RICE UNIVERSITY

MODULATION OF CELL MORPHOLOGY AND FUNCTION USING SYNTHETIC BIODEGRADABLE POLYMERS

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

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ABSTRACT

MODULATION OF CELL MORPHOLOGY AND FUNCTION USING SYNTHETIC BIODEGRADABLE POLYMERS

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Lichun Lu

Synthetic biodegradable poly(DL-lactic-co-glycolic acid) (PLGA) has been fabricated into thin films for use as scaffolds for cell transplantation and guided tissue regeneration. We evaluated the ability of PLGA films to provide a suitable template for directing specific cell growth and tissue formation using cells derived from retinal pigment epithelium (RPE) as an in vitro model. RPE cells were shown to attach to and proliferate on PLGA films during a 7-day culture period. Both polymer composition and initial cell seeding density affected cell growth.

PLGA films were formulated with copolymer ratios of 50:50 and 75:25 and thickness levels of 10 and 100 μm. In vitro degradation of these thin films in simulated body fluid was characterized in terms of mass loss, molecular weight loss, dimensional changes, and morphology over a time course of ten weeks. The PLGA films demonstrated a pattern typical of heterogeneous bulk degradation and exhibited an autocatalytic effect.

To further modulate cellular responses, biodegradable polymers of PLGA were investigated for controlled and sustained delivery of bioactive molecules. Transforming growth factor-β1 (TGF-β1) was incorporated into microparticles of blends of PLGA and poly(ethylene glycol) (PEG) as a model growth factor and released in vitro in buffer solutions simulating body fluids for up to 28 days. The
release of TGF-β1 was affected by the PEG content and buffer pH. Increasing the PEG content or decreasing buffer pH resulted in accelerated PLGA degradation. The release of bioactive molecules from PLGA/PEG microparticles was governed by both diffusion and polymer degradation.

The feasibility of controlling cell morphology and function by modification of substrate surface with defined hydrophobic and hydrophilic domains was assessed. We described the preparation and characterization of model surfaces consisting of glass domains with a diameter of 10 or 50 μm surrounded by octadecyltrichlorosilane self-assembled monolayers. The micropatterned surfaces were shown to affect initial RPE cell attachment and spreading, and modulate the expression of differentiated cell phenotype. A novel biodegradable polymer substrate was developed with micropatterned surfaces composed of PLGA and a block copolymer of PEG and poly(DL-lactic acid) (PDLLA). We demonstrated that engineering of surface chemistry and microstructure of substrates could induce proper cell shape and function.
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<tr>
<td>3-D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>3DP</td>
<td>Three-dimensional printing</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ARMD</td>
<td>Age-related macular degeneration</td>
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<tr>
<td>ASTM</td>
<td>American Standards of Testing Materials</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Computer-aided design</td>
</tr>
<tr>
<td>CAM</td>
<td>Computer-aided manufacturing</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Distilled deionized water</td>
</tr>
<tr>
<td>DETA</td>
<td>(N',3-(\text{Trimethoxysilyl})\text{propyl})diethylenetriamine</td>
</tr>
<tr>
<td>Dil</td>
<td>1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagles medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycol-(b)-aminoethyl ether) (N,N,N',N')-tetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESCA</td>
<td>Electron spectroscopy for chemical analysis</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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FTIR  Fourier transform infrared
FTR  Fourier transform Raman
GF  Gas foaming
GPC  Gel permeation chromatography
GTR  Guided tissue regeneration
HEPES  N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid
IgG  Immunoglobulin G
IPA  Isopropyl alcohol
IPS  Interphotoreceptor space
μCP  Microcontact printing
Mn  Number average molecular weight
Mw  Weight average molecular weight
N.B.  Neutral-buffered
NEXAFS  Near edge X-ray absorption fine structure
NMR  Nuclear magnetic resonance
OTS  Octadecyltrichlorosilane
PBS  Phosphate buffered saline
PCL  Poly(ε-caprolactone)
PDLLA  Poly(DL-lactic acid)
PDMS  Poly(dimethylsiloxane)
PEG  Poly(ethylene glycol)
PET  polyvinylpyrrolidone/EGTA/trypsin
PGA  Poly(glycolic acid)
PI  Polydispersity index
PL  Particulate-leaching
PLA  Poly(lactic acid)
PLGA  Poly(DL-lactic-co-glycolic acid)
PPF   Poly(propylene fumarate)
PSN   Penicillin/streptomycin/neomycin
PVA   Poly(vinyl alcohol)
RCS   Royal College of Surgeons
RGD   Arginine-lysine-aspartic acid
ROS   Rod outer segment
RPE   Retinal pigment epithelium
RWV   Rotating-wall vessel
SAM   Self-assembled monolayer
SC    Solvent-casting
SD    Standard deviation
SEM   Scanning electron microscopy
SFF   Solid freeform fabrication
SIMS  Static secondary ion mass spectrometry
SLS   Selective laser sintering
SPM   Scanning probe microscopy
TBS   Tris-buffered saline
TCPS  Tissue culture polystyrene
Tg    Glass transition temperature
TGF-β1 Transforming growth factor-β1
Tm    melting temperature
UV    Ultraviolet
Xc    Crystallinity
ZO-1  Zonula occluden-1
CHAPTER 1

INTRODUCTION

1.1 Tissue Engineering

Tissue engineering involves the development of functional substitutes to replace missing or malfunctioning human tissues and organs [Langer and Vacanti, 1993]. Most strategies in tissue engineering have focused on using biomaterials as scaffolds to direct specific cell types to organize into three-dimensional (3-D) structures and perform differentiated function of the targeted tissue. Synthetic bioresorbable polymers that are fully degradable into the body's natural metabolites by simple hydrolysis under physiological conditions are the most attractive scaffold materials. These scaffolds offer the possibility to create completely natural tissue or organ equivalents and thus overcome the problems such as infection and fibrous tissue formation associated with permanent implants. The bioresorbable polymers are used as porous scaffolds in tissue engineering in the following ways (Figure 1-1) [Lu and Mikos, 1999a].

1.1.1 Tissue Induction

Tissue induction is the process by which ingrowth of surrounding tissue into a porous scaffold is effected (Figure 1-1a). The scaffold provides a substrate for the migration and proliferation of the desired cell types. For example an osteoinductive material can be used to selectively induce bone formation. This approach has been employed to regenerate several other tissues including skin and nerve.
1.1.2 Cell Transplantation

The concept is that cells obtained from patients can be expanded in culture, seeded onto an appropriate polymer scaffold, cultured and then transplanted (Figure 1-1b). The time at which transplantation takes place varies with a specific application. Usually the cells are allowed to attach to the scaffold, proliferate and differentiate before implantation. A scaffold for bone cell transplantation should be osteoconductive meaning that it has the capacity to direct the growth of osteoblasts in vitro and allow the integration of the transplant with the host bone. This strategy is the most widely used in tissue engineering and has been investigated for the transplantation of many cell types including osteoblasts, chondrocytes, hepatocytes, fibroblasts, smooth muscle cells, and retinal pigment epithelium (RPE).

1.1.3 Prevascularization

The major obstacle in the development of large 3-D transplants such as liver is nutrient diffusion limitation, because cells will not survive farther than a few hundred microns from the nutrient supply. Although the scaffold can be vascularized post-implantation, the rate of vascularization is usually insufficient to prevent cell death inside the scaffold. In this case, prevascularization of the scaffold may be necessary to allow the ingrowth of fibrovascular tissue or uncommitted vascular tissue such as periosteum (layer of connective tissue covering bone) before cell seeding by injection (Figure 1-1c). The prevascularized scaffold will provide a substrate for cell attachment, growth, and function. The extent of prevascularization has to be optimized to allow sufficient nutrient diffusion as well as enough space for cell seeding and tissue growth [Mikos et al., 1993c].
Some complex osseous defects created by bone tumor removal or extensive tissue damage exceed critical size for normal healing and require a large transplant to restore function. A novel strategy is to prefabricate vascularized bone flap by implanting a mold containing bioresorbable polymers with osteoinductive elements onto a periosteal site remote from the defect where prevascularization and ectopic bone formation can occur over a period of time as the scaffold degrades [Thomson et al., 1999]. The created autologous bone can then be transplanted to the defect site where vascular supply can be attached via microsurgery to existing vessels.

1.1.4 In-situ Polymerization

Injectable, in situ polymerizable, bioresorbable materials can be utilized to fill defects of any size and shape with minimal surgical intervention (Figure 1-1d). For instance, poly(propylene fumarate) (PPF) has been developed as an injectable bone cement which hardens within 10 to 15 min under physiological conditions [Peter et al., 1997a]. These materials do not require prefabrication but must meet additional requirements since polymerization or cross-linking reactions occur in vivo. All reagents and products must be biocompatible, and the reaction conditions such as temperature, pH, and heat release should not damage implanted cells or the surrounding tissue.

The hardened material (scaffold) must be highly porous and have interconnected pore structure in order to serve as a suitable template for guiding cell growth and differentiation. This can be achieved by combining a porogen such as sodium chloride crystals in the injectable paste which are eventually leached out leaving a porous polymer matrix. Since the leaching step occurs in vivo, local high salt concentration may lead to high osmolarity
and tissue damage. The amount of porogen incorporated has to be optimized to ensure biocompatibility while enough porosity needs to be achieved to allow sufficient nutrient diffusion and vascularization.

1.1.5 Delivery of Bioactive Molecules

Cellular activities can be further modulated by various soluble bioactive molecules such as cytokines, growth factors, hormones, angiogenic factors, or immunosuppressant drugs. For instance bone morphogenetic proteins (BMPs) have been identified as a family of growth factors that regulate differentiation of bone cells [Ripamonti and Duneas, 1996]. Controlled local delivery of these tissue inductive factors to transplanted and regenerated cells is often desirable. This has led to the concept of incorporation of bioactive molecules into scaffolds for implantation. These factors can be bound into polymer matrix during scaffold processing. Alternatively, synthetic biodegradable microparticles or nanoparticles loaded with these molecules can be impregnated into the substrates. By incorporating BMPs into the injectable paste, PPF can also serve as a delivery vehicle for bone growth factors to induce a bone regeneration cascade. The release of bioactive molecules in vivo is governed by both diffusion and polymer degradation.

1.2 Scaffold Design Criteria

The synthetic polymers must possess unique properties specific to the tissue of interest as well as satisfy some basic requirements in order to serve as an appropriate scaffold. One essential criterion is biocompatibility, i.e. the polymer scaffold should not invoke an adverse inflammatory or immune
response once implanted [Babensee et al., 1998]. Some important factors that
determine its biocompatibility, such as the chemistry, structure, and morphology,
can be affected by polymer synthesis, scaffold processing, and sterilization.
Toxic residual chemicals involved in these processes (e.g. monomers,
stabilizers, initiators, cross-linking agents, emulsifiers, organic solvents) may be
leached out from the scaffold with detrimental effects to the engineered and
surrounding tissue.

The primary role of a scaffold is to provide a temporary substrate to which
transplanted cells can adhere. Most organ cell types are anchorage-dependent
and require the presence of a suitable substrate in order to survive and retain
their ability to proliferate, migrate, and differentiate. Cell morphology correlates
with cellular activities and function; strong cell adhesion and spreading often
favor proliferation while a rounded cell shape is required for cell-specific
function. For example, it has been demonstrated that the use of substrates with
patterned surface morphologies or varied extracellular matrix (ECM) surface
coatings can modulate cell shape and function [Chen et al., 1998; Mooney et
al., 1992; Singhvi et al., 1994a].

For epithelial cells, cell polarity is essential for their function. Polarity
refers to the distinctive arrangement, composition and function of cell surface
and intracellular domains. This typically corresponds to a heterogeneous
extracellular environment. For example, RPE cells have three major surface
domains: the apical surface is covered with numerous microvilli; the lateral
surface is joined with neighboring cells by junctional complexes; the basal
surface is convoluted into basal infoldings and connected to the basal lamina.
The polymer scaffold for RPE transplantation should therefore provide proper
surface chemistry and surface microstructure for optimal cell-substrate
interaction, and along with appropriate culture conditions, be able to induce proper cell polarity [Lu et al., 1998].

Besides cell morphology, the function of many organs is dependent on the 3-D spatial relationship of cells with their ECM. The shape of a skeletal tissue is also critical to its function. Gene expression in cells is regulated differently by two-dimensional (2-D) vs. 3-D culture substrates. For instance the differentiated phenotype of human epiphyseal chondrocytes is lost on 2-D culture substrates but re-expressed when cultured in 3-D agarose gels [Aulthouse et al., 1989]. A polymer scaffold should be easily and reproducibly processed into a desired shape that can be maintained after implantation so that it defines the ultimate shape of the regenerated tissue. A suitable scaffold should therefore act as a template to direct cell growth and ECM formation and facilitate the development of a 3-D structure.

Porosity, pore size, and pore structure are important factors to be considered with respect to nutrient supply to transplanted and regenerated cells. To regenerate highly vascularized organs such as liver, porous scaffolds with large void volume and large surface area to volume ratio are desirable for maximal cell seeding, attachment, growth, ECM production and vascularization. Small diameter pores are preferable to yield high surface area per volume provided the pore size is greater than the diameter of a cell in suspension (typically 10 μm). However, topological constrains may require larger pores for cell growth. Previous experiments have demonstrated optimal pore sizes of 20 μm for fibroblast ingrowth, 20-125 μm for adult mammalian skin regeneration, and 200-400 μm for bone ingrowth [Boyan et al., 1996; Whang et al., 1995]. The rate of tissue invasion into porous scaffold also depends on the pore size and polymer crystallinity [Mikos et al., 1993c; Park and Cima, 1996; Wake et al.,
1994]. Compared to isolated pore structure, interconnected pore network enhances the diffusion rates to and from the center of the scaffold and facilitates vascularization, thus improving oxygen and nutrient supply and waste removal. The vascularization of an implant is a prerequisite for regeneration of most 3-D tissues except for cartilage which is avascular.

