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FABRICATION OF PLIABLE POLYMER SCAFFOLDS FOR TISSUE ENGINEERING AND PARTICULATE EFFECTS ON OSTEOBLAST FUNCTION

by

MARY CONLEY WAKE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE MASTER OF SCIENCE

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ABSTRACT

FABRICATION OF PLIABLE POLYMER SCAFFOLDS FOR TISSUE ENGINEERING AND PARTICULATE EFFECTS ON OSTEOBLAST FUNCTION

by

Mary Conley Wake

Pliable, biodegradable scaffolds with poly(lactic-co-glycolic acid) (PLGA) and poly(ethylene glycol) (PEG) blends have been fabricated using a solvent-casting and particulate-leaching technique. This study investigated the effects of different processing parameters, PLGA copolymer ratio, the PLGA/PEG blend ratio, the initial particulate salt weight fraction, and the salt particle size, on scaffold pliability and pore morphology. A wide range of shear moduli, porosities, and median pore diameters was able to be achieved.

Effects of biodegradable particles of poly(L-lactic acid) (PLLA) and PLGA on rat marrow stromal cells in vitro have also been investigated. This study examined the effects of three particle parameters, concentration, polymer molecular weight, and PLGA copolymer ratio, on osteoblast proliferation and function. The most significant trend observed was due to increasing the concentration of particles, resulting in decreased proliferation and function of osteoblasts throughout the 28 day study.
ACKNOWLEDGMENTS

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<td>ALPase</td>
<td>Alkaline phosphatase activity</td>
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<td>DMA</td>
<td>Dynamic Mechanical Analysis</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>GPC</td>
<td>Gel Permeation Chromatography</td>
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<td>GS</td>
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<td>Mw</td>
<td>Weight average molecular weight</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PE</td>
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<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
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<td>SEM</td>
<td>Scanning electron micrograph</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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CHAPTER 1

INTRODUCTION

1.1 Tissue Engineering

It is the great need for better therapies to treat diseased or malfunctioning organs that motivates scientists and clinicians to study tissue engineering strategies. Every year millions of people suffer from organ or tissue loss and require replacement of the tissue by transplantation or allografts [Langer and Vacanti, 1993]. These therapies are successful in some cases but are also fraught with complications. The transfer of disease and rejection of foreign tissue are potential hazards. The largest complication, however, is the scarcity of donor tissue and organs. For example, every year in the United States approximately 30,000 people die waiting for a liver transplant but as few as 3,000 are recipients because of organ availability [Langer and Vacanti, 1993].

Tissue engineering strategies aim to circumvent the problem of donor scarcity by utilizing a polymer scaffold that acts as a vehicle for transplantation of healthy cells. The polymer scaffold is a temporary template onto which the cells are seeded before the polymer-cell construct is implanted [Vacanti, 1988]. As the cells begin to proliferate and develop, secreting their own extracellular matrix to which they can attach, the polymer scaffold degrades and is eliminated by the body. Figure 1 [Ishaug et al., 1995] illustrates a tissue engineering strategy for bone replacement. A defect in the femur is filled by a polymer
Figure 1-1. Schematic of a bone tissue engineering strategy. A defect in the femur is filled by a polymer scaffold that has been seeded with a patient's own cells that were expanded *in vitro*. 
scaffold that has been seeded with the patient's own bone cells. These bone cells were removed from the patient's healthy tissue and expanded \textit{in vitro} before seeding onto the scaffold.

Several advantages of tissue engineering strategies are readily apparent. For many tissues, an unlimited supply of cells exists because a small number can be expanded \textit{in vitro} before seeding onto the scaffold [Cima et al., 1991]. Also, with the onset of the genetic engineering, abnormalities can be corrected at the genetic level and then healthy cells returned to the body via a polymer-cell construct [Gilbert, 1993]. In addition, a degrading polymer also can be used as a vehicle for drug delivery that can enhance the tissue replacement process [Langer, 1990]. The transplantation of allogeneic cells and xenogeneic cells is a possibility as well [Uludag and Sefton, 1993]. They require the use of immuno-suppressive drugs or protection from the body's immune system with a physical barrier such as the polymer itself. Extensive research has been done on the isolation and encapsulation of pancreatic islets to treat insulin and glucagon deficiencies in diabetic patients [Mikos et al., 1994].

The most promising strategy, however, involves the delivery of autogeneic cells using a temporary polymer scaffold that allows for a completely natural tissue replacement. Restoration of a tissue may be achieved either by transplantation of cells seeded into a porous scaffold (Figure 1-2a) or, in some cases, by relying on ingrowth of tissue and cells(Figure 1-2b and 1-2d). Figure 1-2c illustrates how this process can be facilitated or enhanced by the release of chemotactic agents from the scaffold [Thomson et al., 1995].
Figure 1-2. Four tissue engineering strategies: a) scaffold is seeded with cells prior to implantation for regeneration of tissues such as cartilage, b) prevascularization and subsequent cell seeding to regenerate metabolic organs such as liver, c) as the scaffold degrades, bioactive molecules are released that induce tissue ingrowth, and d) a tubular scaffold lined with cells relies on cell migration as well as vascular tissue ingrowth for survival.
1.2 Scaffold Design Criteria

The design criteria for a successful polymer scaffold for tissue replacement are multi-fold. First, the material must be biocompatible so that it will not illicit an inflammatory response and become encapsulated in fibrous scar tissue. The material must also be biodegradable to allow a completely natural tissue replacement [Cima et al., 1991]. In addition, many cell types are anchorage dependent and require a surface to which they can adhere. An appropriate surface chemistry that mimics the cell's extracellular matrix is necessary for proper cell morphology, growth, and function [Ikada, 1994]. Moreover, the spatial relationship between the cells is very important. It has been shown that polymers with an interconnecting pore network are capable of providing a scaffold in which cells can properly organize themselves [Uyama et al., 1993]. They have a large surface area to volume ratio which is ideal for a cell polymer construct. The material used in the scaffold must have the desired mechanical properties to withstand in vivo stresses [Yaszemski et al., 1996]. The scaffolds must also be easily fabricated into the applicable size and shape [Lu and Mikos, 1996] as well as easily sterilized for implantation [Athanasiou et al., 1995].

1.3 Poly(a-hydroxy esters)
Poly(a-hydroxy esters) such as poly(L-lactic acid) (PLLA) and poly(DL-lactic-co-glycolic acid) (PLGA) are currently being investigated as materials for regeneration of several tissues such as cartilage [Freed et al., 1993], bone [Ishaug-Riley et al., in press], liver [Johnson et al., 1994], and intestine [Mooney et al., 1993; Organ et al., 1992]. These materials meet several scaffold design criteria including a biocompatible and biodegradable polymer matrix that aids in the differentiation and proliferation of desired cell types [Barrera et al., 1993]. They are two of the few biodegradable polymers approved by the Food and Drug Administration for clinical use and have a common application as degradable sutures [Thomson et al., 1995]. PLLA and PLGA degrade by simple hydrolysis as shown in Figure 1-3. The degradation products, lactic and/or glycolic acid, occur naturally in the body and upon formation they are processed through metabolic pathways [Thomson et al., 1995].

PLLA is a crystalline, hydrophobic polymer. The presence of the methyl group hinders the hydrolytic process making degradation very slow. It is also one of the strongest biodegradable polymers known and has therefore found tissue engineering applications in the orthopedics field. PLGA copolymers, with lactic acid concentrations between 30% and 75%, are amorphous and not as strong as PLLA. They degrade much faster and the degradation rate can be controlled by varying the ratio of lactic to glycolic acid in the copolymer. Both of these polymers have proven to be suitable substrates for cell growth, attachment, and differentiation [Ishaug et al., 1994]. They are also easily and reproducibly processed into scaffolds that degrade as need or their support diminishes.
Figure 1-3. Hydrolytic degradation reaction of poly(DL-lactic-co-glycolic acid) into its monomer units lactic acid and glycolic acid.
1.4 Processing Techniques

Several processing techniques exist to manufacture scaffolds for tissue engineering. Some of them include fiber bonding [Cima et al., 1991; Mikos et al., 1993], melt molding [Thomson et al., 1995] and solvent-casting and particulate-leaching [Mikos et al., 1994]. The solvent-casting and particulate-leaching technique is one of the most versatile techniques, allowing easy control of the pore size and porosity of the scaffold. Using this technique, scaffolds with irregular geometries can be formed by laminating individual scaffolds together to form a three-dimensional structure [Mikos et al., 1993]. The solvent-casting and particulate-leaching technique is a two part process as shown in Figure 1-4. First the polymer is dissolved in methylene chloride and combined with salt particles of the desired size range. The entire solution is then cast into a petri dish. After the methylene chloride has evaporated, the solid salt-polymer membrane is immersed in distilled/deionized water and the salt is leached out of the polymer. The membrane dries, leaving a porous polymer scaffold.

1.5 Engineering Scaffold Properties

Each kind of tissue also requires a scaffold with unique physical and mechanical properties. Pore morphology, porosity, degradation rate, and mechanical characteristics are important properties that need to be tailored to
1. Dissolve Polymer, Add Salt, and Cast

2. Allow Solvent to Evaporate

3. Leach Salt from Membrane

4. Dry Porous Polymer Scaffold

Figure 1-4. Schematic of the solvent-casting and particulate-leaching technique. 1) polymer is dissolved in dichloromethane and cast into a petri dish, 2) the solvent evaporates leaving a dry polymer/salt composite membrane, 3) the salt is leached out of the membrane in distilled/deionized water, and 4) the membrane dries leaving a porous polymer scaffold.
the type of tissue being regenerated. For example, pore morphology effects the rate of tissue ingrowth into a scaffold [Mikos et al., 1993; Wake et al., 1995; Wake et al., 1994]. The size of the pores are easily controlled by changing the size of the salt crystals that are leached out of the membranes. Porosity is controlled by the polymer/salt ratio. Scaffolds with large interconnecting pore networks allow rapid advance of tissue, which is necessary for regeneration of tissues such as skin or intestine. Strength of the polymer scaffold is a primary concern in bone regeneration [Yaszemski et al., 1995]. Pliability is an important consideration for intestine and blood vessels, which are tissues that require a tubular scaffold. Other non-tubular soft tissues such as skin require a flexible matrix that can conform to a variety of different contours [Wake et al., 1996].

1.6 Degradation Products - Particle Debris

Not only are the mechanical properties important but understanding and controlling the degradation processes and products are crucial for long term success of a tissue engineering strategy. Poly(a-hydroxy esters) degrade by bulk degradation. The first measurable event that occurs during bulk degradation is the loss of strength because molecular weight decreases [Bos et al., 1991]. Mass is not lost at first because the polymer chains are not soluble in the extracellular fluid until the molecular weight is below 7 kD [Agrawal et al., 1994; Gibbons, 1992]. By this time the material has little strength and the polymer begins to fragment due to local mechanical stresses. When particles are approximately 20 μm they can interact with multinucleated cells or macrophages. Further breakdown of the polymer occurs both inside and
outside of the cells. Inside the cell the oligomers are either processed and/or released and it is at this stage that high concentrations of the polymer degradation products in the cytoplasm can become toxic to the cells. Also, sharp edges can puncture or damage cell membranes [Gibbons, 1992].

