used. The pore walls were mostly smooth and connections between the pores were visible. These foams were stiff enough to be manipulated without damaging the foam. The freeze-drying technique produced a foam with very small rounded pores interconnected with larger void spaces. These foams were very fragile, and had to be manipulated with great care.

The cell-PLGA 75:25 foam interaction was evaluated for matrix elaboration by tetracycline labeling, as described previously for polymeric films, and then further examined by SEM. Both foams prepared from PLGA 75:25 revealed fluorescence labeling at the surface, but not deep within the device, as determined by changing the focal plane during examination by UV light microscopy. Recent studies by Mikos *et al.* have explored cell seeding techniques to overcome this apparent lack of matrix formation within the device (Bostrom and Mikos, 1996).

Bone marrow-derived cells were evident, by SEM, on the outer surface of the polymeric foam but did not appear to interact closely with the pore walls of the three-dimensional device. (Figures 6a, b and c) A sheet of matrix was observed on the surface of both polymeric structures but no matrix was observed within cross-sections of the foam structure.

Discussion

These results confirm published observations (Ishaug *et al.*, 1997) that, from the biodegradable PLGA copolymers chosen for this study, PLGA 75/25 is suitable for the culture of bone marrow-derived cells. The polymer films showed little change in surface morphology during the 14 days of immersion in culture medium without

Figure 6. Morphology of bone marrow-derived cells cultured on a PLGA 75:25 device obtained by the solvent-casting particulate leaching technique: (a) Field width = 430.16 μm; (b) Field width = 339.6 μm and (c) Field width =79.3 μm. Cells proliferated and produced matrix; but no interaction was observed between the cells and the substrate.

cells, as demonstrated by SEM. The crack morphology observed in Figure 1, which may either be an artefact of SEM sample preparation or may represent polymer degradation, was not observed in the presence of cells. The degradation, if any, affected neither the pH of the medium nor cell behaviour in our culture system as cell proliferation and differentiation occurred on PLGA 75:25 surfaces. Thus the difference in cell behaviour observed on the different polymers was not due to changes in pH. Nevertheless, we are aware that measuring the pH of the total volume of a buffered medium may not reflect the local pH changes in the microenvironment at the cell-surface interface. We did not address such changes in the experiments described here.

This lack of pH change is important when interpreting the results of the 3-D configurations of the PLGA 75:25 copolymer employed. The relationship between cells and matrix formation on the surface of the 3-D device was most likely unaffected by polymer surface degradation, as would be expected, in the 28 day period.

The results indicate that substrate hydrophobicity and elemental composition may influence both the number and morphology of cells on the material examined. For example, the number of adherent cells increased and their morphologies were more spread on the more hydrophilic surfaces (i.e., PLGA 50:50) than on the more hydrophobic surfaces (i.e., PLA). Bone matrix elaboration, as indicated by the fluorescent tetracycline label, was not observed on every substrate. While cell

growth on PLGA 50:50 was similar to that on PLGA 75:25 and the TCP control, the lack of fluorescent signal clearly indicated that cell growth cannot, necessarily, be equated with bone matrix production. Thus, the cells on PLGA 50:50 had not differentiated to become osteogenic cells, or, it is possible that the cells differentiated and secreted matrix, but that the underlying polymer produced a sufficiently acidic environment to compromise this newly formed matrix. In order to verify this assumption, micro-environmental pH changes would have to be monitored, but were not included in the work reported here.

These observations are important since alkaline phosphatase (AP) and collagen levels in the culture medium are often used as markers of bone cell activity (Ishaug *et al.*, 1994). Since both the ectoenzyme AP and collagen are expressed by many cells, including fibroblasts and osteoblasts, and given that it is accepted that cell proliferation and differentiation are gene regulated phenomena of reverse onset (Stein *et al.*, 1990), it is essential in developing polymer matrices for bone cell growth that extracellular matrix elaboration, rather than media biochemical markers alone, is taken into consideration. Our experiments demonstrate that PLGA 75:25 is the most suitable polymer, of those tested, for bone matrix elaboration, as illustrated by the tetracycline assay.

The 3-D polymer matrices used in this work showed significantly different morphologies; yet, in each case, differentiated osteogenic cells in the culture system were able to produce an elaborated extracellular mineralized matrix. Light micrographic examination of complete samples was unable to resolve the location of this bone matrix. Thus, the combined light and scanning electron micrography reported herein are important in determining the relationship between the elaborated bone matrix and the underlying substrate. The cells closest to the polymer substrate bridged the surface features created during polymer processing and, as a result, the multilayer cell sheets that accumulated on the material did not themselves follow the detailed geometry of the surface. SEM also demonstrated that the extracellular matrix formed by these cells was present between these multilayered cells, rather than between the cells and the substrate. We were unable to find any evidence of bone-matrix in direct contact with the polymer surface; in all areas examined, the polymer surfaces were occupied by either the nearest cell layer or a gap between the cell layer and the polymer substrate created by the bridging phenomenon described above. These results clearly indicated that, on these foams, no bone tissue could grow within the pores of the foams. All the obtained bone tissue was on the surface of the foams, within the superficial layer of pores. Similar observations are reported by Ishaug *et al.* (1997), in a study where polymer foams were seeded with extremely high cell densities (20x the amount of cells used in the present work) and left in culture for twice as long as described herein. These results emphasize the importance of both polymer chemistry and surface topography in deriving optimal biodegradable polymers for bone tissue engineering.

Conclusions

These *in vitro* observations show that the PLGA 75:25 copolymer, of the series investigated, is the optimal biodegradable polymer for bone matrix elaboration. For the three-dimensional foams that were prepared herein, no bone tissue formed within the pores of the polymer foam; the only bone tissue obtained was localized on the outer surface of the foam structure. In these areas, no direct contact between the bone tissue and the polymer was observed.

Acknowledgments

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Discussion with Reviewer

Reviewer I: The terms mineralization, bone, and bone matrix are different and cannot be used interchangeably. What the authors have observed here is matrix mineralization, not bone formation. Please comment.

Authors: The tetracycline technique which we have described is one which has been developed in this laboratory over the last two years or so and has been presented both to the biomaterials community (Lowenberg *et al.*, 1996) and more recently, using human bone cultures, to the American Society of Bone and Mineral Research (Parker *et al.*, 1997). We have shown that the tetracycline label is specific to the developing mineralizing bone matrix in these culture systems and can, therefore, confidently refer to the bone matrix elaborated in our cultures visualized by this technique.