Mechanical properties of the polymer scaffold should be similar to the tissue or organ intended for regeneration. For load bearing tissues such as bone, the scaffold should be strong enough to withstand physiological stresses to avoid collapse of the developing tissue. Also transfer of load to the scaffold (stress shielding) after implantation may result in lack of sufficient mechanical stimulation to the ingrowing tissue. For the regeneration of soft tissues such as skin, the scaffolds are required to be pliable or elastic. The stiffness of the scaffold may affect the mechanical tension generated within the cell cytoskeleton which is critical for the control of cell shape and function [Chicurel et al., 1998]. A more rigid surface may facilitate the assembly of stress fibers and enhance cell spreading and dividing. Scaffold compliance may also affect cell-cell contacts and aggregation [Moghe, 1996].

Understanding and controlling the degradation process of a scaffold and the effects of its degradation products on the body is crucial for long-term success of a tissue engineered cell-polymer construct. The local drop in pH due to the release of acidic degradation products from some implants may cause tissue necrosis or inflammation. Polymer particles formed after long-term implantation of a scaffold or due to micromotion at the implantation site may elicit an inflammatory response. Microparticles of polymers have been shown to suppress initial rat marrow stromal osteoblast proliferation in culture [Wake et al., 1998]. The mechanism by which the scaffold degrades should also be
considered. For example the degradation products are released gradually by surface erosion. While during bulk degradation, the release of degradation products occurs only when the molecular weight of the polymer reaches a critical value. This late-stage burst effect may cause greater local pH drop.

The rate of scaffold degradation is tailored to allow cells to proliferate and secrete their own ECM while the polymer scaffold vanishes over a desired time period (from days to months) to leave enough space for new tissue growth. Since the mechanical strength of a scaffold usually decreases with degradation time, the degradation rate may be required to match the rate of tissue regeneration in order to maintain the structural integrity of the implant. The degradation rate of a scaffold can be affected by various factors listed in Table 1-1.

The design requirements of a tissue engineering scaffold are specific to the structure and function of the tissue to be regenerated. The polymer scaffold is typically engineered to mimic the natural ECM of the body. ECM proteins play crucial roles in the control of cell growth and function [Hay, 1993; Howe et al., 1998]. However, most synthetic polymer scaffolds do not possess the specific signals (ligand) that can be recognized by cell-surface receptors. It is therefore preferable that the polymer chain has chemically modifiable functional groups onto which sugars, proteins, or peptides can be attached. In addition, polymer-peptide hybrid molecules may be created or the ligand may be immobilized on the scaffold surface to generate biomimetic micro-environment [Shakesheff et al., 1998].

1.3 Synthetic Bioresorbable Polymers
Table 1-1. Factors affecting scaffold degradation.

<table>
<thead>
<tr>
<th>Polymer Chemistry</th>
<th>Scaffold Structure</th>
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<tbody>
<tr>
<td>Composition</td>
<td>Density</td>
</tr>
<tr>
<td>Structure</td>
<td>Shape</td>
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<tr>
<td>Configuration</td>
<td>Size</td>
</tr>
<tr>
<td>Morphology</td>
<td>Mass</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>Surface texture</td>
</tr>
<tr>
<td>Molecular weight distribution</td>
<td>Porosity</td>
</tr>
<tr>
<td>Chain motility</td>
<td>Pore size</td>
</tr>
<tr>
<td>Molecular orientation</td>
<td>Pore structure</td>
</tr>
<tr>
<td>Surface to volume ratio</td>
<td>Wettability</td>
</tr>
<tr>
<td>Ionic groups</td>
<td>Processing method and conditions</td>
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<tr>
<td>Impurities or additives</td>
<td>Sterilization</td>
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*In Vitro*  |  *In Vivo*  |
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<tr>
<td>Degradative medium</td>
<td>Implantation site</td>
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<tr>
<td>pH</td>
<td>Access to vasculature</td>
</tr>
<tr>
<td>Ionic strength</td>
<td>Mechanical loading</td>
</tr>
<tr>
<td>Temperature</td>
<td>Tissue growth</td>
</tr>
<tr>
<td>Mechanical loading</td>
<td>Metabolism of degradation products</td>
</tr>
<tr>
<td>Type and density of cultured cells</td>
<td>Enzyme</td>
</tr>
</tbody>
</table>
The range of physical, chemical, mechanical, and degradative properties which may be achieved using synthetic biodegradable polymers render them extremely versatile as scaffold materials. Their molecular weight and chemical composition may be precisely controlled during polymer synthesis. Copolymers, polymer blends and composites with other materials may be manufactured to give rise to properties that are advantageous over homopolymers for certain applications. Moreover, many polymers can be functionalized by converting end groups or addition of side chains with various chemical groups to obtain polymers that can be self cross-linked or cross-linked with proteins and other bioactive molecules [Behravesh et al., 1999]. By choosing an appropriate processing technique, scaffolds of specific architecture and structural characteristics may be fabricated.

Not all types of currently available synthetic biodegradable polymers can be manufactured into 3-D scaffolds due to their chemical and physical properties and processability (Table 1-2). The most widely utilized scaffold materials are poly(α-hydroxy esters) such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymer poly(DL-lactic-co-glycolic acid) (PLGA) [Lu et al., 1998; Lu and Mikos, 1999b, c]. They have been fabricated into thin films, fibers, porous foams, conduits and investigated as scaffolds for regeneration of several tissues. Furthermore, the lysine groups in poly(lysine-co-lactic acid) provide sites for addition of cell-adhesion sequences such as arginine-lysine-aspartic acid (RGD) peptides [Barrera et al., 1995; Cook et al., 1997].

PPF, an unsaturated linear polyester that can be cross-linked through its fumarate double bonds, has been investigated as a biodegradable bone cement [Peter et al., 1997a]. The cross-linking, mechanical, and degradative properties
Table 1-2. Scaffold materials and their applications.\(^a\)

<table>
<thead>
<tr>
<th>Materials</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly((\alpha)-hydroxy esters)</td>
<td></td>
</tr>
<tr>
<td>Poly(glycolic acid) (PGA)</td>
<td>bone, cartilage, tendon, liver, intestine, urothelium</td>
</tr>
<tr>
<td>Poly(lactic acid) (PLA)</td>
<td>bone, cartilage, nerve</td>
</tr>
<tr>
<td>Poly(DL-lactic-co-glycolic acid) (PLGA)</td>
<td>bone, cartilage, urothelium, RPE, nerve</td>
</tr>
<tr>
<td>PLLA-bonded PLGA fibers</td>
<td>smooth muscle</td>
</tr>
<tr>
<td>PLLA coated with collagen or poly(vinyl alcohol) (PVA)</td>
<td>liver</td>
</tr>
<tr>
<td>PLLA and PEG block copolymer</td>
<td>bone</td>
</tr>
<tr>
<td>PLGA and PEG blends</td>
<td>soft tissue and tubular tissue</td>
</tr>
<tr>
<td>Poly(DL-lactic acid-co-(\varepsilon)-caprolactone)</td>
<td>vascular graft</td>
</tr>
<tr>
<td>Poly(L-lactic acid-co-(\varepsilon)-caprolactone)</td>
<td>meniscal tissue, nerve</td>
</tr>
<tr>
<td>Poly(L-lactic acid)/polyurethane</td>
<td>small-caliber arteries</td>
</tr>
<tr>
<td>Poly(lysine-co-lactic acid)</td>
<td>bone, cartilage, nerve</td>
</tr>
<tr>
<td>Poly((\varepsilon)-caprolactone) (PCL)</td>
<td>drug delivery</td>
</tr>
<tr>
<td>Poly(dioxanone)</td>
<td>bone</td>
</tr>
<tr>
<td>Polyhydroxyalkanoate</td>
<td>cardiovascular</td>
</tr>
<tr>
<td>Poly(phosphates) and poly(phosphazenes)</td>
<td>skeletal tissue, nerve</td>
</tr>
<tr>
<td>Poly(propylene fumarate) (PPF)</td>
<td>bone</td>
</tr>
<tr>
<td>Poly(propylene fumarate-co-ethylene glycol)</td>
<td>cardiovascular</td>
</tr>
<tr>
<td>PPF/(\beta)-tricalcium phosphate</td>
<td>bone</td>
</tr>
<tr>
<td>Pseudo-poly(amino acids)</td>
<td>bone</td>
</tr>
</tbody>
</table>

\(^a\)Adapted from Babensee, et al., 1998.
of an injectable composite scaffold made of PPF and \( \beta \)-tricalcium phosphate have been characterized [Peter et al., 1997b, 1998b, 1999]. Poly(ethylene glycol) (PEG), although non-degradable, is often used to fabricate copolymers or polymer blends to increase the hydrophilicity, biocompatibility, and/or softness of the scaffold. Poly(propylene fumarate-co-ethylene glycol) hydrogels have been developed for cardiovascular applications [Suggs et al., 1997, 1998a, b, 1999].

Poly(\( \varepsilon \)-caprolactone) (PCL) as well as blends and copolymers containing PCL have also been studied as scaffold materials [Suggs and Mikos, 1996]. Poly(phosphates) and poly(phosphazenes) have been processed into scaffolds for bone tissue engineering [Behravesh et al., 1999; Renier and Kohn, 1997]. Pseudo-poly(aminoc acids), in which amino acids are linked by both amide and non-amide bonds (such as urethane, ester, iminocarbonate, and carbonate), are amorphous and soluble in organic solvents and thus processable into scaffolds. The most studied among these polymers are tyrosine-derived polycarbonates and polyacrylates [James and Kohn, 1996]. By structural modifications of the backbone and pendant chains, polymer families with systematically and gradually varied properties can be created.

1.4 Processing Techniques

The technique used to manufacture synthetic bioresorbable polymers into suitable scaffolds for tissue regeneration depends on the properties of the polymer and its intended application. Scaffold processing usually involves (1) heating the polymers above their glass transition or melting temperatures; (2) dissolving them in organic solvents; and/or (3) incorporating and leaching of
porogens (gelatin microspheres, salt crystals, etc.) in water. These processes usually result in decrease in molecular weight and have profound effects on the biocompatibility, mechanical properties, and other characteristics of the formed scaffold. Incorporation of large bioactive molecules such as proteins into the scaffolds and retention of their activity have been a major challenge. The various processing techniques available are discussed in the following sections (Table 1-3).

1.4.1 Fiber Bonding

Fibers provide a large surface area to volume ratio and are therefore desirable as scaffold materials. PGA fibers in the form of tassels and felts have been utilized as scaffolds to demonstrate the feasibility of organ regeneration [Cima et al., 1991b; Vacanti et al., 1991]. However, these fibers lack the structural stability necessary for in vivo uses, which has led to the development of a fiber bonding technique [Mikos et al., 1993a]. With this method, PGA fibers are aligned in the shape of the desired scaffold and then embedded in a PLA/methylene chloride solution. After evaporation of the solvent, the PLA-PGA composite is heated above the melting temperatures of both polymers. PLA is removed by selective dissolution after cooling, leaving the PGA fibers physically joined at their cross-points without any surface or bulk modifications while maintaining their initial diameter. Stipulations concerning the choice of solvent, immiscibility of the two polymers and their relative melting temperatures, restrict the general application of this technique to other polymers.

An alternative method of fiber bonding has also been developed to prepare tubular scaffolds for the regeneration of intestine, blood vessels and ureters [Mooney et al., 1994a, 1996]. In this technique, a nonwoven mesh of
Table 1-3. Currently available polymer processing techniques and examples of scaffolds processed by these techniques.

<table>
<thead>
<tr>
<th>Processing Techniques</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber bonding</td>
<td>PGA fibers; PLA-reinforced PGA fibers</td>
</tr>
<tr>
<td>Solvent-casting/particulate-leaching</td>
<td>PLA, PLGA foams</td>
</tr>
<tr>
<td>Superstructure engineering</td>
<td>PLA, PLGA membranes</td>
</tr>
<tr>
<td>Compression molding</td>
<td>PLA, PLGA foams</td>
</tr>
<tr>
<td>Extrusion</td>
<td>PLA, PLGA conduits</td>
</tr>
<tr>
<td>Freeze-drying</td>
<td>PLGA foams</td>
</tr>
<tr>
<td>Phase separation</td>
<td>PLA foams</td>
</tr>
<tr>
<td>High pressure gas foaming</td>
<td>PLGA foams</td>
</tr>
<tr>
<td>Solid freeform fabrication</td>
<td>Complex 3-D PLA, PLGA structures</td>
</tr>
</tbody>
</table>
PGA fibers is attached to a rotating Teflon cylinder. The scaffolds are reinforced by spray casting with solutions of PLA or PLGA, which results in a thin coat that bonds the cross-points of PGA fibers. The behavior of transplanted cells is therefore determined by the PLA or PLGA coating instead of the PGA mesh. The mechanical strength of the scaffold is provided by both fibers and coating and is designed in such a way to withstand mechanical stresses or compromise degradation of PLA or PLGA. For example, PGA fiber-based matrices alone did not withstand contractile forces exerted by cultured smooth muscle cells, while scaffolds stabilized by spray-coating atomized PLA solution over the sides of the PGA matrices maintained their desired size and shape over 7 weeks in culture [Kim and Mooney, 1998]. This method is very useful for fabrication of thin scaffolds, however, it does not allow the creation of complex 3-D scaffolds since only a thin layer at the surface may be engineered by coating.

1.4.2 Solvent-Casting and Particulate-Leaching

In order to overcome some of the drawbacks associated with fiber bonding, a solvent-casting and particulate-leaching (SC/PL) technique has been developed to prepare porous scaffolds with controlled porosity, surface area to volume ratio, pore size and crystallinity for specific applications [Mikos et al., 1994b]. This method can be applied to PLA, PLGA and any other polymers which are soluble in a solvent such as chloroform or methylene chloride. For example, sieved salt particles are dispersed in a PLA/chloroform solution which is used to cast a membrane onto glass petri dishes. After evaporating the solvent, the PLA/salt composite membranes are heated above the PLA melting temperature and then quenched or annealed by cooling at controlled rates to yield amorphous or semicrystalline foams with regulated crystallinity. The salt
particles are eventually leached out by selective dissolution in water to produce a porous polymer matrix.