Outside of the cell the particles continue to degrade and decrease in size. As lactic and glycolic acid are released, there is danger of a local drop in pH if the area is not well vascularized. This problem can be avoided by modulating the release of lactic and glycolic acid by varying the molecular weights of the starting polymers [Von Recum et al., 1995]. The particles themselves, however, can still illicit an inflammatory response which has been documented in several long term studies investigating poly(lactic acid) and poly(glycolic acid) [Bergsma et al., 1995; Beumer et al., 1994; Bostman et al., 1992; Paivarinta et al., 1993; Suganuma and Alexander, 1993].

1.7 Particle Debris in the Orthopedics Field

Particles of several non-degradable materials used in the orthopedics field have been responsible for the failure of a large number of implants. Most of the studies investigating this phenomenon have been done on materials such as ultra high molecular weight poly(ethylene) (UHMWPE) [Amstutz et al., 1991], poly(methyl methacrylate) (PMMA) [Frondoza et al., 1993; Glant and Jacobs, 1994] cobalt-chromium alloy [Pazzaglia et al., 1986], titanium alloy [Glant and Jacobs, 1994; Shanbhag et al., 1994] and ceramics [Amstutz et al., 1991]. Particles in a spectrum of sizes can be generated from an
implant/implant interface or a bone/implant interface. As many as 20 million to 40 billion UHMWPE particles have been found around failed hip prostheses [Amstutz et al., 1991].

The mechanism by which the particles effect bone resorption or cause implant failure is a much debated topic. A widely accepted theory is that they indirectly effect bone cells via mediators secreted by macrophages [Glant et al., 1993]. The primary secretory products hypothesized to be involved in bone resorption include interleukin-1 (IL-1), tumor necrosis factor-a (TNF-a), prostaglandin E2 (PGE2), and interleukin-6 (IL-6) [Nathan, 1987]. Studies have also been involved in investigating particle effects on other cell types such as fibroblasts [Frondoza et al., 1993] and lymphocytes [Jiranek et al., 1995]. Figure 1-5, adapted from Howie et al. [Howie et al., 1993], illustrates two postulated mechanisms of how particles effect bone: indirectly via macrophages and directly via osteoblasts. Important particle parameters effecting the response of different cell types include size, geometry, concentration, material composition, and surface roughness [Amstutz et al., 1991; Glant et al., 1993; Howie et al., 1993; Lam et al., 1993; Shanbhag et al., 1994].

Degradable particles as well as non-degradable particles can cause bone resorption or illicit an inflammatory response. Therefore, it is important to consider the effects of particles from degradable scaffolds used for bone regeneration strategies as well.

1.8 Osteoblast Isolation and Biology
Figure 1-5. Postulated mechanisms adapted from Howie et al. [Howie et al., 1993] for how particles effect bone resorption. Macrophages phagocytose particles and secrete bone resorbing mediators such as IL-1, TNF-a, PGE-2 and IL-6 which act on the osteoblast or osteoclast. Particles could also have a direct effect on osteoblasts and osteoclasts.
Several methods have been developed to isolate osteoblasts for tissue culture [Nijweide et al., 1981; Peck et al., 1964; Yagiela and Woodbury, 1977]. The method of isolation and the factors added to the media can alter the development and expression of proteins by osteoblasts. Isolated rat marrow stromal cells can be induced to form osteoblast-like cells in culture by the addition of dexamethasone to the media [Aubin et al., 1992]. Ascorbic acid and b-glycerol phosphate are also added to the media for osteoblast cultures because they are necessary for collagen production and mineralization. Though the marrow derived cells must be induced to form osteoblasts, unlike the rat neonatal calvarial cells widely used in vitro osteoblast studies, the marrow isolation method has several advantages. Most importantly, future clinical applications necessitate a marrow isolation because it avoids a complicated surgery and donor site complications such as infection and extensive damage to local tissue. Also, the cells would be the patient’s own, avoiding transfer of pathogens or foreign tissue rejection. Osteoblast-like cells have been harvested from human as well as rat bone marrow [Haynesworth et al., 1992].

Osteoblasts go through three main developmental stages as they mature from the preosteoblast. The first stage is the proliferative phase which is characterized by proliferation and collagen production. The second phase is marked by an increase in alkaline phosphatase production and extracellular matrix development. In the third phase, the mineralization phase, mature osteoblasts mineralize the surrounding matrix. Figure 1-6, adapted from Lian et al. [Lian and Stein, 1992], illustrates the relationship between proliferation and differentiation of the developing rat calvarial osteoblast.
Figure 1-6. The three developmental phases of osteoblasts showing the relationship between proliferation and differentiation. Principal developmental proteins and growth factors which are expressed during each phase are listed: TGF-β, transforming growth factor-beta; Col I, collagen type I; AP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; Mineral, total accumulated calcium.
1.9 Future of Tissue Engineering

Current advances in the medical and science field strongly suggest that tissue engineering with biodegradable polymers may become a widespread therapy or treatment for several diseases and damaged tissue in the future. In order to reach that goal, scientists and clinicians must strive to more perfectly tailor the polymer scaffold to the type of tissue being regenerated. It is imperative to understand the long term degradation processes, products, and product interactions with all local cell types, to ensure long-term success and a completely natural tissue replacement.
CHAPTER 2

OBJECTIVES

The objectives of this thesis were to fabricate pliable biodegradable polymer foams for use in tissue engineering and investigate the effects of biodegradable polymer particles on osteoblast function *in vitro*.

We hypothesize:

1) The structural and mechanical properties of biodegradable polymer foams can be engineered by varying the processing parameters of the manufacturing method.

2) Biodegradable polymer particles in size ranging from 1.0 - 1.5 μm can effect osteoblast proliferation and differentiated function.

To test these hypotheses, we proposed the following specific aims:

1) To fabricate PLGA/PEG blend scaffolds using a solvent-casting and particulate-leaching technique and investigate the effects of four different processing parameters on the pliability, porosity, and pore morphology. The processing parameters that were varied included: copolymer ratio, PLGA/PEG blend ratio, initial salt weight fraction, and salt particle size.
2) To culture rat marrow stromal cells in the presence of biodegradable polymer particles having varying composition (also referred to as copolymer ratio), molecular weight, and concentration, over a period of 28 days. The effects of the particles were isolated by determining proliferation, alkaline phosphatase activity, and mineralization of the cells.
CHAPTER 3

FABRICATION OF Pliable BIODEGRADABLE POLYMER FOAMS TO ENGINEER SOFT TISSUES

3.1 Introduction

Each kind of tissue requires a scaffold with unique physical and mechanical properties. Pore morphology, porosity, degradation rate, and mechanical characteristics are important properties which need to be tailored to the type of tissue being regenerated. For example, pore morphology affects the rate of fibrovascular ingrowth into a scaffold [Wake et al., 1995; Wake et al., 1994]. Those scaffolds with large interconnecting pore networks allow rapid advance of tissue which is necessary for regeneration of tissues such as skin or intestine. Strength of the polymer scaffold is a primary concern in bone regeneration [Thomson et al., 1995; Yaszemski et al., 1995]. Pliability is an important consideration for intestine and blood vessels which are tissues that require a tubular scaffold [Atala et al., 1992; Mooney et al., 1993; Natsume et al., 1993]. Other non-tubular soft tissues such as skin require a flexible matrix that can conform to a variety of different contours.

In this study, the effects of different processing parameters in a solvent-casting and particulate leaching technique [Mikos et al., 1994] on pliability, pore morphology, and porosity have been investigated. Examination of the effect of

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blending poly(ethylene glycol) (PEG) with poly(lactic-co-glycolic acid) (PLGA) polymers has led to the development of pliable three-dimensional biodegradable polymer scaffolds. The impetus for the investigation of PLGA/PEG blends was evidence of enhanced hydrophilicity and flexibility of copolymers of poly(lactic acid) (PLA) and PEG [Cohn and Younes, 1988; Younes et al., 1988]. In addition to the effect of blending PLGA with PEG, the present investigation examined the effects of three other processing parameters on mechanical properties and physical characteristics of porous scaffolds. These include the lactic/glycolic acid copolymer ratio, the initial salt weight fraction, and the salt particle size utilized to fabricate the foams. This chapter describes the effects each of these parameters has on mechanical properties and pore morphology of biodegradable scaffolds. With a better understanding of the main effects of these parameters, a scaffold will be able to be designed that has pore size, porosity and pliability tailored for the type of tissue being regenerated.
3.2 MATERIALS AND METHODS

3.2.1 Materials

PLGA with a lactic/glycolic acid ratio of 75/25 (PLGA 75/25) (Birmingham Polymers, Birmingham, AL), PLGA 50/50 (Medisorb, Cincinnati, OH), and PEG (Aldrich Chemical Company, Milwaukee, WI) were used in various combinations according to Table 3-1 to process the polymer foams. The weight average molecular weights, $M_w$, and polydispersity indices, $PI$, of each of these polymers as determined by gel permeation chromatography were for PLGA 75/25: $M_w = 61900 \pm 800$ and $PI = 2.2 \pm 0.0$ (n=3), for PLGA 50/50: $M_w = 40100 \pm 700$ and $PI = 2.2 \pm 0.0$ (n=3), and for PEG: $M_w = 2700 \pm 700$ and $PI = 1.2 \pm 0.1$ (n=3). Sodium chloride particles (Baxter, Houston, TX) were sieved into two size ranges: 250-500 μm and 500-710 μm.

3.2.2 Experimental Design

The polymer foams were processed with specifications determined by a Resolution IV two-level fractional factorial design (Table 3-1) [Box et al., 1978]. The four variables examined were: 1) the PLGA copolymer ratio, 2) the PLGA/PEG blend ratio, 3) the initial salt weight fraction, and 4) the salt particle size. There were a total of 8 polymer foams in the Resolution IV design. Each foam was a combination of the "high" and "low" levels of the variables listed in
Table 3-1. Combinations of the experimental variables in Resolution IV design.

<table>
<thead>
<tr>
<th>Foam Code</th>
<th>PLGA Copolymer Ratio</th>
<th>PLGA/PEG Blend Ratio</th>
<th>Initial Salt Weight Fraction</th>
<th>Salt Particle Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>F2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>F3</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F4</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F6</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F7</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>F8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3-2. These combinations of the variables are represented by a "+" sign for "high" and a ",-" sign for "low". The Resolution IV design was used to calculate the main effect that each of the four variables had on porosity, median pore diameter, and shear modulus. In addition, two extra foams were made to more completely determine the effect of weight percent PEG on the shear modulus and porosity. One of the foams was a blend of 50 wt% PLGA 75/25 and 50 wt% PEG, 90% initial salt weight fraction, and 500-700 μm salt particle size and the other foam was 100% PLGA 75/25, 90% initial salt weight fraction, and 500-700 μm salt particle size.

Four of the eight foams in the Resolution IV design were processed with a blend of 70% PLGA and 30% PEG. The main effect of 1) the PLGA copolymer ratio, 2) the initial salt weight fraction, and 3) the salt particle size on the amount of PEG left in these foams after processing was determined based on a Resolution III design. The Resolution III design based on these three variables is listed in Table 3-3. The low and high levels of each of the variables are the same as in the Resolution IV design and are listed in Table 3-2.

