
Characterization of osteoblast-like behavior of cultured bone marrow stromal cells on various polymer surfaces

Jay W. Calvert,^{1,5} Kacey G. Marra,² Lisa Cook,³ Prashant N. Kumta,⁴ Paul A. DiMilla,³ Lee E. Weiss⁵

¹*Division of Plastic and Reconstructive Surgery, University of Pittsburgh Medical Center, 6B Scaife Hall*

²*The Institute for Complex Engineered Systems*

³*Chemical Engineering Department*

⁴*Materials Science and Engineering Department*

⁵*The Robotics Institute, Carnegie Mellon University, Pittsburgh, Pennsylvania 15261*

Received 6 July 1999; accepted 18 February 2000

Abstract: The creation of novel bone substitutes requires a detailed understanding of the interaction between cells and materials. This study was designed to test certain polymers, specifically poly(caprolactone) (PCL), poly(D,L-lactic-co-glycolic acid) (PLGA), and combinations of these polymers for their ability to support bone marrow stromal cell proliferation and differentiation. Bone marrow stromal cells were cultured from New Zealand White rabbits and were seeded onto glass slides coated with a thin layer of PCL, PLGA, and combinations of these two polymers in both a 40:60 and a 10:90 ratio. Growth curves were compared. At the end of 2 weeks, the cells were stained for both matrix mineralization and alkaline phosphatase activity. There was no statistically significant difference in growth rate of the cells on any poly-

mer or polymer combination. However, there was a striking difference in Von Kossa staining and alkaline phosphatase staining. Cells on PCL did not show Von Kossa staining or alkaline phosphatase staining. However, in the 40:60 and 10:90 blends, there was both positive Von Kossa and alkaline phosphatase staining. These data indicate that PCL alone may not be a satisfactory material for the creation of a bone substitute. However, it may be used in combination with PLGA for the creation of a bone substitute material. © 2000 John Wiley & Sons, Inc. *J Biomed Mater Res*, 52, 279–284, 2000.

Key words: biomaterials; bone substitutes; bone marrow; osteoblast; mineralization; polymeric biomaterials

INTRODUCTION

Congenital disorders, oncologic surgery, and trauma often leave patients with large bony defects that require reconstruction. Development of an effective bone substitute for the repair of these defects has been a difficult goal to achieve.^{1,2} Current materials and processes have not resulted in a bone substitute capable of repairing large bony defects. The current challenge is to create a readily available, implantable bone substitute that will result in tissue that ultimately functions as true bone tissue. To perform this task, the material from which to fabricate the bone substitute must be chosen carefully.³ Resorbable polymer materials hold the most promise for this purpose and therefore were the subject of this study.^{4,5} Our hypothesis was that resorbable polymers could support cultured bone marrow stromal cell growth and differentiation.⁶ These data could then be used to design three-

dimensional scaffold materials for the creation of a novel bone substitute material.⁷

The most successful bone grafting material to date is autogenous cancellous bone. This substance has osteogenic, osteoconductive, and osteoinductive properties by virtue of the surviving cells, bone collagen, bone mineral, and bone matrix proteins.^{8,9} However, the technique of autogenous bone grafting has many shortcomings. The morbidity at the harvest site can be tremendous with problems, such as pain, infection, and blood loss requiring blood transfusion adding the associated risks of transfusion reaction and blood-borne infection. In addition, the procedure of harvesting the bone graft adds time and cost to the procedure for which the bone graft is required. Allograft bone is a secondary material preferred by many surgeons but it, too, is fraught with problems including inflammatory response, transmission of infections, and resorption of the graft.^{10–12} Allograft is appropriate for some applications, but it is not a panacea.

Many groups have examined the properties of hydroxyapatite-based materials,^{13–15} polymer matrices,^{16,17} and combinations of both of these types of

Correspondence to: J. W. Calvert; e-mail: calvertjw@msx.upmc.edu

materials.^{18–21} This study was designed to evaluate commercially available biopolymers and novel blends of these polymers for their ability to support proliferation of bone-forming cells, as well as their ability to allow the cells to maintain their differentiated state as measured by alkaline phosphatase activity and Von Kossa staining. By evaluating the cells in a two-dimensional system, certain decisions can be made about the osteogenic potential of these materials that will help to formulate three-dimensional constructs for the creation of bone replacement materials.