Highly porous PLA foams with porosities up to 93% and median pore diameters up to 500 μm have been prepared using the above technique [Mikos et al., 1994b; Wake et al., 1994]. Porous PLGA foams fabricated by the same method have been shown to support osteoblasts growth both in vitro and in vivo [Ishaug et al., 1997; Ishaug-Riley et al., 1997, 1998]. The porosity and pore size can be controlled independently by varying the amount and size of the salt particles, respectively. The surface area to volume ratio depends on both initial salt weight fraction and particle size. In addition, the crystallinity which affects both degradation and mechanical strength of the polymer can be tailored to a particular application. A disadvantage of this method is that it can only be used to produce thin wafers or membranes with uniform pore morphology up to 3 mm thick [Wake et al., 1996]. The preparation of thicker membranes may result in the formation of a solid skin layer characteristic of asymmetric membranes. The two controlling phenomena are solvent evaporation of the surface and solvent diffusion in the bulk.

This method has been modified to fabricate tubular scaffolds [Mooney et al., 1994b, 1995a]. Porous PLGA membrane prepared using SC/PL are wrapped around Teflon cylinders, and the overlapping ends are fused together with chloroform. The Teflon core is then removed to leave a hollow tube. Due to the relatively brittle nature of the porous membranes used, this method is limited to tubular scaffolds with a low ratio of wall thickness to inner diameter.

To increase the pliability of the porous membranes, PEG has been blended with PLGA in the SC/PL process [Wake et al., 1996]. Micropores
resulted from dissolution of PEG during leaching are believed to alter the structure of the pore walls and increase the pliability of the scaffold. These membranes can be rolled over into tubular scaffolds with a significantly higher ratio of wall thickness to inner diameter. The membranes fabricated from the polymer blend does not show any macroscopic damage during rolling as is observed for tubes made of PLGA alone.

1.4.3 Superstructure Engineering

Polymer scaffolds with complex 3-D architecture (superstructures) can be formed by superimposing defined structural elements such as pores, fibers, or membranes in orders according to stochastic, fractal or periodic principles [Wintemantel et al., 1996]. This approach may provide optimal spatial organization and nutritional conditions for cells. The coherence of structural elements determines the anisotropic structural behavior of the scaffold. The major concern in engineering superstructures is the spatial organization of the elements in order to obtain desired pore sizes and interconnected pore structure.

A simple example of this technique is membrane lamination to construct foams with precise anatomical shapes [Mikos et al., 1993b]. A contour plot of the particular 3-D shape is first prepared. Highly porous PLA or PLGA membranes with the shapes of the contour are then manufactured using SC/PL. The adjacent membranes are bonded together by coating chloroform on their contacting surfaces. The final scaffold is thus formed by laminating the constituent membranes with the proper order. It has been shown that continuous pore structures are formed with no boundary between adjacent
layers. In addition, the bulk properties of the 3-D scaffold are identical to those of the individual membranes.

1.4.4 Compression Molding

Compression molding is an alternative technique of constructing 3-D scaffolds. In this method, a mixture of fine PLGA powder and gelatin microspheres is loaded in a Teflon mold and then heated above the glass transition temperature of the polymer [Thomson et al., 1995]. The PLGA/gelatin composite is subsequently removed from the mold and gelatin microspheres are leached out. In this way, porous PLGA scaffolds with a geometry identical to the shape of the mold can be produced.

Polymer scaffolds of various shapes can be constructed by simply changing the mold geometry. This method also offers the independent control of porosity and pore size by varying the amount and size of porogen used, respectively. In addition, it is possible to incorporate bioactive molecules in either polymer or porogen for controlled delivery, because this process does not utilize organic solvents and is carried out at relatively low temperatures for amorphous PLGA scaffolds. This manufacturing technique may also be applied to PLA or PGA. However, higher temperatures are required (above the polymer melting temperatures) because these polymers are semicrystalline.

Compression molding can be combined with the SC/PL technique to form porous 3-D foams. The dried PLGA/salt composites obtained by SC are broken into pieces of less than 5 mm in edge length and compression-molded into a desired 3-D shape [Widmer et al., 1998]. The resulted composite material can then be cut into desired thickness. Subsequent leaching of the salt leaves
an open-cell porous foam, with more uniform pore distribution than those obtained by SC/PL for increased thickness.

Highly porous poly(α-hydroxy ester) scaffolds, though desirable in many tissue engineering applications, may lack required mechanical strength for the replacement of load bearing tissues such as bone. Hydroxyapatite and β-tricalcium phosphate are biocompatible and osteoconductive materials and can be incorporated into these foams to improve their mechanical properties. Because the macroscopic mixing of three solid particulates (polymer powder, porogen, and ceramic) is difficult, a combined SC, compression molding, and PL technique described above has been employed to fabricate an isotropic composite foam scaffold of PLGA reinforced with short hydroxyapatite fibers (15 μm in diameter and 45 μm in length) [Thomson et al., 1998]. Within certain range of fiber contents, these scaffolds have superior compressive strength compared to non-reinforced materials of the same porosity.

1.4.5 Extrusion

Various extrusion methods such as ram (piston-cylinder) extrusion, hydrostatic extrusion, or solid-state extrusion (die drawing) have been applied to increase the orientation of polymer chains and thus produce high-strength, high-modulus materials [Ferguson et al., 1996]. More recently, an extrusion process has been successfully combined with the SC/PL technique to manufacture porous tubular scaffolds for guided tissue such as peripheral nerve regeneration [Widmer et al., 1998]. First the dry polymer/salt composite wafers obtained from SC are cut into pieces and placed in a customized piston extrusion tool (Figure 1-2). The tool is then mounted into a hydraulic press and heated to the desired processing temperature. The temperature is allowed to
Figure 1-2. Piston extrusion tool for the manufacture of tubular polymer/salt composite structures: (1) extruded polymer/salt construct, (2) nozzle defining the outer diameter of the tubular construct, (3) tool body, (4) melted polymer/salt mixture, (5) rod defining the inner diameter of the tubular construct, (6) heat band with temperature control, and (7) piston moving the melted polymer/salt mixture. The arrows indicate the attachment points for the forces involved in the extrusion process.
equilibrate and the polymer/salt composite is then extruded by applying pressure. The extruded tubes are cut to appropriate lengths. Finally, the salt particles are leached out to yield highly porous conduits.

The pressure for extrusion at a constant rate is dependent on the extrusion temperature. High temperature may result in thermal degradation of the polymer. The porosity and pore size of the extruded conduits are determined by salt weight fraction, salt particle size, and processing temperatures. The fabricated conduits have an open-pore structure and are suitable for incorporation of cells or microparticles loaded with tissue inductive factors.

1.4.6 Freeze-Drying

Low-density polymer foams have been produced using a freeze-drying technique [Hsu et al., 1997]. Polymer is first dissolved in a solvent such as glacial acetic acid or benzene to form a solution of desired concentration. The solution is then frozen and the solvent is removed by lyophilization under high vacuum. Several polymers including PLGA and PLGA/PPF have been prepared into porous foams with this method. The foams have either leaflet or capillary structures depending on the polymer and solvent used in fabrication. These foams are generally not suitable as scaffolds for cell transplantation. Subsequent compression of the foams by grinding and extrusion can generate matrices with varied densities. Foam density has been shown to determine the kinetics of drug release from these matrices.

An emulsion freeze-drying technique has also been developed to fabricate porous scaffolds [Whang et al., 1995]. In this technique, water is
added to a PLGA/methylene chloride solution and the immiscible phases are homogenized. The created emulsion (water-in-oil) is then poured into a copper mold maintained in liquid nitrogen (−196°C). After quenching, the samples are freeze-dried to remove methylene chloride and water. Using this technique, PLGA foams with porosity in the range of 91-95% and median pore diameters of 13-35 μm with larger pores greater than 200 μm have been made by varying processing parameters such as water volume fraction, polymer weight fraction, and polymer molecular weight. Compared to the SC/PL technique, this method produces foams with smaller pore sizes but higher specific pore surface area, and can produce thick (> 1 cm) foams.

1.4.7 Phase Separation

The ability to deliver bioactive molecules from a degrading polymer scaffold is desirable for tissue regeneration. However, the activity of the molecule is often dramatically decreased due to harsh chemical or thermal environments used in some polymer processing techniques. Using a novel phase separation technique, scaffolds loaded with small hydrophilic and hydrophobic bioactive molecules have been manufactured [Lo et al., 1995]. The polymer is dissolved in a solvent such as molten phenol or naphthalene, followed by dispersion of the bioactive molecule in this homogeneous solution. A liquid-liquid phase separation is induced by lowering the solution temperature. The resulting bicontinuous polymer and solvent phases are then quenched to create a two-phase solid. Subsequent removal of the solidified solvent by sublimation leaves a porous polymer scaffold loaded with bioactive molecules.
The fabricated PLA foams have pore sizes up to 500 μm with relatively uniform distributions. The properties of the foams depend on the polymer type, molecular weight, concentration, and solvent used. It has been shown that proteins such as alkaline phosphatase retain as much as 75% of their activity after scaffold fabrication with the naphthalene system, but the activity is completely lost in the phenol system. Although phenol has a lower melting temperature than naphthalene, it is a more polar solvent and can interact with proteins and weaken the hydrogen bonding within the protein structure, resulting in a loss of protein activity. The phenol system may be useful for the entrapment of small drugs or short peptides instead.

1.4.8 High Pressure Gas Foaming

In the gas foaming (GF) technique, solid disks of PLGA prepared by either compression molding or solvent casting are exposed to high pressure CO₂ (5.5 MPa, 25°C) environment to allow saturation of CO₂ in the polymer [Mooney et al., 1996a]. A thermodynamic instability is then created by reducing the CO₂ gas pressure to ambient level, which results in nucleation and expansion of dissolved CO₂ pores in the polymer particles. PLGA sponges with a porosity of up to 93% and a pore size of about 100 μm have been fabricated. The porosity and pore structure are dependent on the amount of CO₂ dissolved, the rate and type of gas nucleation, and the rate of gas diffusion to the pore nuclei.

The major advantage of this technique is that it involves no organic solvent or high temperature and therefore is promising for incorporating tissue induction factors in the polymer scaffolds. However, the effects of high pressure on the retention of activity of proteins still need to be assessed. In addition, this
process yields mostly non-porous surfaces and a closed pore structure inside the polymer matrix, which is undesirable for cell transplantation. In an improved method, a porogen such as salt particles can be combined with the polymer to form composite disks before gas foaming [Harris et al., 1998]. The expansion and fusion of the polymer particles lead to the formation of a continuous matrix with entrapped salt particles, which are subsequently leached out. The GF/PL process produces porous matrices with predominately interconnected macropores (created by leaching of salt) and smaller, closed pores (created by the nucleation and growth of gas pores in the polymer particles). The fabricated matrices have a more uniform pore structure and higher mechanical strength than those obtained with SC/PL.

1.4.9 Solid Freeform Fabrication

Solid freeform fabrication (SFF) refers to computer-aided design, computer-aided manufacturing (CAD/CAM) methodologies such as stereolithography, selective laser sintering (SLS), ballistic particle manufacturing, and 3-D printing (3DP) for the creation of complex shapes directly from CAD models. SFF techniques, although mainly investigated for industrial applications such as rapid prototyping, offer the possibility to fabricate polymer scaffolds with well-defined architecture because local composition, macrostructure and microstructure can be specified and controlled at high resolution in the interior of the components. These methods build complex 3-D objects by material addition and fusion of cross-sectional layers (2-D slices decomposed from CAD models). In addition, they allow the formation of multi-material structures by selective deposition. Prefabricated structures can also be embedded during material buildup. By carefully controlling the processing
conditions, cells, bioactive molecules, or synthetic vasculature may be included directly into layers of polymer scaffolds during fabrication.

In the SLS technique, a thin layer of evenly distributed fine powder is first laid down [Bartels et al., 1993; Berry et al., 1997]. A computer-controlled scanning laser is then used to sinter the powder within a cross-sectional layer. The energy generated by the laser heats the powder into a glassy state and individual particles fuse into a solid. Once the laser has scanned the entire cross-section, another layer of powder is laid on top and the whole process is repeated.

In 3DP process, each layer is created by adding a layer of polymer powder on top of a piston and cylinder containing a powder bed and the part being fabricated. This layer is then selectively joined where the part is to be formed by ink-jet printing of a binder material such as an organic solvent. The printed droplet has a diameter of 50-80 μm [Griffith et al., 1997]. The printhead position and speed are controlled by the computer. The piston, powder bed, and part are lowered and a new layer of polymer powder is laid on top of the already processed layer and selectively joined. The layered printing process is repeated until the desired part is completed.

The local microstructure within the component can be controlled by varying the printing conditions. The resolution of features currently attainable by 3DP for degradable polyesters is about 200 μm [Griffith et al., 1997]. Using this technique, scaffolds with complex structures may be fabricated [Giordano et al., 1996]. A model drug (dye) with concentration profile specified by a CAD model has been successfully incorporated into a scaffold during the 3DP process, demonstrating the feasibility of producing complex release regimes
using a single drug delivery device [Wu et al., 1996]. The retention of activity of proteins in these devices is yet to be tested. By mixing salt particles in the polymer powder and their subsequent leaching after 3DP process, porous PLGA scaffolds with an intrinsic network of interconnected branching channels have been fabricated for cell transplantation [Kim et al., 1998]. This network of channels and micropores could provide a structural template to guide cellular organization, enhance neovascularization, and increase the capacity for mass transport. Furthermore, multiple printheads containing different binder materials can be used to modify local surface chemistry and structure. Patterned PLA substrates with selective cell-adhesion domains have been fabricated by 3DP [Park et al., 1998].