3.2.3 Processing Method

The polymer foams were fabricated by a modification of a previously reported solvent-casting and particulate-leaching method [Mikos et al., 1994; Wake et al., 1994]. The polymer was dissolved in dichloromethane and then combined with sodium chloride particles of desired size range and cast into 60 mm petri dishes. Foams fabricated with 90 wt% sodium chloride salt were
Table 3-2. High (+) and low (-) levels for the four variables in the Resolution IV fractional factorial design.

<table>
<thead>
<tr>
<th></th>
<th>PLGA Copolymer Ratio</th>
<th>PLGA/PEG Blend Ratio</th>
<th>Initial Salt Weight Fraction</th>
<th>Salt Particle Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Level</td>
<td>75/25</td>
<td>100% PLGA</td>
<td>90%</td>
<td>500-710 μm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0% PEG</td>
<td></td>
</tr>
<tr>
<td>Low Level</td>
<td>50/50</td>
<td>70% PLGA</td>
<td>80%</td>
<td>250-500 μm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30% PEG</td>
<td></td>
</tr>
</tbody>
</table>
Table 3-3. Combinations of the experimental variables in the Resolution III design. The high and low levels of these variables appear in Table 1 and are the same as in the Resolution IV design.

<table>
<thead>
<tr>
<th>Foam Code</th>
<th>PLGA Copolymer Ratio</th>
<th>Initial Salt Weight Fraction</th>
<th>Salt Particle Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>F2</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>F5</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
made with 0.9 g raw polymer material that was dissolved in 6 ml dichloromethane and combined with 8.1 g salt. Foams fabricated with 80 wt% salt were made with 1.8 g raw polymer that was dissolved in 8 ml dichloromethane and combined with 7.2 g salt. The dichloromethane evaporated over 24 hours in a fume hood leaving a polymer/salt composite membrane. After a ten hour vacuum-drying of the membranes, the salt was leached out of the membrane by immersing in distilled/deionized water for 24 hours. The water was changed every 4 hours. The resulting foams were air dried for 24 hours, vacuum dried for 24 hours, and stored under vacuum until subsequent characterization.

3.2.4 Formation of Tubular Foams

Foams prepared with a blend of 70 wt% PLGA 75/25 and 30 wt% PEG with 90 wt% sodium chloride particles ranging from 500-700 μm in diameter were rolled into a tube. They were cut into rectangular sections approximately 3.5 cm long and 1.5 cm wide. The section was then rolled into a tube and ends were sealed by slightly dissolving the edges with chloroform and pressing them together for approximately 15 seconds [Mikos et al., 1993]. The resulting tubular structures were approximately 1 cm in diameter and had a wall thickness of approximately 2 mm.

3.2.5 Mechanical Testing
A dynamic mechanical analyzer (DMA) (TA Instruments 983, Wilmington, DE) was used to obtain shear storage, $G'$, and loss moduli, $G''$, which describe the viscoelastic properties of a polymer. The shear storage and loss moduli are combined to form the complex shear modulus, $G = G' + iG''$. The real part of the modulus, $G'$, represents the energy storage term whereas the imaginary part, $G''$, represents the energy dissipation term. The magnitude, $|G|$, of the complex shear modulus can be obtained by $|G| = \sqrt{G'^2 + G''^2}$. The DMA method employed involved mounting the samples with low mass clamps and tightening the clamps without causing significant sample deformation. The samples were run at 37°C, a frequency of 1 Hz, and an oscillation amplitude of 0.3 mm for one hour at which time values for the moduli were taken [Thomson et al., 1995] All samples were rectangular with approximate dimensions of 2 x 10 x 20 mm. All samples were run in duplicates.

3.2.6 Mercury Intrusion Porosimetry

The pore size distribution of each of the foams was determined by mercury intrusion porosimetry (Quantachrome, AUTOSCAN 500, Syosset, NY). Samples ranged in weight from 0.05-0.35 g. The filling pressure, $P_{\text{min}}$, of the penetrometer was 0.7 psi and the maximum pressure was 50 psi. Reported values of pore diameter ($d_p$) and porosity refer to equivalent cylindrical pores with diameters smaller than 284 μm which was determined from the Washburn equation [Mikos et al., 1994]: $d_p = -4 \gamma \cos \theta / P_{\text{min}}$. The contact angle, $\theta$, of mercury with PLGA 50/50 is 135° and the surface tension, $\gamma$, is 485 dyn/cm at 25°C [Mikos et al., 1994]. The contact angle of mercury with PLGA 75/25 was
assumed equal to 135°. All samples were run in duplicates. Porosity and median pore diameter data as measured by porosimetry should be considered with caution because large pores with diameter greater than 284 μm cannot be detected and therefore are not factored into the foam porosity and median pore diameter results. Thus, gravimetry methods were also utilized to determine porosity.

3.2.7 Gravimetry

The porosity, ε, was determined by calculating the apparent density from volume and mass measurements of specimens of defined geometries:

\[ \varepsilon = 1 - \left( \frac{\rho^*}{\rho} \right). \]

Here \( \rho \) is the polymer skeletal density (1.35 g/ml for PLGA 50/50 [Mikos et al., 1994] and assumed to be also for PLGA 75/25), and \( \rho^* \) is the calculated apparent density. Samples were measured in duplicates.

3.2.8 Scanning Electron Microscopy

Foams were gold coated using a sputter coater (Pelco Sputter Coater 91000, model 3, Ted Pella, Reading, CA) set at 20 mA for a total of 120 s (coating thickness approximately 40 nm). The scanning electron microscope
(JEOL JSM-5300 Scanning Microscope, Boston, MA) was set to 25 kV and was utilized to view the top surface of the foams.

3.2.9 Gel Permeation Chromatography

The molecular weight distributions of the foams were determined by gel permeation chromatography (GPC) using a differential refractometer (Waters, Model 410, Milford, MD). Raw PLGA 75/25, raw PLGA 50/50, and samples made with 100% PLGA 75/25 or 50/50 were dissolved in chloroform and eluted through a Phenogel 5 Guard column (model 1063376, 50 X 7.8 mm, Phenomenex, Torrance, CA) and a Phenogel linear column (model 1063338, 300 x 7.8 mm, 5 micron particles, Phenomenex) at a flow rate of 1 ml/min. Polystyrene standards were used to obtain a primary calibration curve. Mark-Houwink constants for poly(L-lactic acid), $K=5.45 \times 10^{-3}$ ml/g and $\alpha=0.73$, were utilized to determine molecular weights of the samples made with 100% PLGA [Mikos et al., 1994]. The Mark-Houwink constants for PEG, $K=0.206$ ml/g and $\alpha=0.5$, were utilized to determine molecular weights of PEG [Bailey and Koleske, 1976].

Foams fabricated from 70% PLGA and 30% PEG were eluted through a Phenogel 5 Guard column (model 494386, 50 x 7.8 mm, Phenomenex) and a Phenogel column (model 94890, 300 x 7.8 mm, 5 micron particles, Phenomenex) at a flow rate of 1 ml/min. To determine the weight fraction of residual PEG in the foams after processing, a calibration curve based on the
height of PEG peaks at concentrations ranging from 0.01 to 0.10 weight percent was constructed. The height of the PEG peak in the foam samples was then measured and the corresponding concentration in the foam sample determined using the calibration curve. The weight fraction of PEG in the foam was then calculated by dividing the concentration of PEG in the sample by the concentration of the entire polymer in the sample.

3.2.10 Thermogravimetric Analysis

The residual amount of sodium chloride left in the polymer foams was determined by thermogravimetric analysis (TGA) (Perkin-Elmer, Model TGS-2, Newton, MA). Approximately 3-8 mg of material were heated from 25 to 600°C at a constant rate of 10°C/min. The polymer weight fraction was determined by dividing the difference of the sample at 150°C and 600°C by the weight at 150°C [Mikos et al., 1994]. Single measurements were used in the calculation.
3.3 RESULTS

The results of mechanical testing indicate a wide range of shear moduli, 0.91-9.55 MPa, dependent upon the combination of processing parameters (Table 3-4). As the polymer material changed from PLGA 75/25 to PLGA 50/50, a relatively large negative effect, a decrease of approximately 3 MPa in shear storage modulus, G', is exhibited (Figure 3-1a). Decreasing the initial salt weight fraction from 90% to 80% had an equally positive affect on G'. Shear storage modulus also decreased when PEG was added as a raw material. Changing the salt particle size from 500-700 µm to a smaller 250-500 µm particle range had a much less significant decreasing effect on the shear storage modulus.

Similar trends were noted for the shear loss modulus, G'' (Figure 3-1b). The salt weight fraction played the most significant role in changing the value of the loss modulus, increasing it by approximately 1 MPa as the initial salt weight fraction decreased from 90% to 80%. Overall the values for shear loss moduli are less than those for storage moduli.

The magnitude of complex shear modulus, |G|, decreases as the weight percent of PEG in the foams increases (Figure 3-2). This trend is demonstrated for foams made with blends of PLGA 75/25 and PEG with PEG weight fractions of 0, 30, and 50% and fabricated with 90% salt weight fraction and salt particles ranging from 500-700 µm. The first of the two extra foams (fabricated with a blend of 50 wt% PLGA 75/25 and 50 wt% PEG, 90% initial salt weight fraction of particle size between 500-700 µm) had a thickness of 2.42 ± 0.16 and shear
Table 3-4. Results of dynamic mechanical testing. $G'$ and $G''$ are the real and imaginary parts of the complex shear modulus, $G$, and $|G|$ is its magnitude. Samples were run in duplicate and reported as mean ± range.