MATERIALS AND METHODS

Polymers

Poly(caprolactone) (PCL) [Aldrich (M_w 65 K)] and poly(D,L-lactic acid-co-glycolic acid) (PLGA) [M_w 40–75 K (65:35), Aldrich] were all used as received. Molecular weights were determined by gel permeation chromatography (GPC) for PLGA and PCL [PLGA: $M_w = 4.4 \times 10^4$ g/mol, PDI (polydispersity index) = 1.6; PCL: $M_w = 7.5 \times 10^4$ g/mol, PDI = 1.5]. GPC was carried out using a Styrogel column equipped with a Waters 510 programmable pump and a Waters 410 differential refractometer. Molecular weights are relative to monodisperse polystyrene standards (Waters). The solvent used was THF. PCL, PLGA, and two combinations of the aforementioned polymers in a 10:90 and 40:60 ratio were prepared and spin cast on glass microscope slides. The layer of polymer on each slide was transparent and measured to be approximately 100 Å thick. The slides were then dried and sterilized with an ultraviolet light for 90 min. Contact angles were determined using a Rame-Hart goniometer (Model 100-00). The values reported are an average of six to eight measurements on three different samples. Distilled water was used to measure advancing and receding contact angles. Advancing contact angles are determined by placing 5 μ L of water on the surface, then adding 2 μ L. Receding contact angles are determined by removing 2 μ L of water.

Silanization

Silanization creates a more hydrophobic surface that will aid in maintaining polymer adhesion to the slides throughout the duration of the experiment. All microscope slides used in this study were cleaned by rinsing sequentially in the following substances: (a) chromerge for 20 min, (b) doubly distilled water three times, (c) phosphoric acid for 20 min, (d) doubly distilled water for 20 min, (e) 10 mM sodium hydroxide for 10 min, and (f) doubly distilled water. The cleaned slides were dried under N_2 , then placed in a desiccator with two drops of trimethylchlorosilane for 2 h. The slides were rinsed with ethanol, dried under N_2 , and coated with a 10% w/v polymer solution in chloroform using the spin caster at 1000 rpm.

Cell culture and isolation

Bone marrow stromal cells were harvested from 6-week-old New Zealand White rabbits. The rabbits used in this study were being killed for another Institutional Animal Care and Use Committee-approved experiment and the bone marrow cells were taken immediately following the death of the animal. Bone marrow was obtained by harvesting bilateral femora and tibia. The bones were then fractured with a bone cutter under sterile conditions and bone marrow plugs were obtained.²²

The bone marrow plugs were then mixed with 4 mL of Dulbecco's modified Eagle's medium (Gibco Laboratories, NY) and antibiotics (penicillin–streptomycin) in a 15-mL centrifuge tube. The marrow was disaggregated by passing it gently through a 21-gauge intravenous catheter and syringe to create a single cell suspension. The suspension was then centrifuged ($800 \times g$, 5 min). The supernatant was discarded and the process repeated a second time. The cells were then resuspended in tissue culture medium which included DMEM, 15% fetal calf serum (Gibco), antibiotics (penicillin–streptomycin), an antifungal agent (amphotericin B), 10 mM Na β -glycerol phosphate (Sigma), 10^{-10} M dexamethasone (Sigma), and 50 μ g/mL ascorbic acid (Sigma).

Cultures were grown to near confluence in 75-mm² flasks (Fisher Scientific, PA) with media changed every 3 or 4 days. Prior to reaching confluence, the cells were resuspended by exposing them to 2 mL trypsin (Gibco) for 10 s and then adding 10 mL culture medium. Cells were centrifuged again and resuspended in culture medium as described above. Cells concentration was then controlled to 40,000 cells/mL by Trypan blue dye exclusion.

Proliferation assay

The polymer-coated slides were then placed into 60-mm² culture dishes and 4 mL of culture medium was added. One milliliter of the cell suspension was then pipetted into the dish directly over the center of the glass slide, giving a concentration of 8000 cells/mL. Controls were also created with silanized sterilized glass slides and 60-mm² tissue culture dishes.

Cell proliferation rates were calculated by normalization of all cell count data. Lag time counts were eliminated from the counts and natural log counts were used to normalize data amongst all cell counts. Using the equation of a line, the slope represented the proliferation rate.