1.5 Characterization of Scaffolds

Various techniques are available to characterize the fabricated polymer scaffolds (Table 1-4). The molecular weight and polydispersity index of the polymer can be measured by gel permeation chromatography (GPC). Information on chemical composition and structure can be obtained by nuclear magnetic resonance (NMR) spectroscopy, X-ray diffraction, Fourier transform infrared (FTIR), and FT-Raman (FTR) spectroscopy. The thermal properties of the polymer such as glass transition temperature (Tg), melting temperature (Tm), and crystallinity (Xc) can be determined by differential scanning calorimeter (DSC). Porosity and pore size distribution of a porous scaffold are measured by mercury intrusion porosimetry. Scanning electron microscopy (SEM) is the most common method to view the pore structure and morphology. Recently, the 3-D microstructure of porous PLGA matrices has been analyzed
Table 1-4. Characterization of bioresorbable polymer scaffolds.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bulk Properties</strong></td>
<td></td>
</tr>
<tr>
<td>Molecular weight</td>
<td>GPC</td>
</tr>
<tr>
<td>Polydispersity index</td>
<td>GPC</td>
</tr>
<tr>
<td>Chemical composition, structure</td>
<td>NMR, X-ray diffraction, FTIR, FTR</td>
</tr>
<tr>
<td>Thermal properties (Tg, Tm, Xc, etc.)</td>
<td>DSC</td>
</tr>
<tr>
<td>Porosity, pore size</td>
<td>Mercury intrusion porosimetry</td>
</tr>
<tr>
<td>Morphology</td>
<td>SEM, confocal microscopy</td>
</tr>
<tr>
<td>Mechanical properties</td>
<td>Mechanical testing</td>
</tr>
<tr>
<td>Degradative properties</td>
<td><em>In vitro, in vivo</em></td>
</tr>
<tr>
<td><strong>Surface Properties</strong></td>
<td></td>
</tr>
<tr>
<td>Surface chemistry</td>
<td>ESCA, SIMS</td>
</tr>
<tr>
<td>Distribution of chemistry</td>
<td>Imaging methods (e.g. SIMS)</td>
</tr>
<tr>
<td>Orientation of groups</td>
<td>Polarized IR, NEXAFS</td>
</tr>
<tr>
<td>Texture</td>
<td>SEM, AFM, STM</td>
</tr>
<tr>
<td>Surface energy and wettability</td>
<td>Contact angle measurement</td>
</tr>
</tbody>
</table>
by confocal microscopy [Tjia and Moghe, 1998]. Mechanical properties of the scaffolds such as tensile strength and modulus, compression strength and modulus, compliance/hardness, flexibility, elasticity, and stress and strain at yield can be measured using mechanical testing equipment. Some tests require the processing of scaffolds into a particular shape and dimensions specified by American Standards of Testing Materials (ASTM).

The in vitro degradation properties can be evaluated by placing the bioresorbable scaffolds in simulated body fluid, typically pH 7.4 phosphate buffered saline (PBS) [Lu et al., 1999]. The changes in sample weight, molecular weight, morphology, thermal, and mechanical properties can then be measured at various time points until degradation process is completed. This method does not allow the continuous observation of changes within the scaffolds. An in vivo study is often necessary to predict the degradation behavior of the scaffolds for cell transplantation.

Material surfaces, which are usually different from the bulk, play a crucial role in regulating cell response. Electron spectroscopy for chemical analysis (ESCA) and static secondary ion mass spectrometry (SIMS) are the most powerful tools for analyzing surface chemistry and composition. Information on the orientation of chemical groups can be obtained by polarized IR and near edge X-ray absorption fine structure (NEXAFS). Surface morphology can be characterized by SEM, scanning probe microscopy (SPM), and atomic force microscopy (AFM). Surface wettability and energy are assessed by contact angle measurements.

1.6 Cell Seeding and Culture in 3-D Scaffolds
The major obstacles to the in vitro development of 3-D cell-polymer constructs for the regeneration of large organs or defects have been obtaining uniform cell seeding at high densities and maintaining nutrient transport to the cells inside the scaffolds. To achieve desired spatial and temporal distribution of cells and molecular cues affecting cellular function, cell culture conditions should provide control over hydrodynamic and biochemical factors in the cell environment.

1.6.1 Static Culture

The conventional static cell seeding technique involves the placement of the scaffold in a cell suspension to allow the absorption of cells. However, the resulting cell distribution in the scaffold is often not uniform, with the majority of the cells attached only to the outer surfaces [Wald et al., 1993]. Wetting hydrophobic polymer scaffolds with ethanol and water prior to cell seeding allows for displacement of air-filled pores with water and thus facilitates penetration of cell suspension into these pores [Mikos et al., 1994a]. Infiltration with hydrophilic polymers or surface hydrolysis of scaffolds has also been shown to increase the cell seeding density [Gao et al., 1998; Mooney et al., 1995b]. Seeding cells by injection or applying vacuum to ensure penetration of the cell suspension through the 3-D matrix could result in uniform cell seeding initially, however, the uniformity is lost under static culture conditions due to the nutrient and oxygen diffusion limitation within the scaffold.

Several dynamic cell seeding and culture techniques have been developed to ensure uniform cell distribution which will lead to uniform tissue regeneration (Figure 1-3). Compared to static culture conditions, mass transfer rates can be maintained at higher levels and cell growth is not restricted by the
Figure 1-3. Dynamic cell seeding and culture techniques in 3-D scaffolds: (a) spinner flask, (b) rotary vessel, and (c) perfusion system.
rate of nutrient supply under well-mixed culture conditions. These methods can be scaled up and are suitable for cell cultivation using multiple scaffolds.

1.6.2 Spinner Flask Culture

In a spinner flask, 3-D polymer scaffolds are first fixed to needles attached to the cap of the flask, and then exposed to a uniform, well-mixed cell suspension (Figure 1-3a) [Freed et al., 1993b]. Using this method, porous PGA scaffolds have been uniformly seeded with chondrocytes at high yield and high kinetic rate (to minimize the time that cells stay in the suspension) [Vunjak-Novakovic et al., 1998]. Mixing has been found to promote the formation of cell aggregates with sizes of 20-32 μm. The spin rate, however, needs to be well adjusted to minimize cell damage under high shear stress. Spinner flask is also suitable for suspension culture of hepatocyte spheroids which exhibit enhanced liver function compared to monolayer culture in the long-term [Kamihira et al., 1997].

1.6.3 Rotary Vessel Culture

Rotating-wall vessel (RWV) also allows enhanced mass transport and is useful for 3-D cell culture (Figure 1-3b). The polymer scaffolds are loaded into the vessel and a uniform cell suspension is added. Vessel rotation is initiated to allow dynamic cell seeding and increased to maintain high rate of nutrient and oxygen diffusion. Alternately, the scaffolds can be pre-seeded with cells under static conditions before loading [Goldstein et al., 1999]. Several configurations of RWV have been used in microgravity tissue engineering [Freed and Vunjak-Novakovic, 1997].
1.6.4 Perfusion Culture

A flow perfusion culture system has been used for in vitro regeneration of large 3-D tissues and organs (Figure 1-3c) [Glowacki et al., 1998; Griffith et al., 1997; Kim et al., 1998]. The cell-polymer constructs are maintained in a continuous flow condition. The culture medium is pumped from a reservoir through an oxygenator, the cell-polymer constructs, and recirculated back to the reservoir. The flow rate for cell survival is adjusted based on cell mass. The entire perfusion unit is maintained in normal sterile culture conditions. Compared to static culture, medium perfusion has been shown to significantly enhance cell viability and matrix production [Glowacki et al., 1998]. This system is useful for the development of complex tissue structures and study of cell-cell and cell-ECM interactions.

1.6.5 Other Culture Conditions

Ideally the culture conditions should provide all necessary signals that the cells normally experience in vivo for optimal tissue regeneration. For instance mechanical stimulation plays an important role in the differentiation of mesenchymal tissues [Chiquet et al., 1996; Goodman and Aspenberg, 1993]. Application of well-controlled loads may stimulate bone growth into porous scaffolds. The degradation of the scaffolds can be affected by applied strain [Miller and Williams, 1984]. Transwell culture systems that allow the use of different culture media for apical or basal sides are often employed to induce and maintain the polarity of epithelial cells. The growth and function of some retinal cells may be regulated by the light-dark cycle. In some cases, a gradient substrate with spatially controlled wettability or other properties may be desired [Ruardy et al., 1995]. Some cellular chemotactic responses may require the
creation of concentration gradients of growth factors. Temporal presentation of signals is also important. For example, each phase of the differentiation of osteoblasts (proliferation, maturation of ECM, and mineralization) requires different signals [Lian and Stein, 1992; Peter et al., 1998a]. Co-culture of several cell types may be preferred for in vitro organogenesis including angiogenesis.

1.7 Retinal Pigment Epithelium

1.7.1 Anatomy

Retinal pigment epithelium (RPE) is composed of a continuous monolayer of simple cuboidal cells located between choroid capillaries and the neurosensory retina. In a normal eye, RPE cells are hexagonal-shaped and packed together like cobblestones. The size of an RPE cell depends on the age and location. Cells in the macula have a height of 14 μm and a width of 10-14 μm for young people [Zinn and Marmor, 1979]. RPE has a mottled brown color due to the presence of melanin and other pigments including lipofuscin granules, which accumulate with age.

Though simple in appearance, RPE cells have developed a complex structural and functional polarity that allows them to perform highly specialized roles. The RPE cell membrane has distinct apical, basal and lateral surfaces. The apical surface of the cells is covered with two types of microvilli. The long microvilli abut photoreceptor cones (responsible for daytime vision) and the short microvilli interdigitate with photoreceptor rods (responsible for nighttime vision) [Zinn and Marmor, 1979]. When fused around a photoreceptor element,
the microvilli form connected sheets of extended cytoplasm collectively called microplicae. These microvilli communicate with the cells of the neural retina through the interphotoreceptor space (IPS) without having physical connections. The proteins expressed only on the apical surface include various receptor proteins used for signaling across the IPS, such as a rod outer segment (ROS) phagocytosis recognition receptor, retinoid binding proteins, g-glutamyl transpeptidase, and carbonic anhydrase.

The basal surface of the RPE cells is convoluted into numerous basal infoldings, leading to a high surface area suitable for transport properties. It is connected through hemidesmosomes to the underlying Bruch's membrane, which borders the capillary network of the choroid. The cellular elements located on the basal surface are the nucleus, mitochondria, endoplasmic reticula, and lipofuscin. The presence of these cellular organelles as well as basal infoldings indicates high energy functions such as protein output.

The lateral surfaces of adjacent RPE cells are joined by four types of junctions: tight junctions, adherent junctions, desmosomes, and gap junctions [Zinn and Marmor, 1979]. The gap junctions allow the communication along the entire epithelium. The tight junctions separate the apical and basolateral surfaces and contribute to the barrier function of RPE. The tight junctions are connected to a network of cytoskeleton called circumferential ring, which consists actin, myosin, α-actinin, and other molecules. This ring structure and tight junctions are crucial in the maintenance of polarized distribution of membrane proteins.

1.7.2 Physiology
RPE is a multifunctional and indispensable component of the vertebrate retina. Physically, it serves as a barrier to prevent paracellular passage of molecules. Transport of nutrients from the choroid to the photoreceptors and the removal of waste in the opposite direction must occur through the RPE layer itself. Harmful factors such as antibodies, macrophages, and endothelial cells in the choroidal circulation are prevented from entering the neurosensory retina. The presence of these factors could disrupt the retina's fragile structure through inflammation and neovascularization. This helps to create an "immunologically privileged" site in the retina, which may be beneficial for allograft or xenograft tissue transplantation. In addition, the major force for the adhesion of retina to the periphery of the eye is provided by RPE through fluid pumping from the vitreous to the choroid [Zinn and Marmor, 1979]. Minor contribution to the adhesiveness include glycosaminoglycan (secreted by RPE) adhesion and surface energy adhesion between interdigitated microvilli and photoreceptors.

Optically, the melanin granules of the cells absorb excessive light thus preventing the scattering of stray light. This greatly sharpens the resolving power of the retina. Another useful property of these granules is to absorb excess photothermal energy during laser coagulation repair of the eye.

A primary metabolical function of RPE is to phagocytize the rod outer segments, the disks of which are shed at a rate of 80-90 daily [Bok, 1993; Zinn and Marmor, 1979]. As the photoreceptors are stimulated for neuronal signal transmission, they discard the chemical reaction end products in membrane bound packages into the IPS. The RPE cells contain special hydrolytic enzymes to degrade the ROS disks. RPE participates actively in the visual cycle through the regeneration of 11-cis retinal [Bok, 1993; Zinn and Marmor, 1979]. The photoisomerization and reduction of this retinoid into all-trans retinol
by photoreceptor out segments are the first steps in phototransduction. All-trans retinol then leaves the photoreceptors, transverses the IPS, and undergoes re-isomerization to 11-cis retinal and oxidation prior to returning to the photoreceptors across the IPS.

The primary transport function of RPE is the nutrient supply from the choroid to the photoreceptors and the waste removal in the reverse direction. The active transport of molecules and trafficking of membrane proteins are controlled by Na⁺,K⁺-ATPase. This ion pump, together with associated cytoskeletal proteins ankyrin and fodrin, are apically localized in the RPE. This unique feature is shared only with the epithelium of the choroid plexus, as opposed to the basolateral expression in most other epithelia [Bok, 1993]. Na⁺,K⁺-ATPase controls the flux of Na⁺ and K⁺ across the plasma membrane, thereby maintaining the proper balance of these ions in the IPS and establish membrane potentials.