<table>
<thead>
<tr>
<th>Foam Code</th>
<th>Shear Storage Modulus</th>
<th>Shear Loss Modulus</th>
<th>Magnitude of Modulus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$G'$ (MPa)</td>
<td>$G''$ (MPa)</td>
<td>$</td>
</tr>
<tr>
<td>F1</td>
<td>1.25 ± 0.05</td>
<td>0.85 ± 0.04</td>
<td>1.51 ± 0.06</td>
</tr>
<tr>
<td>F2</td>
<td>0.73 ± 0.15</td>
<td>0.54 ± 0.12</td>
<td>0.91 ± 0.19</td>
</tr>
<tr>
<td>F3</td>
<td>2.02 ± 0.38</td>
<td>1.07 ± 0.18</td>
<td>2.28 ± 0.42</td>
</tr>
<tr>
<td>F4</td>
<td>0.87 ± 0.01</td>
<td>0.41 ± 0.01</td>
<td>0.96 ± 0.00</td>
</tr>
<tr>
<td>F5</td>
<td>3.70 ± 0.70</td>
<td>0.67 ± 0.06</td>
<td>3.76 ± 0.70</td>
</tr>
<tr>
<td>F6</td>
<td>0.93 ± 0.22</td>
<td>0.17 ± 0.04</td>
<td>0.95 ± 0.23</td>
</tr>
<tr>
<td>F7</td>
<td>8.90 ± 0.60</td>
<td>3.46 ± 0.22</td>
<td>9.55 ± 0.64</td>
</tr>
<tr>
<td>F8</td>
<td>3.00 ± 0.04</td>
<td>0.48 ± 0.01</td>
<td>3.02 ± 0.03</td>
</tr>
</tbody>
</table>
Figure 3-1. The main effect of four experimental parameters on a) $G'$, the shear storage modulus and b) $G''$, the shear loss modulus. A positive number indicates that the particular parameter had an increasing effect on the moduli as it went from a high level to a low level. A negative number indicates a decrease in the moduli as it went from a high level to a low level. The standard errors of effect on $G'$ and $G''$ were 0.26 and 0.08 respectively ($n=2$).
Figure 3-2. The dependence of magnitude, $|G'|$, of the complex shear modulus on the percentage of PEG in the blend ratio of polymer material used to make porous foams. Foams were fabricated with 90 wt% of sodium chloride particles ranging from 500-700 μm and PLGA 75/25, a blend of 70 wt% PLGA 75/25 and 30 wt% PEG, and a blend of 50% PLGA 75/25 and 50% PEG, respectively. Error bars stand for means ± range for $n=2$. 
moduli of $G' = 0.58 \pm 0.01$, $G'' = 0.10 \pm 0.00$ and $|G| = 0.59 \pm 0.06$ MPa (for $n=2$). The second extra foam (fabricated with 100% PLGA 75/25, 90% initial salt weight fraction of particle size between 500-700 \( \mu \)m) had a thickness of $2.39 \pm 0.12$ and shear moduli of $G' = 1.74 \pm 0.14$, $G'' = 0.53 \pm 0.01$ and $|G| = 1.81 \pm 0.27$ MPa (for $n = 2$). The thickness of all of the foams ranged between 2 and 3 mm (Table 3-5).

Characterization of the foam's pore morphology using mercury porosimetry and gravimetry indicate that foam porosities ranging from 54% to 94% and median pore diameters from 71 to 154 \( \mu \)m were achieved with various combinations of processing parameters (Table 3-6). The initial salt weight fraction has the greatest effect on porosity and median pore diameter (Figures 3-3 and 3-4). Decreasing the salt weight fraction from 90% to 80% decreases the porosity of the resulting foams by nearly 13%. The same decrease in salt weight fraction decreases the median pore diameter approximately 30 \( \mu \)m. The copolymer ratio and salt particle size had approximately equal decreasing effects on the porosity and median pore diameter, approximately 7% and 15 \( \mu \)m, respectively. The porosity increases as the weight percent PEG in the PLGA/PEG blend increases (Figure 3-5). Foams used to illustrate this trend were fabricated with a copolymer of PLGA 75/25, 90% initial salt weight fraction, and salt particles ranging from 500-700 \( \mu \)m. The extra foam fabricated with 50 wt% PEG had a porosity equal to $0.942 \pm 0.000$ and a median pore diameter of $154 \pm 1 \mu m$ ($n=2$). The other foam fabricated with 100% PLGA 75/25 had a porosity equal to $0.889 \pm 0.010$ ($n = 2$).

To further characterize the foams, the amount of residual PEG and residual salt after processing was determined. GPC studies indicated small
Table 3-5. Foam thickness (n=2), residual PEG weight fractions (n=3) and salt weight fractions (n=1) in foams. Foams F3, F4, F7, and F8 were made with 100% PLGA and so no PEG is present.

<table>
<thead>
<tr>
<th>Foam Code</th>
<th>Foam Thickness (mm)</th>
<th>Residual PEG Weight Percent</th>
<th>Residual Salt Weight Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>2.76 ± 0.03</td>
<td>4.02 ± 0.08</td>
<td>6.1</td>
</tr>
<tr>
<td>F2</td>
<td>2.24 ± 0.11</td>
<td>3.40 ± 0.10</td>
<td>4.7</td>
</tr>
<tr>
<td>F3</td>
<td>2.81 ± 0.07</td>
<td>N/A</td>
<td>39.8</td>
</tr>
<tr>
<td>F4</td>
<td>3.06 ± 0.13</td>
<td>N/A</td>
<td>10.0</td>
</tr>
<tr>
<td>F5</td>
<td>2.41 ± 0.04</td>
<td>2.94 ± 0.03</td>
<td>0.6</td>
</tr>
<tr>
<td>F6</td>
<td>2.36 ± 0.13</td>
<td>2.53 ± 0.04</td>
<td>4.7</td>
</tr>
<tr>
<td>F7</td>
<td>2.91 ± 0.04</td>
<td>N/A</td>
<td>1.9</td>
</tr>
<tr>
<td>F8</td>
<td>2.78 ± 0.06</td>
<td>N/A</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Table 3-6. Porosity and median pore diameter of prepared foams. Samples were run in duplicate and reported as mean ± range.

<table>
<thead>
<tr>
<th>Foam Code</th>
<th>Porosity*</th>
<th>Porosity†</th>
<th>Median Pore Diameter* (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.763 ± 0.004</td>
<td>0.858 ± 0.008</td>
<td>103 ± 1</td>
</tr>
<tr>
<td>F2</td>
<td>0.862 ± 0.012</td>
<td>0.895 ± 0.004</td>
<td>120 ± 22</td>
</tr>
<tr>
<td>F3</td>
<td>0.539 ± 0.003</td>
<td>0.531 ± 0.020</td>
<td>95 ± 7</td>
</tr>
<tr>
<td>F4</td>
<td>0.704 ± 0.059</td>
<td>0.894 ± 0.006</td>
<td>104 ± 2</td>
</tr>
<tr>
<td>F5</td>
<td>0.767 ± 0.008</td>
<td>0.850 ± 0.000</td>
<td>71 ± 7</td>
</tr>
<tr>
<td>F6</td>
<td>0.790 ± 0.009</td>
<td>0.919 ± 0.008</td>
<td>154 ± 8</td>
</tr>
<tr>
<td>F7</td>
<td>0.704 ± 0.067</td>
<td>0.798 ± 0.000</td>
<td>104 ± 7</td>
</tr>
<tr>
<td>F8</td>
<td>0.820 ± 0.002</td>
<td>0.891 ± 0.002</td>
<td>144 ± 12</td>
</tr>
</tbody>
</table>

* determined by mercury porosimetry
† determined by gravimetry
Figure 3-3. The main effect of four experimental parameters on porosity as measured by gravimetry studies. A positive number indicates that the particular parameter had an increasing effect on the porosity as it went from a high level to a low level. A negative number indicates a decrease in the porosity as it went from a high level to a low level. The standard error of effect was 0.01 (n=2).
Figure 3-4. The main effect of four experimental parameters on median pore diameter as measured by mercury intrusion porosimetry. A positive number indicates that the particular parameter had an increasing effect on the median pore diameter as it went from a high level to a low level. A negative number indicates a decrease in the median pore diameter as it went from a high level to a low level. The standard error of effect was 7.3 (n=2).
Figure 3-5. The dependence of porosity on the percentage of PEG in the blend ratio of polymer material used to make porous foams. Foams were fabricated with 90 wt% of sodium chloride particles ranging from 500-700 μm and PLGA 75/25, a blend of 70 wt% PLGA 75/25 and 30 wt% PEG, and a blend of 50% PLGA 75/25 and 50% PEG, respectively. Porosity was determined by gravimetry studies. Error bars stand for means ± range for n=2.
amounts of residual PEG (2-4 wt% of the foam mass) in the foams initially fabricated with 30 wt% PEG and 70 wt% PLGA (Table 3-5). Changing from PLGA 75/25 to PLGA 50/50 has an increasing effect, approximately 1%, on the weight percent of residual PEG in the foams (Figure 3-6). Likewise, decreasing initial salt weight fraction from a 90% to 80% also increases the amount of residual PEG. Decreasing the salt particle size from 500-750 μm to 250-500 μm decreases the residual PEG in the foam. Residual salt amounts as determined by TGA are relatively minimal in all of the foams except for the foam F3 which was fabricated with PLGA 50/50 with 80 wt% salt particles ranging in size from 250-500 μm as indicated in Table 3-5.
Figure 3-6. The main effect of three experimental parameters on weight percent of residual PEG in the foams made with blends of 70 wt% PLGA and 30 wt% PEG. A positive number indicates that the particular parameter had an increasing effect on the PEG weight percent as it went from a high level to a low level. A negative number indicates a decrease in the PEG weight percent as it went from a high level to a low level. The standard error of effect was 0.03.
3.4 DISCUSSION

We have analyzed the effects of different processing parameters on shear moduli, median pore diameter, and porosity of biodegradable foams for engineering soft tissues. The parameters investigated were 1) the lactic/glycolic acid copolymer ratio, 2) the PLGA/PEG blend ratio, 3) the initial salt weight fraction, and 4) the salt particle size. Combining these parameters in various ways allows the pliability and pore morphology of a scaffold to be tailored to the requirements of a particular tissue that is being regenerated.

Mechanical properties were greatly influenced by the initial salt weight fraction. A higher initial salt weight fraction results in a more porous foam which will be less stiff and thus have a lower shear modulus. Shear storage modulus is less for PLGA 50/50 than PLGA 75/25. Changing the salt particle size from 500-700 μm to a smaller 250-500 μm particle range had a much less significant decreasing effect on the shear storage modulus. When PEG was added as a raw material, a decrease of shear storage modulus was noted. The majority of PEG, a water-soluble polymer, was removed from the foams when the salt/polymer composite membranes were immersed in distilled/deionized water. Tiny vacancies on the pore surfaces of foams made with a PLGA/PEG blend are attributable to the PEG (Figure 3-7). On the contrary, the pores of foams made with 100% PLGA are relatively smooth. The micropores on the pore walls together with any residual PEG make the entire macroscopic structure of the foam more pliable. The pliability increases with increasing PEG content, however the foam made with 50% PLGA and 50% PEG was very fragile and
Figure 3-7. Scanning electron micrographs of the top surfaces of biodegradable foams fabricated with 90 wt% of sodium chloride particles ranging from 500-700 μm in diameter and (a) PLGA 75/25, or (b) a blend of 70 wt% PLGA 75/25 and 30 wt% PEG showing an interconnected pore morphology.
would not be suitable as a scaffold for tissue regeneration because it does not maintain its mechanical integrity.

The foam porosity also increases when the amount of PEG in the blend increases. The increase in porosity is attributable to the disappearance of PEG during the salt leaching step of the foam processing. Porosity is also affected by the salt weight fraction. This result is expected because the salt is leached out of the foam during processing. Most of the salt was removed from the foams except for that in foam F3. We believe the combination of parameters used to make F3, 100% PLGA 50/50, 80 wt% salt particles ranging in size from 250-500 μm, made it difficult for all of the salt to be leached from the foam. Porosities measured by mercury porosimetry were lower than those determined by gravimetry because pores above 284 μm could not be detected. All experimental parameters had a decreasing effect on median pore diameter as they changed from the high level to the low level. Though salt particle size is the primary parameter used to control the size of the pore, the effect was small, only decreasing the median pore diameter by 15 μm as the range decreased from 500-700 to 250-500 μm. The small effect of salt particle size is due also to the mercury porosimetry method not detecting pores with diameters larger than 284 μm.