Measurement of proliferation

The proliferation of cells on different polymeric substrata was measured by counting the number of cells in 10 randomly chosen fields at intervals of 2, 24, 48, 72, 120, and 168 h after plating. Individual 750- μ m² fields were examined using phase-contrast optics at a magnification of $\times 10$ using an IMT-2 inverted microscope. For each sample the sum of the number of cells present in individual fields at $t = 24, 48, 72, 120,$ and 168 h after plating, $N(t)$, was normalized to the

sum of the number of cells present in individual fields 2 h after plating, N_o , to determine a relative cell number,

$$\frac{N(t)}{N_o}$$

as a function of time in culture. Mean and standard deviation of the mean were calculated from triplicate platings per type of substratum.

The temporal dependence of relative cell number was assessed by applying a first-order model with a lag phase prior to growth for the proliferation of cells:

$$N(t) = N_o e^{k(t - t_{lag})}$$

where k is the rate of proliferation and t_{lag} is the time associated with attachment, spreading, and adjustment of cells to a substratum prior to growth. Means and standard deviations for k and t_{lag} were determined for each type of substratum using a linear regression analysis applied to experimental data for the natural logarithm of

$$\frac{N}{N_o}$$

at 24, 48, and 72 h.²³ Values for the probability of agreement between experimental data and the theoretical model, Q , for each type of substratum were determined based on values of χ^2 with degrees of freedom $\nu = 1$. Means and standard deviations for k and t_{lag} among different substrata were compared using analysis of the variance with $\alpha = 0.05$.

Alkaline phosphatase assay

Alkaline phosphatase activity was obtained using a standardized kit (Sigma). The staining was performed after 10 days of subculture on the polymer-coated glass slides. Control cells were also stained. In addition, polymers not seeded with cells were stained as negative controls. Activity was evaluated as either positive or negative. Positive activity was assigned a subjective score of positivity on a scale of 0–5. Two independent parties performed all of the observations and were blinded to the nature of the polymer.

Von Kossa stain

Matrix mineralization was evaluated by the use of Von Kossa stain. After we washed the cells multiple times with deionized water, we added 5 mL 2% silver nitrate solution to the specimens. Treated specimens were placed in the dark for 15 min and then exposed to a bright light for 15 min. Polymer slides without cells were also treated as negative controls. Staining was evaluated as either positive or negative by two independent observers.

Statistical analysis

A microcomputer (Model 410CDT; Toshiba Satellite Pro) was used to perform statistical analysis using Microsoft Ex-

cel standard software. To detect difference among groups and intragroup variation, we used analysis of the variance to compare the proliferation rates with α set at 0.05. Differences in alkaline phosphatase staining were compared using a two-tailed paired t test. Statistical significance is represented by $p < .05$.

RESULTS

Polymer surface characterization

Contact angle measurements were taken on thin films spun cast onto glass slides. A Rame-Hart goniometer Model 100-00) was used. The values reported are an average of six to eight measurements on at least three different samples. Table I indicates the contact angle values for the homopolymers and polymer blends. Although the hydrophobicity of the polymer blends was similar, pure PCL appeared to be the most hydrophilic and demonstrated a significant difference in advancing contact angle ($p < .05$). (Table I)

Cell proliferation assay

Cell proliferation rates were fairly similar among all samples and repetitions of this experiment. There was no statistically significant difference among any of the surfaces. Figure 1 shows all of the proliferation rates compared to one another, with error bars representing standard error of the mean. Analysis of the variance was performed and revealed that there was no statistically significant difference in the proliferation rate of the bone marrow stromal cells on any of the polymers or polymer combinations (Fig. 1).

Alkaline phosphatase staining

The control cells grown on glass slides stained heavily for alkaline phosphatase activity in all cases with an observer score of 4.8 ± 0.4 . This score was statisti-

TABLE I
Contact Angles of Polymer Surfaces

Polymer	Advancing Contact Angle (\pm SD)	Receding Contact Angle (\pm SD)
PCL	73 (2.5)*	53 (4.0)
PLGA	77 (5.1)	51 (2.1)
PCL:PLGA (10:90)	79 (1.5)	54 (5.0)
PCL:PLGA (40:60)	81 (4.1)	53 (3.0)

*Statistically significant.