1.7.3 Pathology

The importance of RPE in the maintenance of normal functions and health of the neural retina can not be overemphasized. Loss any of the functions discussed above leads to certain retinal disorders. The most widespread disease due to RPE dysfunction is age-related macular degeneration (ARMD). It is the leading cause of blindness in people over age 55 and is thought to affect over 30% of people over 65 [Peyman et al., 1991]. ARMD accounts for 16,000 new cases of severe loss of vision each year. The most common condition is the dry, atrophic type, which starts with gradual loss of central vision in one eye and eventually involves both eyes [Zinn and Marmor, 1979]. The RPE cells become less pigmented, shorter, are missing in
places and piled up in others. Also deposits (drusen) form between the RPE and the Bruch's membrane leading to distortion. The wet or exudative type has all the symptoms described above, but in addition includes hemorrhagic areas in and around the macula [Peyman et al., 1991]. The hemorrhage does not resorb and fibrovascular tissue invasion from the choroid and retinal blood vessels can result in rapid loss of central vision.

Another common condition involving RPE is retinitis pigmentosa, a group of disorders characterized by slow degeneration of the photoreceptors with hereditary transmission. RPE degeneration is present in many other retinal dystrophies such as Stargardt's disease, pattern dystrophies, choroideremia, and photic maculopathy. Retinal detachment resulting from the loss of adhesive function of the RPE can also cause photoreceptor degeneration.

1.7.4 PRE Tissue Engineering

There are currently no successful treatment for the retinal dystrophies caused by RPE degeneration. Although it is possible to slow the onset of the late stage symptoms, it is usually inadequate to prevent blindness. In the past decade or so, the idea of replacing aged and/or diseased RPE with healthy RPE graft has been extensively investigated in Royal College of Surgeons (RCS) rat [Gaur et al., 1992; LaVail et al., 1992; Li and Turner, 1988, 1991; Little et al., 1996; Lopez et al., 1989; Seaton et al., 1994; Sheedlo et al., 1989; Yamamoto et al., 1993], a well-known animal model of hereditary retinal degeneration, and other animals [El Dirini et al., 1992; Gouras et al., 1985; Lane et al., 1989; Lopez et al., 1987; Sheng et al., 1995; Wongpachedchai et al., 1992]. The promising results obtained from these studies demonstrated that RPE transplantation may provide a cure for the retinal diseases previously mentioned.
However, most of the methods involve the injection of an RPE cell suspension into the subretinal space. The random placement of these polar cells does not always result in RPE regeneration. Here we propose a potentially more efficient therapy employing tissue engineering strategies (Figure 1-4). The RPE cells are harvested from the patients and cultured onto a biodegradable substrate, such as a very thin PLGA film. Growth factors or immunosuppressant drugs can also be incorporated into the scaffolds or desired surface micropatterns can be engineered onto the scaffold surface for further modulation of cell growth and function. After the cells reach confluence in vitro, the cell-polymer construct is then implanted into the subretinal space. The cells can establish associations with the surrounding tissues and rescue degenerating photoreceptors.

1.8 Future of Tissue Engineering

Significant progress has recently been made to optimize the engineering of tissue and organ analogues. However, many challenges remain in the engineering of 2-D and 3-D tissues and organs for clinical use. Nevertheless, many advances have been made in synthetic polymer chemistry, scaffold processing methods, and tissue culture techniques. These may eventually allow the generation of long-term functional complex cell-polymer constructs with precisely controlled local environment such as material microstructure, nutrient and growth factor concentration, and mechanical forces.
Figure 1-4. Schematic of an RPE tissue engineering strategy.
CHAPTER 2
OBJECTIVES

The objectives of this thesis were to modulate cell morphology and function utilizing synthetic biodegradable polymers as scaffolds for cell culture or as vehicles for controlled delivery of growth factors.

We hypothesized that:

1) Synthetic biodegradable polymer films can serve as suitable substrates for in vitro RPE cell culture.

2) The polymer composition and film thickness can affect in vitro film degradation.

3) Biodegradable microparticles can serve as carriers for controlled delivery of growth factors.

4) Desired micropatterns engineered onto substrate surfaces can enhance the expression of differentiated RPE cell phenotype.

5) Synthetic biodegradable polymers can be fabricated into substrates with desired surface micropatterns.

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

1) To fabricate thin PLGA films of few microns using a solvent casting technique and investigate the effects of PLGA copolymer ratio on
RPE cell attachment, proliferation, morphology, and tight junction formation in vitro.

2) To evaluate the effects of PLGA copolymer ratio and film thickness on the in vitro degradation of thin PLGA films in simulated body fluids and characterize the films in terms of mass loss, molecular weight loss, dimensional changes, and morphology over a time course of 10 weeks.

3) To encapsulate transforming growth factor β1 (TGF-β1) and fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) into blends of PLGA and PEG using a double-emulsion-solvent-extraction technique and study the effects of PEG content and buffer pH on the protein release kinetics and degradation of PLGA in vitro during a 28-day period.

4) To fabricate model substrates with defined chemical domains of micrometer scale using a microcontact printing technique and investigate the effects of surface micropatterning and pattern size on the morphology, cytoskeleton organization, attachment, and proliferation of cultured RPE cells.

5) To test the feasibility of using biodegradable PLGA and PEG/PLA for controlling RPE cell attachment and proliferation and their fabrication into micropatterned surfaces using the microcontact printing technique.
CHAPTER 3

RETINAL PIGMENT EPITHELIUM CELL CULTURE ON THIN BIODEGRADABLE POLY(DL-LACTIC-CO-GLYCOLIC ACID) FILMS

3.1 Introduction

Given the strategic placement between choroid capillaries and neurosensory retina, retinal pigment epithelium (RPE) plays a key role in the maintenance of the normal functions of the photoreceptors [Bok, 1993]. It constitutes part of the blood-retina barrier through the formation of tight junctions. The RPE is responsible for the transport of many substances from the choroid to the retina and others in the reverse direction. The melanin granules of the RPE improve visual clarity by absorbing excessive light. RPE cells participate actively in the visual cycle through the metabolism of vitamin A. They also carry out phagocytosis of rod outer segments shed daily.

Alteration in RPE structure and function is implicated in a variety of hereditary and degenerative retinal diseases. The idea of replacing aged and/or diseased RPE with healthy RPE graft has been extensively investigated in animal models [Gaur et al., 1992; LaVail et al., 1992; Li and Turner, 1988; Little et al., 1996; Lopez et al., 1989; Sheng et al., 1995; Yamamoto et al., 1993]. RPE transplants have been shown to survive well in the subretinal space and prevent photoreceptor degeneration to some extent. These results raise the possibility that some blinding disorders in humans, such as age-related macular degeneration (ARMD) and Stargardt's disease, which result from RPE degeneration, may be amenable to treatment by RPE transplantation [Algvere et al., 1994; Peyman et al., 1991].
Virtually all of the existing therapies involve the injection of an RPE cell suspension into the subretinal space [Gaur et al., 1992; LaVail et al., 1992; Li and Turne, 1988; Little et al., 1996; Lopez et al., 1989; Yamamoto et al., 1993]. With this method, the transplant is usually composed of isolated groups of cells that seldom resemble an epithelial monolayer. Moreover, the random placement of these cells could not always result in RPE regeneration, since RPE cells are polar with distinct apical/basal characteristics. It is therefore desirable to transplant an organized sheet of RPE cells with appropriate orientation.

There have been several reports describing attempts to transplant patches of RPE with [Bhatt et al., 1994] or without collagen support [Algvere et al., 1994; Peyman et al., 1991; Sheng et al., 1995]. The disadvantages associated with collagen include thickness of the substrate, poor degradability and poor permeability. Without the support, however, it is more difficult to handle the cells during transplantation and the polarity of the patch is uncertain after injection.

We propose the use of biodegradable polymers as temporary substrates for RPE cell culture and subsequent transplantation. Such a polymer scaffold must be biocompatible and allow the attachment of RPE cells in vitro because they are anchorage-dependent cells that require a supportive matrix to survive and grow. It must also provide a suitable substrate for the proliferation and maintenance of differentiated functions of RPE cells. The material should also act as a template to guide cell orientation and organization. In addition, it must be processable into thin films with controlled thickness to allow precise manipulation of the cell-polymer construct during surgery, yet prevent retinal distortion, minimize the diffusion limitation of nutrients, and minimize the
quantity of degradation products. Finally, the polymer scaffold should degrade over a desired time period after implantation, leaving space for the regenerated RPE layer to re-establish association with the underlying Bruch's membrane.

Poly(α-hydroxy esters), such as poly(DL-lactic-co-glycolic acid) (PLGA), fulfill most of the material design requirements. PLGA copolymers are biocompatible, biodegradable, easily processable, and have been utilized in many tissue engineering applications [Cima et al., 1991a; Freed et al., 1993a; Ishaug et al., 1994; Langer and Vacanti, 1993; Lu and Mikos, 1996; Organ et al., 1992]. The biocompatibility of PLGA at the intraocular site has also been demonstrated [Giordano et al., 1993]. Recently, thin PLGA films manufactured in our laboratory have been shown to allow the attachment of human fetal RPE cells in vitro [Giordano et al., 1997; Thomson et al., 1996]. Moreover, it is feasible that by incorporation of certain growth factors or drugs into the polymer matrix, PLGA films can also serve as carriers for short-term drug delivery to modulate the cell-polymer construct [Langer, 1990].

An investigation of the effects of PLGA copolymer ratio and culture conditions on RPE cell attachment, proliferation, morphology, and structure in vitro was needed in order to elucidate the important parameters in the design of a novel RPE/PLGA graft before RPE transplantation could be attempted in vivo. Such an investigation is the focus of this study. Human D407 RPE cell line cells were cultured on PLGA films; these cells have been shown to retain many of the morphological, structural and functional characteristics of in vivo RPE cells [Davis et al., 1995]. In this study, we determined: (1) whether thin films of PLGA of few microns could be fabricated, (2) whether PLGA copolymer ratio would affect cell attachment and proliferation in vitro, (3) whether initial cell seeding
density would affect cell attachment, and (4) whether PLGA copolymer ratio would affect cell morphology and the formation of normal tight junctions *in vitro*.

3.2 MATERIALS AND METHODS

3.2.1 PLGA Film Fabrication

The biodegradable polymers used in this study were 50:50 PLGA (Medisorb®, Alkermes, Cincinnati, OH) and 75:25 PLGA (Birmingham Polymers, Birmingham, AL). The ratios 50:50 and 75:25 designate the copolymer ratios of lactic to glycolic acid. The weight average molecular weight of the polymers were 47,500 [polydispersity index (PI) = 1.87] for 50:50 PLGA and 75,200 (PI = 1.68) for 75:25 PLGA, as measured by gel permeation chromatography. PI is equal to the ratio of weight average to number average molecular weight (Mw/Mn).

The polymers were manufactured into thin films using a solvent casting technique [Thomson et al., 1996], with modifications. Briefly, 17.5 mg of 50:50 PLGA (15 mg for 75:25 PLGA) were dissolved in 1 mL of chloroform (Sigma Chemical Co., St. Louis, MO). The solution was then cast onto glass coverslips (diameter 12 mm) (Fisher Scientific, Pittsburgh, PA) placed on a leveled table in the fume hood. Approximately 70 μL of solution (75 μL for 75:25 PLGA) were used to completely cover each glass. Partially covered samples were left in the hood for 20 h to allow the solvent to evaporate and further vacuum-dried for 24 h. The films were stored in a desiccator over anhydrous CaSO₄ (W.A. Hammond Drierite Company Ltd., Xenia, OH) under vacuum until use.
The films for scanning electron microscopy (SEM) evaluation were soaked in distilled deionized water (ddH₂O) for 90 min, lifted off the glass with a razor blade, and subsequently air- and vacuum-dried. The samples were then gold-coated using a sputter coater (Pelco Sputter Coater 91000, model 3, Ted Pella, Redding, CA) set at 20 mA for a total time of 120 s (coating thickness approximately 40 nm) before observation with the SEM (JEOL JSM-5300 Scanning Microscope, Boston, MA) operated at 20 kV.

The wettability of the PLGA films and control surfaces of glass and 12-well polystyrene tissue culture plates (TCPS) (Fisher) was assessed by the captive drop method. The water contact angles were measured at room temperature under air, using a goniometer (Model 100-00, Rame-Hart, Mountain Lakes, NY). A small drop of ddH₂O was introduced on the surfaces using a micrometer syringe. The drop size was gradually increased until a dynamic contact interface was achieved. The contact angles thus obtained were of the advancing type.

3.2.2 RPE cell culture

A human D407 RPE cell line was kindly provided by Dr. Richard C. Hunt (Department of Ophthalmology, University of South Carolina Medical School, Columbia, SC) [Davis et al., 1995]. The cells were cultured with Dulbecco's modified eagles medium (DMEM) (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 25 μg/mL gentamicin (Sigma) in a 37°C humidified atmosphere of 5% CO₂. When 90% confluency was reached after about 4 days, the cells were passaged with PET - a trypsin solution containing polyvinylpyrrolidone, 0.0125% ethyleneglycol-bis(b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.0625% trypsin (T-8253),
and 26 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) in 1.125% NaCl (all Sigma). Cells between passages 54 and 59 were used in these experiments.

3.2.3 Cell attachment and proliferation

Thin films (on glass) of 50:50 and 75:25 PLGA were placed in 12-well plates and sterilized by exposure to ultraviolet (UV) light for 1 h on each side prior to cell culture. In the attachment study, 200 µL of cell suspension were placed on top of each PLGA film and 1 mL of diluted solution (dilution factor = 0.68) was added to each TCPS well (diameter 22.1 mm) to obtain the same initial cell seeding density of 27,000 cells/cm². The cultures were placed in the incubator for 1.2, 2, 4, 6, 8 and 10 h. Upon removal, films were transferred to new wells and the cultures were washed with Mg²⁺- and Ca²⁺-free phosphate buffered saline (PBS) (Gibco) and trypsinized. Aliquots of dissociated cell suspensions were analyzed on a Coulter counter multisizer (Model 0646, Coulter Electronics, Hialeah, FL) and only particles between 6.4 and 24.3 µm in diameter were counted.