In addition to salt, approximately 2-4 wt% residual PEG remains in the foams after processing. There was more PEG, approximately 1 wt%, in foams fabricated with PLGA 50/50 than with PLGA 75/25. We think the more hydrophilic nature of the PLGA 50/50 as opposed to the PLGA 75/25 allows the molecule to form a more homogeneous mixture in solution with the PEG, a hydrophilic molecule. The PEG is then trapped evenly throughout the polymer
scaffold when the solvent evaporates and is less accessible to the water. Increasing the initial salt weight fraction increases porosity thus decreasing the amount of residual PEG because water has more access to the inner structure of the scaffold. In the same manner, increasing the surface area per unit volume ratio by decreasing the salt particle size from 500-750 μm to 250-500 μm allows water more access to the scaffold and decreases the amount of residual PEG.

As evidence of the enhanced pliability of the foams, we were able to shape a porous foam into a tubular structure without any macroscopic damage to the foam as illustrated in Figure 3-8. Foams that did not contain PEG were not pliable and would break if rolled or bent. Previous work using tubular scaffolds for intestinal regeneration in rats utilized biodegradable poly(α-hydroxy esters) [Mooney et al., 1995; Mooney et al., 1993]. The polymer was processed into porous films thin enough to be rolled into a tube. The ability to form a tubular structure necessitated the use of very thin films approximately 0.3 mm thick [Mooney et al., 1995]. Enhanced pliability of the three dimensional foams allows thick walled tubes suitable for regeneration in larger animals to be constructed. They have great potential as scaffolds for regeneration of a tissue such as vascular graft on which multiple layers of cells, smooth muscle cells and endothelial cells, could be cultured and subsequently formed into a tubular structure. A thick-walled tube fabricated with the method reported in the present study could also provide increased mechanical support for regeneration of a tubular tissue such as esophagus which requires a sturdy yet pliable scaffold [Natsume et al., 1993].
Figure 3-8. Photomicrographs of a tubular foam fabricated with 90 wt% sodium chloride particles ranging from 500-700 μm in diameter and a blend of 70 wt% PLGA 75/25 and 30 wt% PEG shown (a) in cross-section and (b) at an angle.
We think that the use of the blend of PLGA and PEG enables the formation of flexible polymer scaffolds. We believe the PEG is leached out of the scaffold during processing leaving tiny micropores that make the walls of the foam more pliable. The removal of PEG from a PLGA/PEG blend with water is in agreement with previous studies investigating blends and copolymers of PLA and PEG [Cohn and Ycunes, 1988]. The initial salt weight fraction has the most significant effect on porosity, pore size, and shear modulus; however, none of the foams made with only PLGA were able to be rolled into a tube without breaking. We believe that the incorporation of PEG into foams will be very valuable to the field of soft tissue engineering which requires a more pliable structure to provide the template for tissue ingrowth.
3.5 CONCLUSIONS

The results of the two level fractional factorial design indicate that a wide range of shear moduli, porosities, and pore diameters can be achieved by changing processing parameters in a solvent-casting and particulate-leaching method. The initial salt weight fraction has the most significant effect on the mechanical and physical properties of the foams. Foams processed with the 90% initial salt weight fraction have lower shear moduli than those processed with 80%, they are more porous, and have higher median pore diameter. The resulting foams are more pliable and have a large interconnecting pore network. Using PLGA 50/50 rather than PLGA 75/25 lowers the porosity, pore diameter, and shear modulus. Moreover, using salt particles in the range of 500-700 μm rather than those in the 250-500 μm range increases the porosity and pore diameter.

Fabricating biodegradable scaffolds from blends of PLGA and PEG makes the foams more pliable. The removal of PEG during processing most likely alters the structure of the foam walls leaving tiny micropores that lower the modulus of the entire material. We were able to shape a foam into tubular structures without any macroscopic damage to the pore morphology. We attribute this to the utilization of PEG in the foam processing. These foams can be used for a variety of tissue engineering applications. The ability to make more pliable porous polymers with a large interconnecting network could potentially be very valuable to the field of tissue engineering. Pliable polymer substrates with a large pore configuration hold great promise for regeneration of
soft tissues that require rapid and complete vascularization such as skin, intestine, or vascular graft.
CHAPTER 4

EFFECTS OF BIODEGRADABLE POLYMER PARTICLES ON
OSTEOBLASTS IN VITRO

4.1 INTRODUCTION

Understanding the degradation processes of biodegradable scaffolds and the effects that their degradation products have on the body is crucial for long term success of a tissue engineered cell-polymer construct. Biodegradable particles formed after long-term implantation of poly(a-hydroxy esters) or due to micromotion at the implantation site can illicit an inflammatory response [Bergsma et al., 1995; Beumer et al., 1994; Bostman et al., 1992; Paivarinta et al., 1993; Sugaluna and Alexander, 1993].

It has been widely documented that particles of materials used in the orthopedics field have been responsible for the failure of a large number of implants [Amstutz et al., 1991; Glant and Jacobs, 1994; Shanbhag et al., 1994]. A widely accepted theory regarding the role particles play in bone resorption is that the phagocytosis of particles by macrophages results in the release of bone resorbing mediators [Amstutz et al., 1991; Frondoza et al., 1993; Pazzaglia et al., 1986]. To fully understand the role of particles in the bone resorbing/regenerating process, however, it is important to examine particle interactions with other cells in the local environment in addition to macrophages. In this study, we chose to isolate the effects of particles on the osteoblast, a cell involved in the formation and maintenance of bone
[Buckwalter et al., 1995]. By ascertaining the effects that different types of biodegradable particles have on osteoblast proliferation and phenotype expression, we will be able to better choose a scaffold material for use in regenerating bone tissue.
4.2 MATERIALS AND METHODS

4.2.1 Materials

Poly(L-lactic acid) (PLLA) and poly(lactic-co-glycolic acid) (PLGA 50/50) (Birmingham Polymers, Birmingham AL) were used in various combinations to manufacture biodegradable polymer particles. The weight average molecular weights ($M_W$) and polydispersity indices (PI) of these degradable polymers as determined by gel permeation chromatography were: high $M_W$ PLLA: $M_W = 62,300 \pm 4700$, PI = 2.3 $\pm$ 0.3; low $M_W$ PLLA: $M_W = 3600 \pm 1000$, PI = 2.0 $\pm$ 0.3; high $M_W$ PLGA 50/50: $M_W = 48600 \pm 5300$, PI = 2.1 $\pm$ 0.4; low $M_W$ PLGA 50/50: $M_W = 3000 \pm 900$, PI = 2.3 $\pm$ 0.2 (n=3). Non-degradable high density poly(ethylene) (PE) particles were donated by Shamrock Technologies (Newark, NJ).

4.2.2 Fractional Factorial and Statistical Design

The biodegradable polymer particles were manufactured with specifications determined by a Resolution III two-level fractional factorial design (Table 4-1) [Box et al., 1978]. The three variables examined were: 1) the copolymer ratio, 2) the molecular weight, and 3) the concentration of the particles. There were a total of four biodegradable polymer particle types in the Resolution III design. Each particle type was a combination of the “high” and
Table 4-1. Combinations of the experimental variables in the Resolution III design.

<table>
<thead>
<tr>
<th>Code</th>
<th>Particle</th>
<th>PLGA</th>
<th>Molecular Weight</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P3</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P4</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
the "low" levels of the variables listed in Table 4-2. These combinations of the variables are represented by a "+" sign for high and a "-" sign for low. PLLA and PLGA 50/50 were chosen to be the high and low levels of the copolymer ratio category that represents a difference in composition. PLLA is crystalline whereas PLGA 50/50 is amorphous. The high level of the molecular weight category (>40,000 Mw) was chosen to represent particles of scaffolds that have formed due to micromotion or local forces at the implant site. The low level (<4,000) was chosen to represent particles that would form at the final stages of degradation of the polymer scaffolds. The high level of the concentration category (212,000 particles/ml) was chosen to correspond to one particle per osteoblast at confluency (53,000 cells/cm²) and the low level was one-tenth of the concentration. The Resolution III design was used to calculate the main effect that each of the variables had on [³H]-thymidine incorporation, cell number, alkaline phosphatase activity, and mineralization of osteoblasts in culture. Osteoblasts were also cultured with no particles as well as cultured with non-degradable PE particles added in both the low and high concentrations.

Particles made with the above specifications were exposed to the cells beginning at two different time points. In one study particles were added the day after seeding (Day 1) into 24-well tissue culture polystyrene (TCP) dishes (Fisher Scientific, Springfield, NJ). The particle containing media that was exposed to the cells was removed from the wells every two days throughout the 28 day study. It was replaced with fresh media containing particles that had been fabricated on Day 1 and had been concentrated and suspended in media until ready for use. In a second parallel study, particle containing media was
Table 4-2. High (+) and low (-) levels for the three variables in the Resolution III fractional factorial design.

<table>
<thead>
<tr>
<th></th>
<th>Copolymer Ratio</th>
<th>Molecular Weight</th>
<th>Concentration (particles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Level</td>
<td>PLLA</td>
<td>&gt;40,000</td>
<td>212,000</td>
</tr>
<tr>
<td>Low Level</td>
<td>PLGA</td>
<td>&lt;4,000</td>
<td>21,200</td>
</tr>
<tr>
<td></td>
<td>50/50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
not exposed to the cells until Day 14. The media was also changed in the same manner every two days throughout the duration of the 28 day study.

In addition to isolating trends due to changing particle parameters with the fractional factorial design, single factor analysis of variance (ANOVA) was used to assess the statistical significance of results as well. First, each of the samples (control, P1, P2, P3, P4, PE[high], and PE[low]) were compared to the same sample type over time. Second, the statistical significance of the presence of particles was assessed by comparing control samples exposed to no particles with samples exposed to a low concentration of 1) degradable PLLA particles (P2) and 2) non degradable PE particles (PE[low]) at each time point. Only samples exposed to the low concentration of one of the types of biodegradable particles (PLLA, chosen arbitrarily over PLGA) was included to avoid incorrectly interpreting effects due to changing parameters such as molecular weight and copolymer ratio in this analysis. Third, the statistical significance of particle material was assessed by comparing wells exposed to high concentrations of three different particle types (P1, P4, and PE[high]) at each time point. Scheffe's method was used for multiple comparison tests at a significance level of 95%. A two-tailed unpaired t-test was used to evaluate the significance of particles added to samples at varying stages of development of the osteoblast (particles added beginning on Day 1 vs. Day 14). All samples were run in triplicate.

4.2.3 Biodegradable Particle Preparation Method
The polymer particles were manufactured by a single-emulsion-solvent-extraction technique adapted from Gref [Gref et al., 1994] and Maruyama [Maruyama et al., 1994]. Briefly, 25 mg of the polymer were dissolved in 1 ml dichloromethane. The polymer solution was added to 20 ml of 0.3% aqueous PVA that had been filtered through a 0.2 μm filter. Under sterile conditions the solution was emulsified with a vortexer followed by fine emulsification with an ultrasonicator at 60 watts for 30 seconds. The solution was then added to 20 ml of 2% isopropanol (also filtered through a 0.2 μm filter) and was maintained on a magnetic stirrer for 1 hour. The dichloromethane was extracted from the alcohol phase and the dissolved polymer precipitated forming the particles. The solution was centrifuged at 45 g for 10 min. The supernate was removed and the particles were resuspended in sterile distilled/deionized H2O. Centrifuging and washing particles was repeated two times. The particles were finally suspended in 5 ml media and sterilized with ultraviolet light (UV) overnight.