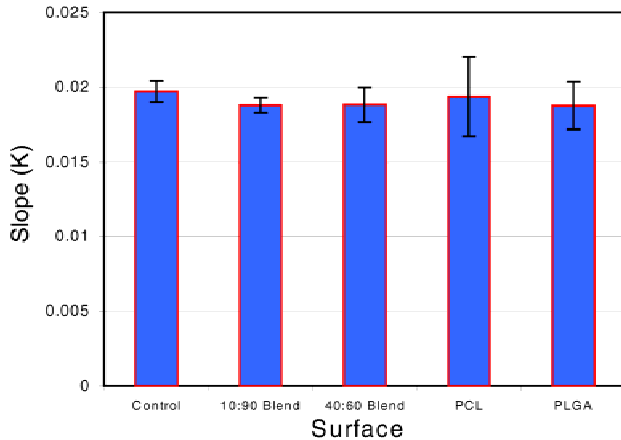


Figure 1. Comparison of proliferation rates of bone marrow stromal cells on surfaces. In all cases there was no statistically significant difference between proliferation rates for any of the various polymer surfaces compared with each other and with controls.

cally higher than all other scores, showing that the cells grew best on the control (glass) surface. In all cases, $p < .001$. The cells grown on PCL demonstrated no alkaline phosphatase activity, with a 0 rating by the observers on all slides. This was statistically lower than all other surface, with $p < .001$ in all cases. The PLGA, PCL/PGLA in a 10:90 percent ratio, and the PCL/PGLA in a 40:60 percent combination stained with similar intensity with no statistical difference in their scores ($p > .10$ in all cases). However, the 10:90 blend was the highest scoring surface in this study compared to the other polymer surfaces, although no statistical difference was appreciated (Table II).

Von Kossa staining

There was positive Von Kossa staining of cells after ten days on all samples except for the PCL specimens. The typical appearance of colonies of cells stained positively with the Von Kossa stain was seen on all samples at all time points with the exception of all polycaprolactone specimens. The amount of Von

Kossa staining was not quantified further. (Table II and Figure 2)

DISCUSSION

This study was performed to determine which polymers and polymer blends could be used to create a novel biomaterial for bone tissue engineering. The materials chosen are commercially available, U.S. Food and Drug Administration approved, and relatively inexpensive. An attempt was made to quantify the cell reaction to the polymers by examining the proliferation rates of bone marrow stromal cells on the materials compared to controls. Osteoblast-like activity was assessed using the alkaline phosphatase staining as per other previous investigators.²⁴ In addition, matrix deposition and mineralization of the polymers and blends were analyzed by Von Kossa staining.

The rate of proliferation was not significantly different for any of the polymers or their combinations. Therefore, this result does not limit our choice of polymers for our new scaffold material because no single polymer distinguished itself as an inhibitor of proliferation. Thus, all polymers are candidates for a novel bone tissue-engineering scaffold from the standpoint of cell proliferation.

In contrast, the osteoblast phenotype was affected by the biomaterials. PCL was the material least capable of retaining the osteoblastic phenotype, as evidenced by the low level of alkaline phosphatase activity in those samples. Interestingly, the 10:90 combination of the PCL and the PLGA best approximated the control cells. PLGA has been shown to be osteoconductive,^{25,26} and although we have shown PCL not to be phenotypically osteogenic, we can obtain an osteoconductive material by combining the two polymers. Some of the possible factors that contribute to this observed phenomenon include the following: compatibility of the blends, hydrophobicity of the polymers, and acidity of the degradation products of the polymers. The polymer blends are anticipated to be compatible with 10% PCL²⁷ and incompatible with 40% PCL. Blend incompatibility can lead to phase separation of the polymers and crystallization of the PCL. The crystallinity and amorphous natures of the polymers may lead to different cell behaviors. Although the hydrophobicity of the polymer blends is similar, pure PCL appears to be the most hydrophilic. The osteoblast-like cells appear to prefer more hydrophobic surfaces. Finally, the acidity of the PLGA degradation products is higher than that of pure PCL. This may affect the behavior of the cells as well.

The ability of the polymers to support matrix deposition and mineralization is somewhat difficult to assess in such a short study. Because of the hydropho-

TABLE II
Histologic Staining of Tissue Culture Samples

	Alkaline Phosphatase	Von Kossa Staining
Control	4.8 ± 0.4	Positive
Poly(caprolactone)	0*	Negative
Poly(D,L-lactic-co-glycolic acid)	3 ± 0.6	Positive
PCL:PGLA (40:60 ratio)	2.3 ± 0.9	Positive
PCL:PGLA (10:90 ratio)	3.2 ± 0.9	Positive

*Statistically significant.