To assess the effect of initial cell seeding density on attachment, cells were plated on 50:50 PLGA films at different densities of 8,500, 15,000, 19,000, 25,000, and 44,000 cells/cm². Cell attachment was determined as described above after 3, 5 and 8 h in culture.

In a cell proliferation study, cells were seeded on both PLGA films and control TCPS as described in the attachment study, with an initial density of 25,000 cells/cm². The cultures were left in the incubator for 8 h before the original media were replaced with 1 mL of fresh culture medium for each well.
Media were changed daily for the first 3 days and then twice a day due to high metabolic needs of the cells. The media used for each well were also increased to 1.5 mL. Cell proliferation was determined by cell counts after 1, 3, 5 and 7 days in culture.

3.2.4 Specimen preparation for SEM

RPE cells were seeded on both PLGA films and control surface glass at a density of 25,000 cells/cm². After 4 h in culture, the cells were fixed with 2.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (pH 7.4) (Sigma) for 15 min and then rinsed with 0.1 M cacodylate buffer. The cells were stained with cold 1% osmium tetroxide (Polysciences, Warrington, PA) for 30 min and rinsed with cold ddH₂O. The samples were stored in a deep freezer (-80°C) and freeze-dried for 24 h before SEM observation.

3.2.5 Light microscopy

RPE cells subject to morphological evaluation were seeded on both PLGA films and TCPS and glass controls at a density of 25,000 cells/cm². The cells were viewed under a Diaphot inverted phase contrast microscope (Nikon, Garden City, NY) after 5 days of culture.

3.2.6 Immunocytochemistry of ZO-1

The formation of tight junctions between adjacent RPE cells was assessed by staining for zonula occluden-1 (ZO-1), a 225 kD peripheral membrane protein associated with tight junctions [Barry et al., 1995]. RPE cells were first seeded on both PLGA films and control glass coverslips placed in 24-
well plates, with an initial seeding density of 12,000 cells/cm². After confluent monolayers or a multilayer (on 50:50 PLGA) was obtained, cells were fixed with 4% paraformaldehyde (Sigma) in PBS for 30 min and rinsed three times with PBS. The cultures were then quenched with 10 mM glycine (Sigma) in PBS for 5 min, rinsed in PBS, permeabilized with 0.5% Igepal (Sigma) in PBS for 3 min, and blocked in 5% bovine serum albumin (BSA) (Sigma) in PBS for 30 min. Following a rinse in PBS (0.05% Igepal was added for this and subsequent solutions), the cells were incubated with 25 µg/mL rabbit anti-ZO-1 antibody (Zymed Laboratories, Inc., So. San Francisco, CA) in PBS for 1 h, rinsed three times in PBS, incubated with 5 µg/mL FITC-conjugated goat anti-rabbit IgG antibody (Zymed) in PBS, and finally rinsed twice with PBS. The cultures were then mounted in Vectashield (Vector Laboratories, Burlingame, CA) before examination under a confocal microscope (Zeiss LSM Axiocam, Carl Zeiss, Germany). Depth projection micrographs were constructed from 16 horizontal image sections through the culture.

3.2.7 Statistical analysis

All samples were collected in triplicates, except for the contact angle measurement (n = 9) and cell proliferation study (n = 8) and expressed as means ± standard deviations (SD). Single factor analysis of variance (ANOVA) was used to assess statistical significance of results. Scheffé’s method was employed for multiple comparison tests at a significance level of 95% and 99%.

3.3 RESULTS

3.3.1 PLGA films
Using the solvent casting technique described, polymer films of 50:50 and 75:25 PLGA were manufactured. The films had extremely smooth surfaces and were largely non-porous [Figure 3-1(a,b)]. They had a thickness of less than 10 μm [Figure 3-1(c,d)] which was uniform over most of the area. The advancing contact angles of 50:50 and 75:25 PLGA films were 59.3 ± 1.5º and 63.7 ± 1.5º, respectively (Figure 3-2). The films had moderate hydrophobicity as compared to hydrophilic glass (19.3 ± 2.5º) and hydrophobic TCPS (89.7 ± 5.5º) which are commonly used control surfaces for cell culture.

3.3.2 Cell attachment and proliferation

The dynamics of RPE cell attachment on 50:50 and 75:25 PLGA films was similar to that observed for TCPS control surface over an 8-h period (p > 0.05) (Figure 3-3). The percentage of attached cells increased over time and by 8 h after cell seeding, 98.0 ± 1.3% and 100.6 ± 7.2% of the plated cells attached to 50:50 and 75:25 PLGA films respectively (98.1 ± 2.5% for TCPS control). The subsequent decrease in cell number at 10 h on PLGA films was due to insufficient media left, since only 200 μl of cell solution were placed on each film. With this particular seeding configuration, the film was completely covered with cell solution which did not overflow. Cells were therefore only allowed to attach to PLGA and no cells were found to migrate to TCPS in the attachment and proliferation study. The optimal time for changing media was about 6 to 8 h post-seeding when the cells had already attached to the substrates.

Various initial cell seeding densities ranging from 8,500 to 44,000 cells/cm² were used to assess their effect on attachment to 50:50 PLGA films (Figure 3-4). The percentage of cell attachment increased significantly (p < 0.01) over an 8-h period for all the seeding densities used (Figure 3-4a). The
Figure 3-1. Scanning electron micrographs of top surfaces of (a) 50:50 and (b) 75:25 PLGA, and cross-sections of (c) 50:50 and (d) 75:25 PLGA films prepared by a solvent casting technique.
Figure 3-1. (Continued).
Figure 3-2. Advancing water contact angles of 50:50 and 75:25 PLGA, and control glass and TCPS. Error bars represent means ± SD for n = 9.
Figure 3-3. Percent of plated RPE cells that attached to 50:50 and 75:25 PLGA films, and control TCPS, with an initial seeding density of 27,000 cells/cm², as a function of in vitro culture time. Error bars represent means ± SD for n = 3.
Figure 3-4. RPE cells that attached to 50:50 PLGA expressed as (a) percent of cell attachment and (b) cell density, with different initial cell seeding densities, as a function of *in vitro* culture time. Error bars represent means ± SD for n=3.
optimal seeding density was 25,000 cells/cm², and the cell attachment for this
density was significantly higher than others ($p < 0.05$) for all time points tested.
At 8 h, cell attachment was significantly different between all cell seeding
densities ($p < 0.01$). Cell proliferation also occurred for the optimal seeding
density during this 8-h period, and 125.9 ± 2.5% of plated cells were attached to
the substrate. The cell density (31,200 ± 600 cells/cm²) was even higher than
28,200 ± 600 cells/cm², which was achieved using a much higher initial density
of 44,000 cells/cm² (Figure 3-4b).

RPE cell proliferation on PLGA films was studied after 1, 3, 5, and 7 days
in culture (Figure 3-5). Cell number increased significantly ($p < 0.01$) over the
7-day period on all the substrates tested. By day 7, cell numbers had increased
to 1122,700 ± 31,500 and 1004,500 ± 165,500 cells/cm² on 50:50 and 75:25
PLGA, respectively, corresponding to about 45-fold and 40-fold increases as
compared to the initial cell seeding density. About 34-fold increase was
observed for control TCPS. At all time points tested, the following order of cell
number on the substrates was observed: 50:50 PLGA > 75:25 PLGA > TCPS.
However, no significant difference between these substrates ($p > 0.05$) was
found except between 50:50 PLGA and TCPS at day 7. The cell densities on all
substrates studied exceeded confluency numbers (565,900 cells/cm² based on
a cell diameter of 15 μm) after 7 days and an RPE multilayer was observed in
each case. The experimentally determined confluent density was 538,400
cells/cm², very close to the estimated value.

3.3.3 Cell morphology

The three-dimensional morphology of RPE cells on 50:50 and 75:25
PLGA [Figure 3-6(b-d)] was similar to that on control surface glass (Figure 3-6a).
Figure 3-5. Proliferation kinetics of RPE cells cultured \textit{in vitro}, with an initial seeding density of 25,000 cells/cm\(^2\), on 50:50 and 75:25 PLGA films, and control TCPS. Error bars represent means ± SD for \(n = 8\).
Figure 3-6. Scanning electron micrographs of RPE cells on (a) glass, (b) 50:50, and (c, d) 75:25 PLGA after 4 h of cell seeding, with an initial density of 25,000 cells/cm². Magnification (a-c) x 350 and (d) x 2000.
Figure 3-6. (Continued).
During the first 4 h in culture, cells that just attached to the substrates retained a rounded morphology. Some cells already spread and were more elongated. Higher magnification view of cells attached to a 75:25 PLGA film (Figure 3-6d) revealed numerous filopodia projecting towards adjacent cells. Lamellipodia and filopodia were observed at the leading edge of a more spread cell. These structures can assemble into adhesion plaques that serve to hold the cell to the substratum. The approximate length of the majorities of spread RPE cells was 50 μm, and the diameter for rounded ones was 15 μm.

RPE cells grown to confluence on 50:50 and 75:25 PLGA films showed the characteristic cobblestone epithelial morphology (Figure 3-7). The cells were cuboidal in shape and their nuclei were centrally located. Occasional binucleated cells undergoing cell division were observed. Cells had lost pigmentation after extensive subculture, which was consistent with previously reported for this cell line [Davis et al., 1995]. Cell morphology on 75:25 PLGA was similar to TCPS and glass controls (data not shown), while cells on 50:50 PLGA appeared slightly different due to the underlying textures of the film surface.

3.3.4 Formation of tight junctions

The ability for RPE cells to form normal tight junctions in vitro was evaluated by immunocytochemical staining of ZO-1. Confocal depth projection micrographs of confluent monolayers of cells cultured on glass, 75:25 and 50:50 PLGA films demonstrated cells expressed ZO-1 protein mainly at the surfaces of cell-cell contact [Figure 3-8(a-c)]. The tight junctions between adjacent RPE cells were organized in a belt-like network. They were apically localized, as indicated by the relatively uniform green color of the confocal
Figure 3-7. RPE cells grown to confluence in vitro on (a) 50:50 and (b) 75:25 PLGA films after 5 days of culture, with an initial seeding density of 25,000 cells/cm². Magnification x 200.
Figure 3-8. Confocal micrographs of RPE cells cultured on (a) control glass, (b) 75:25, and (c,d) 50:50 PLGA films, with an initial seeding density of 12,000 cells/cm², and immunologically stained with ZO-1 after forming (a-c) monolayer or (d) multilayer.
micrographs. The cells cultured on all the substrates formed confluent monolayers and a cobblestone morphology was observed in each case. However, cell loss was evident on PLGA films probably due to the lift-off of some cells during extensive rinsing procedures. After prolonged culture, cells formed a multilayer characteristic of cell line cells, and the tight junctions were expressed at different levels (Figure 3-8d).

3.4 DISCUSSION

This study was conducted to answer the following questions: (1) Could thin films of PLGA of few microns be fabricated? (2) Did PLGA copolymer ratio affect RPE cell attachment and proliferation in vitro? (3) Did initial cell seeding density affect cell attachment? (4) Did PLGA copolymer ratio affect cell morphology and the formation of normal tight junctions in vitro?

Both 50:50 and 75:25 PLGA films less than 10 μm thick were manufactured using the modified solvent casting technique. The film thickness was more accurately controlled than that previously reported by varying the copolymer ratio, the concentration of the casting solution, and the total amount of polymer used for each coverslip [Thomson et al., 1996]. Thinner films may be obtained by using more dilute casting solutions. The fabricated films also had more uniform thickness and smooth surfaces. This was primarily because the rate of solvent evaporation was significantly decreased by partial coverage of samples during preparation. The PLGA films were moderately hydrophobic as measured by water contact angles.

To answer the second question, we cultured RPE cells on both 50:50 and 75:25 PLGA films. No significant effect of copolymer ratio on in vitro cell
attachment and proliferation was observed. Cell proliferation on 50:50 PLGA tended to be slightly higher than on 75:25 PLGA during a 7-day culture period. Both PLGA films were similar to TCPS control as substrates for RPE cell growth in vitro.

Similar PLGA films were previously used to culture human fetal RPE cells [Giordano et al., 1997]. Cell attachment on PLGA films was also comparable to that on TCPS, and cell proliferation on the films was similar to each other. However, cell proliferation on the films was significantly higher than on TCPS ($p < 0.05$). The discrepancy between the results of that study and the present one is primarily due to the difference in cell type and initial seeding densities.

A range of initial cell seeding densities between 8,500 and 44,000 cells/cm² was used to determine their effect on cell attachment. The optimal seeding density for RPE cells to attach to 50:50 PLGA was about 25,000 cells/cm². At very low cell seeding densities, the interactions between adjacent cells were limited and intercellular signaling pathways may be blocked. This may lead to cell death and decrease in cell attachment. At very high seeding densities, on the other hand, cell viability was also decreased due to limited space available for the attachment and spreading of these anchorage-dependent cells. The maximal initial cell seeding density was estimated to be 40,000 cells/cm² based on a cell length of 50 μm at two perpendicular directions.

Cell morphology was not affected by the PLGA copolymer ratio. In both cultures, RPE cells assumed characteristic cuboidal morphology when packed together, similar to that observed for in vivo cells [Feeney, 1978]. These cells may lack some of the structures and functions observed for in vivo cells [Davis
et al., 1995]. Nevertheless, they overcome the problem of limited supply and heterogeneity of human cells, and the ease of culture and fast growth render them useful for in vitro studies.