4.2.4 Particle Characterization

The molecular weight distributions of the particles were determined by gel permeation chromatography (GPC) using a differential refractometer (Waters, Model 410, Milford, MD). The biodegradable particles were dissolved in chloroform and eluted through a Phenogel 5 guard column (model 1063376, 50 X 7.8 mm, Phenomenex, Torrance, CA) and a Phenogel linear column (model 106338, 300 x 7.8 mm, 5 micron particles, Phenomenex) at a flow rate of 1 ml/min. Polystyrene standards were used to obtain a primary calibration
curve. Mark-Houwink constants for poly(L-lactic acid), K=5.45x10^{-3} \text{ ml/g and } \alpha=0.73, were utilized to determine molecular weights of the samples made with PLGA 50/50 [Mikos et al., 1994]. The size and number of particles were determined using a Coulter Multisizer (Coulter Electronics, Hialeah, FL) with a 19 \text{ \mu m} aperture orifice tube.

Particles were viewed with a scanning electron microscope (JEOL JSM-5300 Scanning Microscope, Boston, MA) set at 25 kV. Particles were prepared for viewing using a gold sputter coater (Pelco Sputter Coater 91000, model 3, Ted Pella, Reading, CA) set at 20 mA for a total of 120 s (coating thickness approximately 40 nm).

4.2.4 Marrow Stromal Cell Isolation, Seeding, and Culture

Stromal osteoblast-like cells were obtained from the marrow of young adult male Sprague-Dawley rats (6 weeks old, 150 g) by a method adapted by Ishaug [Ishaug-Riley et al., in press] from Maniatopoulos [Maniatopoulos et al., 1988]. After euthanasia, the rat femurs were excised, cleaned of soft tissue, and soaked in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, NY) containing 250 mg/mL gentamicin sulfate (GS) (Sigma Chemical, St. Louis, MO). The metaphyseal ends were then cut off and the marrow was flushed out with 5 mL of primary media [DMEM containing 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah), and 25 mg/mL gentamicin sulfate (GS)] into a sterile petri dish. The cell suspension was pipetted up and down for 5 min to break up cell clumps and then centrifuged at 400 g for 10 min.
The resulting cell pellets were resuspended in 12 mL primary media and plated in T-75 flasks (cells from 2 femurs per flask). After 3 days, flasks were rinsed with phosphate buffered saline (PBS) and the primary media was changed every other day until Day 10. Cells were then enzymatically lifted from the flask using a 625 mg/mL solution of trypsin. The cells were concentrated by centrifugation at 400g for 10 min and resuspended in a known amount of media. Cells were counted by Coulter Counter and diluted to concentrations of 1.06 x 10^5 cells/ml in complete media consisting of DMEM supplemented with 10% FBS, 8 mg/mL GS, 10 mM Na-b-glycerol phosphate (Sigma), and 50 mg/mL L-ascorbic acid (Sigma) containing 10 nM dexamethasone (Sigma) [Rickard et al., 1994]. Aliquots of 0.5 ml were added to 24 well tissue culture polystyrene (TCPS) wells, resulting in a seeding density of 2.5 x 10^4 cells/cm^2. Cell seeding marks the beginning of the experiment and is referred to as Day 0.

4.2.5 DNA Assay

Cell count was determined by doing a fluorimetric DNA assay adapted from West et al. [West et al., 1985]. After 1, 4, 7, 14, 21, and 28 days, the cells were harvested by rinsing with PBS and freezing at -80°C until assayed. Cell standards were prepared and frozen at -80°C by centrifuging known aliquots of cells (25,000 - 625,000 cells) at 400g for 10 min and freezing the pellets for assay at a later time. DNA standards were prepared on the day of the assay from a stock of DNA solution. The DNA solution was a highly polymerized calf thymus DNA (type I, Sigma) at a concentration of 50 μg/ml. Aliquots of the DNA solution (0-160 μl) were used as the standards. The cell samples were thawed
and scraped from the bottom of the tissue culture wells with disposable cell scrapers (Fisher Scientific, Springfield, NJ) into 1.4 ml of the ethylenediaminetetraacetic acid (EDTA) solution (pH 12.3). A volume of 1.4 ml of EDTA was also added to thawed cell standards and DNA standards. All standards and samples were put in a 37°C water bath and cooled on ice until placed in the assayed. Also, 200 μl of 1 M KH₂PO₄ was added to each sample and standard to adjust the pH to 7.0. Subsequently, 1.5 ml of the fluorescent dye solution [200 ng/mL Hoechst 33258 dye (Polysciences, Warrington, PA), 100 mM NaCl, and 10 mM Tris adjusted to pH 7.0] were added to the samples and standards. Fluorescence emissions at 455 nm were read with the excitation set at 350 nm on a fluorescence spectrophotometer (series 2, SLM Amino Bowman, Urbana, IL). The DNA content of the samples was then determined by comparing the absorbance of the samples to the absorbance of both the DNA and cell standards.

4.2.6 Thymidine Incorporation

DNA synthesis was quantified by [³H]-thymidine incorporation using a method adapted from Wu et al [Wu et al., 1996]. Briefly, 7 μCi of [³H]-thymidine were added to each well 24 hours prior to the assay time point (Days 1, 4, 7, 14, 21, and 28). At the time of the assay, the cells were washed with PBS three times, removed from the wells and added to 10 ml aqueous/non-aqueous scintillation cocktail (ICN Biomedicals, Irvine, CA). The samples were sonicated for 5 min (Ultrasonik 300, J.M. Ney, Bloomfield, CT) and counted using a β-counter (Miniaxi-b tricarb 4000 series Packard, Meriden, CT).
4.2.7 Alkaline Phosphatase Activity

Alkaline phosphatase was determined by using an ALPase kit (Diagnostic Kit 245, Sigma) [Ishaug et al., 1994]. Cell samples were harvested at 1, 4, 7, 14, 21, and 28 days, rinsed with PBS, and frozen at -80°C until assayed. The cells were thawed and scraped from the bottom of the tissue culture well using disposable cell scrapers into 1 ml Tris buffer solution. All samples were sonicated (Ultasonik 300, J.M. Ney, Bloomfield, CT) for 4 min at 110 watts (50/60 Hz) on ice. A volume of 20 μl of each sample was added to 1 ml of p-nitrophenyl phosphate solution (16 mmol/L) (Diagnostic Kit 245, Sigma) at 25°C for 5 minutes. P-nitrophenol is produced in the presence of alkaline phosphatase and can be measured with a spectrophotometer at 405 nm. The absorbance was measured every min for five min and the slope of absorbance versus time was used to calculate the alkaline phosphatase activity.

4.2.8 Histology and Histomorphometry

Wells were fixed and stained with a Von Kossa silver nitrate solution that demonstrates mineralized tissue [Ishaug et al., 1994]. Briefly, the cell cultures were fixed in 10% neutral buffered formalin for 15 min and rinsed with distilled-deionized water. The fixed samples were then stained with filtered 5% silver nitrate while being exposed to bright daylight for 30 min, and counterstained with filtered 0.5% safranin-O for 30 s. As a result, calcium phosphate deposits
stain black. Images of the wells were captured using a RGB color video camera with a Jena 250-CF stereo light microscope (Micro-Tech Instruments, Dallas, TX). Images were routed through a digital acquisition system (Quick Capture, Data Translation, Marlboro, MA) and analyzed with NIH Image 1.55 software. The mineralized tissue was black and thus distinguishable from gray non-mineralized tissue (Figure 4-1a). The rectangular field was 640 x 480 pixels wide and was the largest rectangular field that could fit in a circular well. The number of pixels representing black mineralized tissue were used to calculate the percent of mineralized tissue in the entire field (Figure 4-1b).
Figure 4-1. Determination of extent of mineralization. (a) Image is captured of cells challenged with low concentration of low molecular weight PLLA particles and stained with Von Kossa stain that demonstrates mineralization. (b) Image is segmented by gray-level intensity to isolate number of pixels that represent mineralization (shown in black).
4.3 RESULTS

4.3.1 Particle Characterization

The weight average molecular weight ($M_W$) and polydispersity indices (PI) of the four different biodegradable polymer particle types were determined by gel permeation chromatography. The high molecular weight PLLA particles were $M_W = 60,500 \pm 6,000$, PI = 2.5 ± 0.3, and the low molecular weight PLLA particles were $M_W = 3,600 \pm 600$, PI = 1.9 ± 0.2. The high molecular weight PLGA 50/50 particles were $M_W = 47,800 \pm 4900$, PI = 2.2 ± 0.2, and the low molecular weight PLGA 50/50 particles were $M_W = 3000 \pm 400$, PI = 2.4 ± 0.2. The high and low molecular weight PLLA particles were 1.25 ± 0.10 μm and 1.13 ± 0.14 μm in diameter, respectively. The high and low molecular weight PLGA 50/50 particles were 1.20 ± 0.15 μm and 1.18 ± 0.20 μm in diameter, respectively. The non-degradable high density PE particles were 4.12 ± 0.21 μm in diameter. Particles were spherical in shape as illustrated in the scanning electron micrograph of particles manufactured with high molecular weight PLLA (Figure 4-2).

4.3.1 Cell Count

4.3.1.1 Absolute Values
Figure 4-2. Scanning electron micrograph of biodegradable particles fabricated with high molecular weight PLLA particles (P1).
On Day 1, the cell count as determined by the DNA Fluorimetric Assay was 57900 ± 8100 cells/cm². By Day 4, the cell count had nearly doubled for all samples. Figure 4-3 illustrates the number of cells/cm² for Days 4, 7, 14, 21, and 28 for all samples in which particles were exposed to the cells beginning on Day 1. For each time point, the samples exposed to the low concentration of particles (P2 and PE[low]) were not statistically different from those without particles (control) (p=0.05). Cell counts for those samples exposed to high concentrations of particles made of different materials (P1, P4, and PE[high]) were not statistically different either. The number of cells on Day 1 (57900 ± 8100 cells/cm²) was significantly lower than the number for each sample type (except P4) at subsequent time points (p=0.05). The cell counts on Day 1 were not significantly different than cell counts for sample P4 (cells exposed to high concentration of low Mₙ PLGA 50/50 particles) on Days 4, 7, 14, and 21 but were different from cell counts on Day 28 (p=0.05). Cell counts for each sample type on Days 4, 7, 14, 21, and 28 were not significantly different from one another as well (p=0.05).