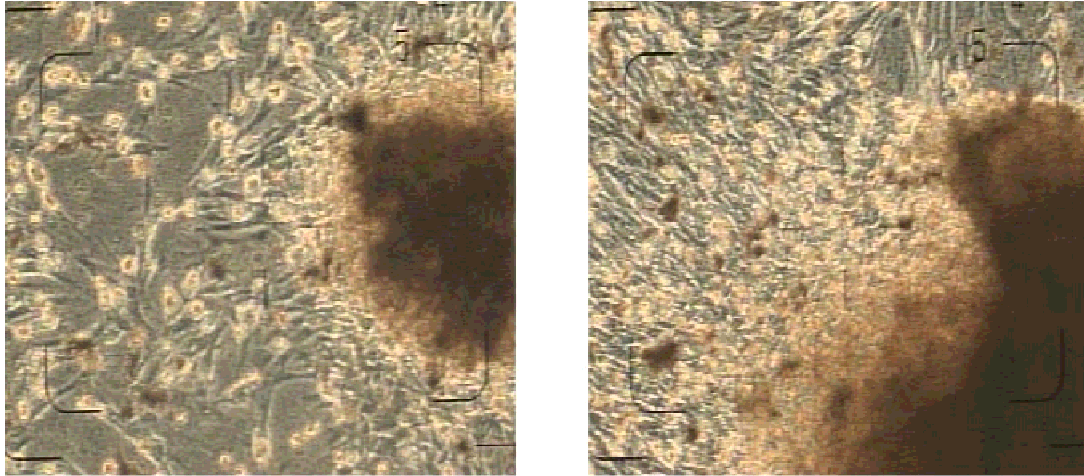


Figure 2. Bone marrow stromal cells with Von Kossa stain. The PLGA:PCL 10:90 blend is shown on the right; control cells are on the left.

bicity of the polymers, the films would remain adhered to the glass slides for only 14 days maximum. Therefore, Von Kossa staining was performed at 10 days and the results are difficult to interpret. The degree of staining could not adequately be quantitatively assessed, and so a designation of positive or negative was made. The fact that PCL was the sole polymer surface demonstrating no Von Kossa staining may show that this polymer alone would not be useful for the creation of an osteogenic scaffold material. However, the staining took place early in the process of matrix deposition and mineralization. Yet, cells on other polymers did show positive staining at this time, and this cannot be overlooked because this characteristic is desirable for the creation of a bone substitute material.

From these data it appears that the optimal polymer for further investigation is the PLGA/PCL blend in a 10:90 ratio. Further study is currently being conducted to examine the properties of these blends when three-dimensional constructs are manufactured. Additional data will also need to be collected regarding the cellular response to the three-dimensional forms of these polymers and polymer blends, as there may be drastically different results in osteoblastic activity and the ability to cause the synthetic matrices to become bone. This thinking may also be extrapolated to the expected *in vivo* properties of these materials. This paradigm for examination of novel biomaterials as potential bone substitutes allows for a systematic evaluation of a material with the opportunity for intervention at all levels of investigation.

CONCLUSIONS

Investigation of thin films of polymers on silanized glass slides and characterization of the films using

contact angle goniometry were performed. The polymer blends appear to have superior osteoconductive properties compared to the homopolymers. The homopolymers and the blends are hydrophobic. Cultured bone marrow stromal cells were seeded onto the films and proliferation rates were not statistically different between polymer species. Mineralization was determined using Von Kossa and alkaline phosphatase staining. Blends of PCL and PLGA seem to be the most osteogenic. We are using these two-dimensional data to prepare three-dimensional scaffolds for use in bone tissue engineering.

The authors thank Mark P. Mooney, Ph.D., for assistance with the bone marrow cell harvest.