In addition to cell growth, differentiation of RPE cells cultured on 50:50 and 75:25 PLGA films was indicated by the formation of normal tight junctions in vitro. The tight junctions between adjacent RPE cells form a barrier to the paracellular passage of ions and metabolites [Bok, 1993; Chang et al., 1997; Rizzolo and Li, 1993]. They are also important in defining the apical and basolateral membrane domains and thus maintain proper cell orientation due to polarized distribution of plasma membrane proteins. The apical localization of tight junctions in vitro was consistent with the expression pattern reported for in vivo RPE cells [Koh, 1989].

RPE cells have been cultured on various other substrates including extracellular matrix (ECM) proteins such as collagen types I, III and IV, fibronectin, laminin, gelatin, Matrigel®, and vitronectin, oligopeptides such as GRGD and YIGSR, and poly(L-lysine) [Kennedy and Frank, 1993; Wagner et al., 1995; Zhou et al., 1993]. Direct comparisons of the results between these studies are difficult to make mainly due to the difference in RPE cell type, the initial cell seeding density, and the presence of growth activators or inhibitors. Fibronectin, laminin, and collagen type I were found to have similar or slightly better attachment affinity for RPE cells compared to TCPS control. Unlike these natural polymers which degrade by enzyme digestion, PLGA films degrade by simple hydrolysis into lactic and glycolic acid, and their degradation rates would not vary from one person to another if used for transplantation.

The in vitro degradation of PLGA films has also been studied in our laboratory (next chapter). After 7 days in PBS, about 90% of weight and 95% of
Mw remained for both 50:50 and 75:25 PLGA films. Similar PLGA degradation was expected during the cell proliferation study. The release of acidic degradation products did not seem to inhibit cell proliferation mainly due to the frequent change of culture media and the small quantity produced. The results from degradation study also showed that PLGA films retained sufficient strength and flexibility after 7 days and therefore can be used as carriers for RPE transplantation. Although the fabricated PLGA films were non-porous initially, they became microporous as degradation proceeded and gradually transformed into meshes with macropores. Therefore, diffusion would not be limited for PLGA/RPE transplants.

3.5 CONCLUSIONS

Human RPE cell line cells were cultured on synthetic biodegradable polymers as an in vitro model for methods to be used for RPE transplantation. RPE cells attached to, and proliferated on, both 50:50 and 75:25 PLGA films examined during a 7-day culture period. Initial cell seeding density affected attachment and there was an optimal value for 50:50 PLGA. Cultured RPE cells also retained their phenotype, as indicated by cuboidal morphology and apical expression of tight junctions at confluence. PLGA films are therefore promising substrates for RPE cell culture and transplantation.
CHAPTER 4

IN VITRO DEGRADATION OF THIN POLY(DL-LACTIC-CO/GLYCOLIC ACID) FILMS

4.1 INTRODUCTION

Synthetic biodegradable polymers have become very important as biomaterials for applications in tissue engineering and controlled drug delivery. Among these materials, poly(DL-lactic-co-glycolic acid) (PLGA) copolymers have been widely utilized either as temporary scaffolds for cell transplantation to regenerate various tissues or as carriers for delivery of bioactive molecules [Langer and Vacanti, 1993]. They can be easily processed into desired configuration and their physical, chemical, mechanical, and degradative properties can be engineered to fit a particular need [Lu and Mikos, 1996]. The biocompatibility of PLGA has also been demonstrated in many biological sites [Lu and Mikos, 1996].

PLGA copolymers in the form of thin films have been previously shown to provide suitable substrates for human retinal pigment epithelium (RPE) cell culture in vitro (chapter 3). RPE cells are essential in the maintenance of normal functions of photoreceptors [Bok, 1993]. Alteration in RPE structure and function is implicated in a variety of hereditary and degenerative retinal diseases, including age-related macular degeneration (ARMD) and Stargardt’s disease [Sheng et al., 1995]. Thin PLGA films may therefore be useful as temporary carriers for subretinal implantation of organized sheets of RPE. In addition, they are potentially applicable in guided tissue regeneration (GTR). PLGA films can serve as barriers to seal off a maxillofacial defect to prevent
other tissues from interfering with the regeneration of periodontal ligament and alveolar bone [Linde et al., 1993]. This has a further beneficial effect due to the osteoconductivity of PLGA [Ishaug et al., 1997].

Degradation properties are of crucial importance in biomaterial selection and design. The rate of degradation may affect a range of processes such as cell growth, tissue regeneration, drug release, and host response. PLGA has been known to degrade by simple hydrolysis of the ester bonds into lactic and glycolic acid, which are removed from the body by normal metabolic pathways (Figure 4-1). PLGA molecular weight, copolymer ratio, specimen size and configuration, and environment conditions have been identified as important factors affecting the kinetics of degradation [Agrawal et al., 1994, 1997; Göpferich, 1996; Li et al., 1990; Miller et al., 1977; Park, 1995; Reed and Gilding, 1981; Vert et al., 1994; Zhang et al., 1997]. However, the degradation of thin PLGA films has not been studied in detail. This work evaluates the effect of film thickness on the in vitro degradation behavior of PLGA thin films. Sample weight loss, molecular weight loss, dimensional, and morphological changes were investigated during 10 weeks of degradation in simulated body fluids.

4.2 MATERIALS AND METHODS

4.2.1 Raw Materials

Poly(DL-lactic-co-glycolic acid) (PLGA) of copolymer ratios of 75:25 (Birmingham Polymers, Birmingham, AL) and 50:50 (Medisorb®, Alkermes, Cincinnati, OH) were used in this study. The weight average molecular weights
Figure 4-1. Hydrolytic degradation reaction of poly(DL-lactic-co-glycolic acid) into its monomer units lactic acid and glycolic acid.
were 67,700 ± 1,100 [polydispersity index (PI) = 3.8 ± 0.2] for PLGA 75:25 and 43,900 ± 900 (PI = 4.1 ± 0.7) for PLGA 50:50, as determined by gel permeation chromatography. PI is equal to the ratio of weight average to number average molecular weight (Mw/Mn).

4.2.2 Film Fabrication

PLGA films were manufactured using a solvent casting technique as previously described [Lu et al., 1998]. Briefly, PLGA solution in chloroform (Aldrich Chemical Co, Milwaukee, WI) with known concentration was prepared. A certain amount of this solution was then cast onto a glass coverslip (diameter 12 mm) (Fisher Scientific, Pittsburgh, PA) placed on a leveled table in the fume hood. The samples were air-dried for 20 h and subsequently placed under high vacuum (10 μm Hg) for 24 h to remove any remaining solvent. Four types of PLGA films prepared using this method are detailed in Table 4-1.

The films (on glass) were then soaked in distilled deionized water (ddH₂O) for 90 min, and then carefully lifted off the glass with a razor blade. The films were subsequently air- and vacuum-dried to obtain the initial samples.

4.2.3 Experimental Design

The initial PLGA films were placed in glass scintillation vials (Fisher) each containing 20 mL of 0.2 M phosphate buffered saline (PBS) (Gibco, Grand Island, NY) (pH = 7.4). The samples were stored in a 37°C environment with shaking (~ 100 rpm) for various time periods up to 10 weeks. The PBS was changed every 8 h for the first 2 days, daily for the remainder of the week, on day 10 and day 14, and then weekly for the rest of the time frame. The pH of the
Table 4-1. Four types of PLGA films used in the degradation study prepared by a solvent casting technique.

<table>
<thead>
<tr>
<th>Film Code</th>
<th>PLGA Copolymer Ratio</th>
<th>Concentration of Casting Solution (mg/mL)</th>
<th>Volume of Casting Solution per Coverslip (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>75:25</td>
<td>15</td>
<td>75</td>
</tr>
<tr>
<td>F2</td>
<td>75:25</td>
<td>88</td>
<td>115</td>
</tr>
<tr>
<td>F3</td>
<td>50:50</td>
<td>17.5</td>
<td>70</td>
</tr>
<tr>
<td>F4</td>
<td>50:50</td>
<td>110</td>
<td>115</td>
</tr>
</tbody>
</table>
PBS was monitored during the course of degradation. At the end of each time point, five samples were removed from PBS and the diameter was measured immediately. These samples were then air-dried overnight and vacuum-dried for 24 h. The weight and Mw (and PI) of these samples were then recorded using five and three samples, respectively. All measurements were expressed as means ± standard deviation (SD) relative to the initial values.

4.2.4 Scanning Electron Microscopy (SEM)

Cross-sections of initial samples were gold-coated using a sputter coater (Pelco Sputter Coater 91000, model 3, Ted Pella, Redding, CA) set at 20 mA for a total time of 120 s (coating thickness approximately 40 nm). The sections were then observed with the SEM (JEOL JSM-5300 Scanning Microscope, Boston, MA) operated at 20 kV. Degraded samples were air- and vacuum-dried, and their surfaces were prepared for observation with the SEM.

4.2.5 Gel Permeation Chromatography (GPC)

The molecular weights of the initial films and degraded samples were determined using the GPC equipped with a differential refractometer (Waters, Model 410, Milford, MA) and an absorbance detector (Waters, Model 486). The samples were dissolved in chloroform (Aldrich) and eluted through a Phenogel 5 guard column (Model 1063376, 50 x 7.8 mm, 5 µm particle diameter, Phenomenex, Torrance, CA) and a Phenogel 5 linear column (Model 106338, 300 x 7.8 mm, 5 µm particle diameter, Phenomenex) at a flow rate of 1 mL/min. Polystyrene standards (Polysciences, Warrington, PA) were used to obtain a primary calibration curve. The values of the Mark-Houwink constants for PLLA,
K = 5.45 x10^3 mL/g and α = 0.73, were utilized to determine the molecular weights of PLGA samples [Mikos et al., 1994b].

4.3 RESULTS

4.3.1 Initial films

Using the solvent casting technique previously described, four types of PLGA films were manufactured (Table 4-2). The weight average molecular weight (Mw) and polydispersity indices (PI) of these films were determined after fabrication. Polymer degradation during processing resulted in decrease in Mw and Mn, and increase in PI relative to the values of raw materials. For the same polymer composition, the thin films had much lower Mn than the corresponding thick ones, indicating increased degradation of the thin films during sample preparation. The thickness levels were 5 to 10 μm for thin PLGA films, and 85 to 100 μm for thick films. All the films maintained a diameter of 12 mm after fabrication.

4.3.2 Weight Loss

The dynamics of weight loss for all the PLGA films was similar to each other (Figure 4-2). Initially the weight remained relatively constant for several weeks, then a dramatic decrease in mass was observed. Thin PLGA 75:25 films (F1) maintained 89.0 ± 1.2% of the day 0 value after 6 weeks of degradation in PBS, and only 64.4 ± 1.9% remained by 10 weeks (Figure 4-2a). The weight for thick PLGA 75:25 films (F2) was 84.0 ± 8.0% at 6 weeks, which reduced to 28.9 ± 1.9% by 10 weeks, due to significant decrease in mass after 6
<table>
<thead>
<tr>
<th>Film Code</th>
<th>Weight (mg)</th>
<th>Mw (Da)</th>
<th>Mn (Da)</th>
<th>PI</th>
<th>Thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.25±0.02</td>
<td>62,900±2,400</td>
<td>4,300±550</td>
<td>14.8±1.9</td>
<td>5-10</td>
</tr>
<tr>
<td>F2</td>
<td>12.76±0.87</td>
<td>58,200±1,900</td>
<td>9,200±1,200</td>
<td>6.4±0.8</td>
<td>85-100</td>
</tr>
<tr>
<td>F3</td>
<td>1.35±0.01</td>
<td>37,300±1,000</td>
<td>4,200±500</td>
<td>8.9±1.3</td>
<td>5-10</td>
</tr>
<tr>
<td>F4</td>
<td>13.35±0.35</td>
<td>34,300±300</td>
<td>7,600±150</td>
<td>4.5±0.1</td>
<td>85-100</td>
</tr>
</tbody>
</table>
Figure 4-2. Percent of weight remaining compared to day 0 values as a function of degradation time for (a) PLGA 75:25 and (b) PLGA 50:50 films: F1 (□), F2 (○), F3 (○), and F4 (△). The different types of PLGA films are presented in Table 4-1. Error bars represent means ± SD for n = 5.
weeks (Figure 4-2a). Thick PLGA 50:50 films (F4) also showed faster mass loss than thin films (F3). After 6 weeks of degradation, 75.8 ± 4.3% and 34.6 ± 1.8% of day 0 mass remained for F3 and F4, respectively (Figure 4-2b). No further measurements were performed for F3 due to the breakage of thin PLGA 50:50 films during retrieval. In addition, PLGA 50:50 films (F3 or F4) showed faster and greater mass loss compared to PLGA 75:25 films (F1 or F2) with similar thickness level.

4.3.3 Molecular Weight Loss

Unlike the profile for mass loss, the molecular weight of all the PLGA films decreased immediately after placement in PBS, and continued to decrease throughout the time course (Figure 4-3). Significant loss of Mw was observed for PLGA 75:25 films, with only 22.1 ± 4.5 and 6.4 ± 0.3% remained for F1 and F2, respectively, after 10 weeks of degradation (Figure 4-3a). The Mw of F4 also decreased to 11.3 ± 0.6% of the day 0 value. The Mw of F3 was measured only up to 8 weeks (20.0 ± 0.5%). In addition, thick films (F2 or F4) showed faster and greater decrease in Mw for both PLGA 75:25 and 50:50 than thin ones (F1 or F3). At 4 weeks, 68.9 ± 8.9% of Mw at day 0 remained for F1, and only 39.0 ± 4.0% left for F2. Similarly, 31.1 ± 3.2 and 11.0 ± 0.1% remained for F3 and F4, respectively. The molecular weight loss for PLGA 50:50 films was also faster than PLGA 75:25 films, provided the film thickness was similar.