Figure 4-4 illustrates the number of cells/cm² for Days 21 and 28 for all samples in which the particles were exposed to the cells beginning on Day 14. For both time points, the samples without particles (control) were not significantly different from those exposed to low concentrations of particles (P2 and PE[low]) (p=0.05). Cell counts for those samples exposed to high concentrations of particles made of different materials (P1, P4, and PE[high]) were not different either (p=0.05). Cell counts for each sample type on Day 21 and 28 were not significantly different from one another as well (p=0.05). There
Figure 4-3. Cellularity of osteoblasts cultured with particles beginning on Day 1, expressed as cells/cm². The cells were exposed to no particles (control) as well as a variety of degradable (P1-P4) and non-degradable (PE[low] and PE[high]) particle types. Error bars stand for means ± standard deviation for n=3.
Figure 4-4. Cellularity of osteoblasts cultured with particles beginning on Day 14, expressed as cells/cm². The cells were exposed to no particles (control) as well as a variety of degradable (P1-P4) and non-degradable (PE[low] and PE[high]) particle types. Error bars stand for means ± standard deviation for n=3.
was not a significant difference in cell count between those samples to which particles were added on Day 1 and those to which particles were added on Day 14 (p=0.05).

4.3.1.2 Fractional Factorial Design

Figure 4-5 illustrates the main effects that changing the particle parameters has on the cell count for samples challenged with particles beginning on Day 1. On Day 4 there was no significant effect due to changing the copolymer ratio from PLGA 50/50 to PLLA, due to changing the molecular weight from the low to the high value, or due to changing the concentration from the low to the high value because the standard error of effect was greater than each of the individual main effects. However, on Day 7, 14, 21, and 28, changing the concentration of particles exposed to the cells from the low to high value significantly decreases the cell count by the amount indicated on each graph, respectively. On Days 14 and 21, changing the copolymer ratio from PLGA 50/50 to PLLA has an increasing effect on cell count. For the samples challenged with particles beginning on Day 14, there is no significant effect of changing the particle parameters on either Day 21 or Day 28 (Figure 4-6).

4.3.2 Thymidine Incorporation

4.3.2.1 Absolute Values
Figure 4-5. The main effect of three particle parameters on the cell count for osteoblasts challenged with particles beginning on Day 1. A positive number indicates that the particular parameter had an increasing effect on the cell number as it went from a low level to a high level. A negative number indicates a decrease in the cell number as it went from a low level to a high level. Error bars stand for the standard error of effect.
Figure 4-6. The main effect of three particle parameters on the cell count for osteoblasts challenged with particles beginning on Day 14. A positive number indicates that the particular parameter had an increasing effect on the cell number as it went from a low level to a high level. A negative number indicates a decrease in the cell number as it went from a low level to a high level. Error bars stand for the standard error of effect.
The thymidine incorporation in counts per minute per cell for samples in which particles were added on Day 1 is illustrated in Figure 4-7. Significantly more thymidine is incorporated into each sample on Day 1 than on Days 4, 7, 14, 21, or 28 (p=0.05). For each time point, the samples without particles (control) were not significantly different from samples challenged with low concentrations of particles (P2 and PE[low]) (p=0.05). Thymidine incorporation for those samples exposed to high concentrations of particles made of different materials (P1, P4, and PE[high]) were not different either with one exception (p=0.05). On Day 1, the thymidine incorporation for samples exposed to the high concentration of low Mₘₚ PLGA 50/50 particles (P4) was significantly lower than that of samples exposed to the high concentration of PE particles (PE[high]).

Thymidine incorporation on Days 21 and 28 for all samples challenged with particles beginning on Day 14 is illustrated in Figure 4-8. On both Days 21 and 28, the samples without particles (control) were not significantly different from those with low concentration of particles (P2 and PE[low]) (p=0.05). Thymidine incorporation for those samples in which particles of different materials (P1, P4, and PE[high]) were exposed in high concentrations to the osteoblasts were not statistically different from each other (p=0.05). There is not a significant difference in thymidine incorporation between those samples to which particles were added on Day 1 and those to which particles were added on Day 14 (p=0.05).
Figure 4-7. Thymidine incorporation of osteoblasts cultured with particles beginning on Day 1, expressed as counts per minute per cell. Thymidine incorporation is an indicator of cellular proliferation. The osteoblasts were exposed to no particles (control) as well as a variety of degradable (P1-P4) and non-degradable (PE[low] and PE[high]) particle types. Error bars stand for means ± standard deviation for n=3.
Figure 4-8. Thymidine incorporation of osteoblasts cultured with particles beginning on Day 14, expressed as counts per minute per cell. Thymidine incorporation is an indicator of cellular proliferation. The osteoblasts were exposed to no particles (control) as well as a variety of degradable (P1-P4) and non-degradable (PE[low] and PE[high]) particle types. Error bars stand for means ± standard deviation for n=3.
4.3.2.2 Factorial Design

On Day 1 there is a significant decrease in thymidine incorporation as the concentration of particles exposed to the cells changed from the low to the high value (Figure 4-9). On Day 4, a significant decrease in thymidine incorporation was detected as the copolymer ratio of the particles was changed from PLGA 50/50 to PLLA. Day 7 showed the same change as Day 4. No significant effects due to particle parameters were apparent on Day 14. On Day 21 there was an increase in thymidine incorporation as the particle type was changed from the low molecular weight to the high molecular weight. Day 28 showed the same results as Day 21.

For the samples in which particles were not exposed to the cells until Day 14, there were no significant effects on thymidine incorporation due to changing the particle parameters on Day 21 (Figure 4-10). On Day 28, an increase in thymidine incorporation was apparent as the concentration of particles was changed from the low to the high value.

4.3.3 Alkaline Phosphatase Activity

4.3.3.1 Absolute Values
Figure 4-9. The main effect of three particle parameters on the thymidine incorporation for osteoblasts challenged with particles beginning on Day 1. Thymidine incorporation is an indicator of cellular proliferation. A positive number indicates that the particular parameter had an increasing effect on the thymidine incorporation as it went from a low level to a high level. A negative number indicates a decrease in the thymidine incorporation as it went from a low level to a high level. Error bars stand for the standard error of effect.
Figure 4-10. The main effect of three particle parameters on the thymidine incorporation for osteoblasts challenged with particles beginning on Day 14. Thymidine incorporation is an indicator of cellular proliferation. A positive number indicates that the particular parameter had an increasing effect on the thymidine incorporation as it went from a low level to a high level. A negative number indicates a decrease in the thymidine incorporation as it went from a low level to a high level. Error bars stand for the standard error of effect.
ALPase activity expressed as \( \mu \text{mole/min/cell} \) increased over time for all samples in which cells were challenged with particles beginning on Day 1 as shown in Figure 4-11. On days 4, 7, 14, and 21, the ALPase activity of samples without particles (control) was not significantly different from those challenged with low concentrations of particles (P2 and PE[low]) \((p=0.05)\). By day 28, however, the ALPase activity of the control samples (no particles) was significantly higher than those of samples exposed to the low concentrations of particles (P2 and PE[low]) \((p=0.05)\). ALPase activity for those samples exposed to high concentrations of particles of different materials (P1, P4, and PE[high]) were not statistically significant either with the exception of one time point \((p=0.05)\). On Day 21, the ALPase activity of samples exposed to the high concentration of particles made with both PLLA (P1) and PLGA 50/50 (P4) was significantly lower than that of samples exposed to the high concentration of PE particles (PE[high]) \((p=0.05)\).

Figure 4-12 illustrates the ALPase activity for Days 21 and 28 for all samples in which the particles were exposed to the cells beginning on Day 14. For both time points, the ALPase activity of samples without particles (control) was not significantly different from those with low concentration of particles (P2 and PE[low]) \((p=0.05)\). ALPase activity for those samples in which particles of different materials (P1, P4, and PE[high]) were exposed to the cells in high concentrations were not statistically significant either \((p=0.05)\). There is not a significant difference in ALPase activity between those samples to which particles were added on Day 1 and those to which particles were added on Day 14 \((p=0.05)\).
Figure 4-11. Alkaline phosphatase activity of osteoblasts cultured with particles beginning on Day 1, expressed as μmole/min/cell (x10^7). The osteoblasts were exposed to no particles (control) as well as a variety of degradable (P1-P4) and non-degradable (PE[low] and PE[high]) particle types. Error bars stand for means ± standard deviation for n=3.
Figure 4-12. Alkaline phosphatase activity of osteoblasts cultured with particles beginning on Day 14, expressed as μmole/min/cell (x10^7). The osteoblasts were exposed to no particles (control) as well as a variety of degradable (P1-P4) and non-degradable (PE[low] and PE[high]) particle types. Error bars stand for means ± standard deviation for n=3.
4.3.3.2 Factorial Design

On Day 4 there is a significant decrease in ALPase activity as the copolymer ratio of the particles is changed from PLGA 50/50 to PLLA as shown in Figure 4-13. Day 7 shows an increase in ALPase activity as the concentration of particles is changed from the low to the high value. Day 14 shows the opposite effect, a significant decrease in ALPase activity due to a change from the low concentration to the high concentration of particles. In addition, on Day 14, there is a significant increase in ALPase activity as the molecular weight of the particles exposed to cells is changed from the low to the high level. On Day 21 there is also a decrease in alkaline phosphatase activity due to the change in concentration of the particles from the low to the high value.

Figure 4-14 illustrates the main effects on ALPase activity of changing the particle parameters on those samples to which the cells were not challenged with particles until Day 14. There were no significant effects on Day 21 due to changing the particle parameters. On Day 28, however, a significant decrease was detected in ALPase activity as the concentration of particles was changed from the low to the high value.

4.3.5 Mineralization

4.3.5.1 Absolute Values
Figure 4-13. The main effect of three particle parameters on the alkaline phosphatase activity for osteoblasts challenged with particles beginning on Day 1. A positive number indicates that the particular parameter had an increasing effect on the alkaline phosphatase activity as it went from a low level to a high level. A negative number indicates a decrease in the alkaline phosphatase activity as it went from a low level to a high level. Error bars stand for the standard error of effect.
Figure 4-14. The main effect of three particle parameters on the alkaline phosphatase activity for osteoblasts challenged with particles beginning on Day 14. A positive number indicates that the particular parameter had an increasing effect on the alkaline phosphatase activity as it went from a low level to a high level. A negative number indicates a decrease in the alkaline phosphatase activity as it went from a low level to a high level. Error bars stand for the standard error of effect.
The percent mineralization of all samples to which particles were exposed beginning on Day 1 is shown in Figure 4-15. The mineralization increases over time for all samples. The percent mineralization of samples on Day 28 is significantly higher than samples on Day 14 (p=0.05). For each time point, the percent mineralization of samples without particles (control) was not different from those exposed to low concentrations of particles (P2 and PE[low]) (p=0.05). Percent mineralization of those samples exposed to high concentrations of particles made of different materials (P1, P4, and PE[high]) were not significantly different either (p=0.05).

Figure 4-16 illustrates the percent mineralization for Days 21 and 28 for all samples in which the particles were exposed to the cells beginning on Day 14. For both time points, the percent mineralization of samples without particles (control) was not different from those exposed to low concentrations of particles (P2 and PE[low]) (p=0.05). Percent mineralization for those samples exposed to high concentrations of particles made of different materials (P1, P4, and PE[high]) was not different either (p=0.05).