References

1. Byrd SH. Implantation: Bone, cartilage and alloplasts. *Selected Readings in Plastic Surgery* 1988;5:2-15.
2. Hollinger JO, Brekke J, Gruskin E, Lee D. Role of bone substitutes. *Clin Orthop* 1996;324:55-65.
3. Lane JM, Sandhu HS. Current approaches to experimental bone grafting. *Orthop Clin NA* 1986;18:213-225.
4. Langer RL, Vacanti JP. Tissue engineering. *Science* 1993;260:920-925.
5. Huatan H, Collett JH, Attwood D, Booth C. Preparation and characterization of poly(epsilon-caprolactone) polymer blends for delivery proteins. *Biomaterials* 1995;16:1297-1303.
6. Eaglesom CC, Ashton BA. The osteogenic capacity of bone marrow cells. *Cell Biol Int Rep* 1980;4:742.
7. Boyan BD, Hummert TW, Dean DD, Schwartz Z. Role of material surfaces in regulating bone and cartilage cell response. *Biomaterials* 1996;17:137-146.
8. Johnson KD, August A, Sciadini MF, Smith C. Evaluation of ground cortical autograft as a bone graft material in a new canine bilateral segmental long bone defect model. *J Orthop Trauma* 1996;10:28-36.

9. Urist MR. Bone formation by autoinduction. *Science* 1965;150:893–899.
10. Kakiuchi M, Ono K. Defatted, gas-sterilised cortical bone allograft for posterior lumbar interbody vertebral fusion. *Int Orthop* 1998;22:69–76.
11. Segur JM, Torner P, García S, Combalía A, Suso S, Ramón R. Use of bone allograft in tibial plateau fractures. *Arch Orthop Trauma Surg* 1998;117:357–359.
12. Virolainen P, Vuorio E, Aro HT. Different healing rates of bone autografts, syngeneic grafts, and allografts in an experimental rat model. *Arch Orthop Trauma Surg* 1997;116:486–491.
13. Holmes RE. Bone regeneration within a coralline hydroxyapatite implant. *Plast Reconstr Surg* 1979;63:626–632.
14. Laczka-Osyczka A, Laczka M, Kasugai S, Ohya K. Behavior of bone marrow cells cultured on three different coatings of gel-derived bioactive glass-ceramics at early stages of cell differentiation. *J Biomed Mater Res* 1998;42:433–442.
15. Schmitz JP, Hollinger JO, Milam SB. Reconstruction of bone using calcium phosphate bone cements: A critical review. *J Oral Maxillofac Surg* 1999;57:1122–1126.
16. Damien CJ, Parsons JR. Bone graft and bone graft substitutes: A review of current technology and applications. *J Appl Biomater* 1991;2:187–208.
17. Andriano KP, Tabata Y, Ikada Y, Heller J. In vitro and in vivo comparison of bulk and surface hydrolysis in absorbable polymer scaffolds for tissue engineering. *J Biomed Mater Res* 1999;48:602–612.
18. Cornell CN, Lane JM. Current understanding of osteoconduction in bone generation. *Clin Orthop* 1998;355(Suppl): S267–S273.
19. Marra KG, Szem JW, Kumta PN, DiMilla PA, Weiss LE. In vitro analysis of biodegradable polymer blend/hydroxyapatite composites for bone tissue engineering. *J Biomed Mater Res* 1999;47:324–335.
20. Garcia AJ, Ducheyne P, Boettiger D. Effect of surface reaction stage on fibronectin-mediated adhesion of osteoblast-like cells to bioactive glass. *J Biomed Mater Res* 1998;40:48–56.
21. Maruyama M, Ito M. *In vitro* properties of a chitosan-bonded self-hardening paste with hydroxyapatite granules. *J Biomed Mater Res* 1996;32:527–532.
22. Maniatopoulos C, Sodek J, Melcher AH. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res* 1988;254:317–330.
23. Press WH, Flannery BP, Teukolsky SA, Vetterling WT. Numerical recipes. New York: Cambridge University Press, 1986. 818 pp.
24. Ishaug SL, Yaszemski MJ, Bizios R, Mikos AG. Osteoblast function on synthetic biodegradable polymers. *J Biomed Mater Res* 1994;28:1445–1453.
25. Ishaug SL, Crane GM, Miller MJ, Yasko A, Yaszemski MJ, Mikos AG. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J Biomed Mater Res* 1997;36:17–28.
26. Attawia MA, Herbert KM, Laurencin CT. Osteoblast-like cell adherence and migration through 3-dimensional porous polymer matrices. *Biochem Biophys Res Commun* 1995;213:639–644.
27. Rocha G, Gross R, McCarthy S. Miscibility studies of blends of poly(glycolic acid) with other biodegradable polymers. *Proc Am Chem Soc Polym Chem* 1992;33:454–456.