On Day 21, samples to which a high concentration of PE particles were added beginning on Day 1 had significantly less mineralization than those samples to which a high concentration of PE particles was added beginning on Day 14 (p=0.05). On Day 28, all of the samples to which a high concentration of particles was added (P1, P4, and PE[high]) beginning on Day 1 were also significantly less mineralized than those to which the same particle types were added beginning on Day 14 (p=0.05) (Figure 4-17).
Figure 4-15. Mineralization of osteoblasts cultured with particles beginning on Day 1, expressed as a percentage of entire culture area. The osteoblasts were exposed to no particles (control) as well as a variety of degradable (P1-P4) and non-degradable (PE[low] and PE[high]) particle types. Error bars stand for means ± standard deviation for n=3.
Figure 4-16. Mineralization of osteoblasts cultured with particles beginning on Day 14, expressed as a percentage of entire culture area. The osteoblasts were exposed to no particles (control) as well as a variety of degradable (P1-P4) and non-degradable (PE[low] and PE[high]) particle types. Error bars stand for means ± standard deviation for n=3.
Figure 4-17. Digitized images of cells stained with a Von Kossa stain that demonstrates mineralized tissue. Cells were exposed to a high concentration of high molecular weight PLLA particles (P1) beginning on (a) Day 1 and (b) Day 14.
The change in percent mineralization of the samples due to varying the particle parameters of those added to samples beginning on Day 1 is illustrated in Figure 4-18. There is a significant decrease in mineralization due to changing from the low concentration to the high concentration of particles on Days 14, 21, and 28.

For those samples to which particles were exposed beginning on Day 14, the main effects of changing particle parameters from the low level to the high level is illustrated in Figure 4-19. There is a significant increase in mineralization on Day 21 due to changing the copolymer ratio from PLGA 50/50 to PLLA. Day 28 shows no significant effects on mineralization due to changing the particle parameters.
Figure 4-18. The main effect of three particle parameters on the mineralization for osteoblasts challenged with particles beginning on Day 14. A positive number indicates that the particular parameter had an increasing effect on the mineralization as it went from a low level to a high level. A negative number indicates a decrease in the mineralization as it went from a low level to a high level. Error bars stand for the standard error of effect.
Figure 4-19. The main effect of three particle parameters on the mineralization for osteoblasts challenged with particles beginning on Day 16. A positive number indicates that the particular parameter had an increasing effect on the mineralization as it went from a low level to a high level. A negative number indicates a decrease in the mineralization as it went from a low level to a high level. Error bars stand for the standard error of effect.
4.4 DISCUSSION

The effects of biodegradable polymer particles on osteoblasts were determined by exposing cells to four different types of biodegradable polymer particles over a period of 28 days. The particles were made of PLGA with varying combinations of copolymer ratio, molecular weight, and concentrations in order to ascertain the main effect that each of these parameters has on osteoblast cell number, thymidine incorporation, alkaline phosphatase activity, and mineralization. In addition, osteoblasts were challenged with both high and low concentrations of non-degradable poly(ethylene) particles. Cells were also challenged with particles at different stages of development (particles added beginning on Day 1 vs. Day 14).

4.4.1 Cell Count

The cell count for all samples increased over the first four days and then proliferation markedly slowed, making the cell count level off over the duration of the study. This pattern of cell proliferation on tissue culture poly(styrene) has been documented previously [Ishaug et al., 1994]. The fractional factorial design indicates that there is a decreasing effect on the cell count as the concentration of particles increases. This is observed from Day 7 onward for those samples to which particles were exposed beginning on Day 1. Increasing the concentration of particles, however, does not effect those samples to which particles were exposed beginning on Day 14. The particles must therefore
interfere with the osteoblast proliferation which has been documented to occur in the first few days after plating the cells [Ishaug et al., 1994].

4.4.2 Thymidine Incorporation

Thymidine incorporation was greatest on Day 1 when the osteoblasts were proliferating. When proliferation slowed (by Day 4), the thymidine incorporation dropped dramatically. The effects due to the particles that are meaningful to interpret occur on Day 1 when the osteoblasts are actively incorporating thymidine. There was a decrease in thymidine incorporation that occurred due to the increase in particle concentration. This supports the data from the DNA assay where a decrease in cell number occurred due to increasing the concentration of the particles.

4.4.3 Alkaline Phosphatase Activity

ALPase activity of all samples increased over time. This type of enzyme activity has also been documented in previous studies of osteoblasts plated on TCPS [Ishaug et al., 1994], as well as PLGA films [Ishaug et al., 1994] and foams [Ishaug-Riley et al., in press]. It was not until Day 28 that the samples that were not challenged with particles had significantly more ALPase activity than those challenged with particles (P2 and PE[low]). It is reasonable to expect that the effects of the addition of particles would not manifest themselves until later
time points when alkaline phosphatase is being actively secreted [Lian and Stein, 1992]. By Day 28, the alkaline phosphatase is nearly 10-fold that of Day 4 and the cells have increased the synthesis of alkaline phosphatase enough that the effects due solely to addition of particles determined using ANOVA can be detected.

The fractional factorial design allows the trends effecting ALPase activity due to changing particle concentration from the low to the high value to be isolated. The effect changes from increasing ALP activity to decreasing ALP activity over the duration of the study. On Day 7, increasing the concentration of particles has an increasing effect on ALPase activity. This effect is likely due to the fact that the cells are still in the proliferative phase [Lian and Stein, 1992]. On Day 7, as mentioned in the results regarding cell number, the increase in concentration of particles directly causes a decrease in cell number. Because of the reciprocal relationship between osteoblast proliferation and differentiation (refer to Figure 1-6), the increase in alkaline phosphatase is likely a consequence of the decrease in cell number.

By Day 14 and 21, however, the osteoblasts are firmly in the matrix development and maturation phase which is marked by the increased expression of alkaline phosphatase. The effects of particles are directly reflected in alkaline phosphatase activity of the samples. Increasing the concentration of particles decreases osteoblast production of alkaline phosphatase. Supporting evidence of the effect of increasing concentration of particles exposed to osteoblasts is the measurable decrease in ALPase activity on Day 28 of those samples to which particles were exposed beginning on Day 14.
There was also a statistically significant difference on Day 21 between samples exposed to high concentrations of particles made of differing materials. Those made with biodegradable particles (P1 and P4) had significantly less ALPase activity than those made with non-degradable PE particles (PE[high]). This was an isolated incident, however, that did not occur at any other time point in either study.

4.4.4 Mineralization

Two factors effected most the extent of mineralization. The first factor was when the particles were exposed to the osteoblasts (beginning on Day 1 vs. Day 14). By Day 28, those samples challenged with a high concentration of particles beginning on Day 1 had significantly less mineralization than those challenged only after Day 14. Evidence of this phenomenon was also seen as early as Day 21 in samples challenged with a high concentration of PE particles. The second factor that effected the mineralization was the concentration of the particles. To those samples exposed to the particles beginning on Day 1, there was a significant decrease in mineralization with increasing concentration of particles. This trend was not observed, however, with those samples that were not exposed to particles until Day 14. The preparation and process by which osteoblasts mineralize their surrounding matrix is complex, involving the secretion and maintenance of many factors and enzymes [Lian and Stein, 1992]. Reduced mineralization is likely due to the culmination of effects of particles hindering osteoblast growth and production of several of these proteins throughout the course of their development.
This study is the first to examine the effects of biodegradable PLGA particles of a variety of copolymer ratios, molecular weights, and concentrations on osteoblasts in vitro. By controlling particle parameters determined by a fractional factorial design, we were able to identify trends which may help us determine what materials are best to use as scaffolds for the tissue engineering of bone. Though no significant trends effecting osteoblast proliferation or phenotype expression were detected due to changing the copolymer ratio or molecular weight, it was apparent that increasing the concentration of the particles decreased cell count, thymidine incorporation, alkaline phosphatase activity, and mineralization at various time points throughout the study.

Many studies documenting the inflammatory effects of particles in the body are in the literature [Adamson and Bowden, 1990; Amstutz et al., 1991; Frondoza et al., 1993; Glant and Jacobs, 1994; Pazzaglia et al., 1986; Shanbhag et al., 1994]. A majority of them focus on the interaction of the foreign particles with macrophages. This study was designed to examine some of the particle parameters that have proven to be important in particle/macrophage interactions such as concentration and the material of which the particles are made. Other parameters not examined in this study that might be important are particle size and shape.

In previous studies, the concentration of particles has also been shown to be a key factor effecting macrophage viability and secretion of bone resorbing factors such as IL-1, PGE\(_2\), and IL-6 [Amstutz et al., 1991; Shanbhag et al., 1994]. Disagreement persists about the mechanism by which the particles effect the macrophages but several studies suggest that the cascade of events resulting in the secretion of bone resorbing factors begins with the phagocytosis
of the particles by macrophages. Other studies have been done examining the
effects of particles on other cells in the local environment such as fibroblasts
and lymphocytes [F尔ndoz'a et al., 1993]. It is also known that osteoclasts
readily phagocytose particles and debris in addition to their bone resorbing
capabilities, but is still under debate as to whether the osteoblast has this
capability [Saad et al., 1996].

The mechanism by which the particles exposed to the osteoblasts in this
study hinder the proliferation, alkaline phosshpatase activity, or mineralization of
the osteoblasts is not known at this time. Though increasing the concentration
of particles magnifies these effects, we have no evidence of osteoblast damage
due to phagocytosis or hypoxia because of particles blocking oxygen diffusion.
There was also no evidence of a pH drop due to particle degradation that would
prevent the cells from functioning normally. It could be that there is simply a
threshold amount of foreign material that can coexist with healthy cells without
adversely effecting their growth and function.
4.4 CONCLUSIONS

Having isolated the trend that increasing particle concentration decreases cell count, thymidine incorporation, alkaline phosphatase, and mineralization of osteoblasts, further studies examining the effects of particle concentration are needed. A more expanded investigation of the mechanism by which osteoblasts respond to particles is recommended in future work. Suggestions include the examination of the effects of particles on other proteins and phenotypic markers of osteoblasts such as osteocalcin or osteopontin. It is also possible that the particles might induce the osteoblasts to secrete bone resorbing mediators such as PGE$_2$ which would stimulate osteoclasts in a physiologic environment [Hughes-Fulford and Lewis, 1996]. Determination of the phagocytic capabilities of osteoblasts is important. This is because, in addition to the particles ability to induce the release of inflammatory mediators in the physiologic environment, the toxicity of the particle material itself must not be overlooked. Toxicity of the particle depends on its interactions with the osteoblast, its size, its solubility in the body's extra-cellular as well as intra-cellular fluids, and its inherent adverse effects on cellular functions [Amstutz et al., 1991].

To practically apply knowledge of the effects of particles on osteoblasts we must also closely examine the degradation processes of the biodegradable scaffolds in vivo, focusing on those environments prone to particle formation. Questions to be answered include: "How many particles are formed over the course of the degradation process?", "What is their size distribution and shape?", "What is their molecular weight upon formation?". When these
questions are answered, a better designed scaffold for bone regeneration, meeting both the short and long-term design criteria, will be fabricated.
REFERENCES