The gel permeation chromatograms of the degraded PLGA 50:50 films suggested that degradation proceeded heterogeneously for both thin and thick films (Figure 4-4). Similar chromatograms were observed for PLGA 75:25 and are not shown. Thin PLGA 50:50 (F3) had a broad single peak at 33,300 before degradation, while bimodal peaks at 19,100 and 2,000 were observed for films
Figure 4-3. Percent of Mw remaining compared to day 0 values as a function of degradation time for (a) PLGA 75:25 and (b) PLGA 50:50 films: F1 (□), F2 (○), F3 (○), and F4 (Δ). The different types of PLGA films are presented in Table 4-1. Error bars represent means ± SD for n = 3.
Figure 4-4. Gel permeation chromatograms of (a) thin and (b) thick PLGA 50:50 films showing the changes in the molecular weight distribution during degradation: at day 0 (1), after 21 days (2), and 56 days (3).
after 21 days of degradation in PBS, and 11,700 and 700 after 56 days (Figure 4-4a). This trend was not prominent for thick PLGA 50:50 films (F4) (Figure 4-4b), probably due to the overlapping of peaks with close molecular weights. However, a secondary peak was detected at 700 in addition to the main peak at 3,700 on day 56. These results indicate the presence of both fast and slowly degrading domains, corresponding to the inner and surface film layers. Faster peak shift towards lower molecular weight for F4 further supports the observation that thick films degraded faster than thin ones. In addition, the broad molecular weight distribution in the range of a few hundred to thousand were seen for day 0 films, compared to the narrower distribution of the raw material. The presence of secondary peaks for degraded samples in this range suggested that the change in molecular weight distribution during film processing followed the same mechanism of sample degradation in aqueous solution.

The polydispersity indices of the PLGA films also changed during degradation (Figure 4-5). Thin PLGA films (F1 or F3) exhibited much broader molecular weight distribution than corresponding thick ones (F2 or F4) after processing. The PI of F1 and F3 increased to a maximum of 26.0 ± 2.0 and 11.7 ± 1.4 after 7 days, and decreased gradually to 9.4 ± 1.0 (10 weeks) and 4.5 ± 0.5 (8 weeks) respectively. Initial increase in PI was resulted from random chain scission during degradation, and the subsequent decrease was due to the decrease in Mw and the release of some of the degradation products. Little variation of PI was measured for thick films, and the PI of F2 and F4 decreased to 4.0 ± 0.2 and 3.9 ± 0.0, respectively, after 10 weeks.
Figure 4-5. Variation of polydispersity indices of PLGA films with degradation time: F1 (□), F2 (○), F3 (◊), and F4 (△). The different types of PLGA films are presented in Table 4-1. Error bars represent means ± SD for n = 3.
4.3.4 pH Variation

Little change in pH of the PBS solution was measured for up to 8, 6, 6, and 3 weeks for PLGA film types F1, F2, F3, and F4, respectively (Figure 4-6). This was followed by a rapid drop in pH, due to the release of acidic polymer degradation products to the solution. The time course of rapid pH drop corresponded roughly to the dramatic weight loss showed in Figure 4-2.

4.3.5 Dimensional Change

All the fabricated PLGA films had an diameter of 12 mm (the diameter of the coverslip used for solvent casting). The diameter of thin PLGA 75:25 films (F1) decreased gradually during degradation, with relatively faster decrease after 6 weeks (Figure 4-7a). Slight decrease in diameter was also observed for thick PLGA 75:25 films (F2) for the first 4 weeks, followed by rather rapid decrease up to 8 weeks. Slight swelling of F2 samples then occurred, which was not seen for F1 samples due to the short time frame. By 10 weeks, the diameter had decreased to 87.6 ± 3.5 and 79.2 ± 7.6% of the initial value for F1 and F2, respectively. Relatively rapid initial shrinkage of both PLGA 50:50 films was measured (Figure 4-7b). Slight swelling for thin films (F3) was also seen, followed by rapid decrease in diameter. Significant swelling occurred at 6 weeks, probably due to the rapid release of degradation products. This phenomenon was also observed for thick films (F4) at an earlier stage of 4 weeks, followed by little change in sample diameter up to 10 weeks. The measurable sample diameter at the end of degradation was 9.7 ± 0.3 and 11.1 ± 0.4 mm for F3 (6 weeks) and F4 (10 weeks), respectively.
Figure 4-6. Variation of pH of the PBS solution with degradation time for (a) PLGA 75:25 and (b) PLGA 50:50 films: F1 (□), F2 (○), F3 (○), and F4 (△). The different types of PLGA films are presented in Table 4-1. Error bars represent means ± SD for n = 5.
Figure 4-7. Variation of film diameter with degradation time for (a) PLGA 75:25 and (b) PLGA 50:50 films: F1 (□), F2 (○), F3 (○), and F4 (△). The different types of PLGA films are presented in Table 4-1. Error bars represent means ± SD for n = 5.
4.3.6 Film Morphology

The gross appearance of all the PLGA films changed over time during degradation (Figure 4-8). The initially transparent films became whitish due to water absorption. Thin and thick PLGA 75:25 films (F1 and F2) appeared whitish after 42 and 7 days respectively. Thin PLGA 50:50 films (F3) remained clear until 28 days. Thick PLGA 50:50 films (F4) appeared whitish as early as 1 day in PBS. This change was reversible after vacuum-drying until day 21, after which the absorbed water was believed to be bound to the polymer matrix [Burg and Shalaby, 1997]. All the films became more stiff and brittle after placement in PBS. However, F1 and F2 remained intact after 28 days while F3 and F4 started to break since day 21 and day 10 respectively. The fragments of F3 could not be fully retrieved for further investigation beyond 6 weeks except for Mw measurement. Thick films (F2 and F4) changed to loose structures with macro pores at late stages of degradation.

All four types of PLGA films were initially non-porous with smooth surfaces, as examined under scanning electron microscopy (representing micrograph for F2 is shown in Figure 4-9a). However, extensive micropores were developed during degradation. The cross-section of F2 revealed numerous pores in the range of 10 to 40 µm after 42 days of degradation (Figure 4-9b). However, the top surfaces of both F1 and F2 were non-porous [Figure 4-9 (a,b), respectively]. The PLGA 50:50 films were more brittle compared to 75:25 films. The porous inner layers of F3 and F4 films were revealed in Figure 4-9 (e,f), respectively, due to the breakage of some part of the surface layer. Part of the non-porous skin can be clearly seen in Figure 4-9f. The inner layer of F4 exhibited pores of a broad size ranging from a few to 100
Figure 4-8. Gross appearance of dried PLGA films (F1, F2, F3, and F4) at day 0, after 14, 28, and 70 days of degradation in PBS. The different types of PLGA films are presented in Table 4-1.
Figure 4-9. Scanning electron micrographs of four types of PLGA films: cross-sections of F2 at (a) day 0 and (b) after 42 days of degradation in PBS, and top surfaces of (c) F1, (d) F2, (e) F3, and (f) F4 on day 42. The different types of PLGA films are presented in Table 4-1. The micrographs (a) and (b) were taken at an angle to show also the top surfaces (T) of the films.
μm. These results further suggested the heterogeneous bulk degradation of the PLGA films.

4.4 DISCUSSION

This study was designed to measure the changes in sample weight, molecular weight, dimension, and morphology of thin PLGA films over a 10-week period of in vitro degradation in PBS to determine the effects of copolymer ratio and film thickness on degradation. Specifically, four types of PLGA films of copolymer ratios of 75:25 and 50:50 with thickness levels of 10 and 100 μm were used for investigation.

Changing the PLGA copolymer ratio from 75:25 to 50:50 without altering the film thickness increased degradation. The higher content of hydrophilic glycolic units in PLGA 50:50 facilitated the absorption and diffusion of water and thus hydrolysis of the ester bonds. This was consistent with the results published by other investigators using different specimen configurations [Miller et al., 1977; Reed and Gilding, 1981].

The effects of film thickness on the degradation profiles of both PLGA 75:25 and 50:50 films depended on the material treatment. Degradation occurred during film fabrication due to the sample exposure to air (solvent casting) and water (film lifting). Thin PLGA films degraded faster than thick ones with the same copolymer ratio, since thin films had a greater surface area to volume ratio and thus greater extent of water uptake. This was confirmed by the observation that at day 0, thin films (F1 or F3) had much broader molecular weight distribution than the corresponding thick films (F2 or F4). In addition, although the processed thin films had slightly higher Mw, their Mn were
significantly lower than the thick ones. These results also suggested that for thin PLGA films, the fabrication process should be carefully controlled since it has a great effect on the film degradation.

Increasing the film thickness level from 10 to 100 μm, however, resulted in faster degradation in PBS over the 10-week period for both PLGA 75:25 and 50:50 films. This was evident from accelerated weight and molecular weight loss profiles. The faster degradation of thick films can be explained by the greater extent of autocatalytic effect. The intermediate degradation products were trapped inside the film before their molecular weights decreased to a critical value of about 1,100 and became soluble in water [Park, 1995]. The accumulation of carboxylic groups over time in the specimen center led to faster central degradation, and therefore overall film degradation. This was less evident for thin films as expected. The difference in the initial molecular weight was not taken into account by using the normalized weight and molecular weight relative to the day 0 values.

All the PLGA films degraded by heterogeneous bulk degradation. The bimodal GPC curves may be explained by the presence of slowly degrading surface layers and fast degrading central layers, which was confirmed by their differential morphology under SEM. This phenomenon has been previously demonstrated using large parallelepiped specimens of 2 mm thick [Li et al., 1990]. The difference in degradation between specimen surface and center typically results in an empty shell with a thickness of about 200 μm after several weeks of degradation. In this study, much thinner PLGA films with thickness levels of 10 and 100 μm were used, and the autocatalytic effect was still observed.
A qualitative model based on diffusion-reaction phenomena was previously proposed for the degradation of poly(α-hydroxy esters) [Grizzi et al., 1995]. The model claims that heterogeneous bulk degradation, and thus skin-layer formation, occurs only when the size of the specimen is above a critical value. The critical thickness (or diameter) for poly(DL-lactic acid) (PDLLA) films (or microspheres) was determined to be 200-300 μm [Grizzi et al., 1995]. This conclusion was based mainly on the observation of uni-modal molecular weight distribution during degradation when sample size was smaller than the critical value.

The results obtained from our study do not support this model. This may be because of the different polymer used. However, other studies using PDLLA and PLGA microspheres did not agree with this model either [Chen et al., 1997b; Park, 1994, 1995]. Heterogeneous bulk degradation was observed for PDLLA and PLGA 50:50 microspheres of diameter less than 10 μm [Park, 1994, 1995]. In addition, the same phenomenon was demonstrated for both PLGA 75:25 and 50:50 microspheres of diameter ranging from 50 to 70 μm [Chen et al., 1997b].

Degradation properties of thin PLGA films are important for their applications. Based on Mw, the half-lives of F1, F2, F3, and F4 are 6, 3, 3, and 2 weeks respectively. Thin PLGA 50:50 could be used as temporary carriers for subretinal implantation of organized sheets of retinal pigment epithelium. These films would limit the distortion of retina and minimize the amount of degradation products released. Compared to thin PLGA 75:25 films which were also shown as suitable substrates for RPE cell culture in vitro [Lu et al., 1998], their rapid degradation would be beneficial to allow the re-association of regenerated RPE layers with the surrounding tissues. In addition, all
biodegradable PLGA films are potentially useful as barriers in guided tissue regeneration. The selection of a particular film for the regeneration of periodontal ligament or alveolar bone depends mainly on its degradation rate. Significant amount of tissue needs to be regenerated before complete polymer degradation.

4.5 CONCLUSIONS

Poly(DL-lactic-co-glycolic acid) films were formulated with varying copolymer ratios and thickness levels for an in vitro degradation study. Both parameters were seen to have a significant effect on the weight loss and molecular weight loss during 10 weeks of degradation in PBS. PLGA 50:50 degraded faster than 75:25 films with similar thickness. Increasing the thickness levels from 10 to 100 μm accelerated both the weight and molecular weight loss over this time frame. All the PLGA films degraded by heterogeneous bulk degradation. The differential morphology of the porous inner layer and non-porous surface layer was due to autocatalysis in the specimen center. These thin PLGA films may be potentially useful in retinal pigment transplantation and guided tissue regeneration.
CHAPTER 5
DELIVERY OF TRANSFORMING GROWTH FACTOR-β1 FROM POLY(DL-LACTIC-CO-GLYCOLIC ACID)/POLY(ETHYLENE GLYCOL) MICROPARTICLES

5.1 INTRODUCTION

Recent advances in tissue engineering have offered the possibility to create completely natural living tissue and replace failing or malfunctioning organs [Langer and Vacanti, 1993]. Controlled release of bioactive molecules such as cytokines, growth factors, hormones, angiogenic factors, immunosuppressant drugs, or inhibitors of cell proliferation has become an important aspect of tissue engineering [Lanza et al., 1997]. Controlled delivery of these molecules allows modulation of cellular growth and tissue formation.

Transforming growth factor β1 (TGF-β1) has been studied as a potential induction factor for bone tissue engineering [Anderson and Shive, 1997; Beck et al., 1991; Gombotz et al., 1993; Hock et al., 1990; Hollinger and Leong, 1996; Nimni, 1997; Noda and Camilliere, 1989; Rosier et al., 1998]. TGF-β1, a member of the TGF-β superfamily, is a disulfide-linked homodimer consisting of two 112 amino acid residue polypeptides and has a molecular weight of approximately 25,000 Da. It is a multifunctional protein that regulates many aspects of cellular activity including cell proliferation, differentiation, and extracellular matrix metabolism, in a time- and concentration-dependent fashion [Nimni, 1997]. Controlled release of TGF-β1 to bone defect may therefore be beneficial to induce a bone regeneration cascade.
Microparticles made of biodegradable polymers have been widely utilized as vehicles for drug delivery. They can be implanted at the afflicted site during surgery or injected as a suspension to the injured area. Alternatively, the microparticles can be impregnated into polymer scaffolds and then transplanted. Among these polymers, poly(DL-lactic-co-glycolic acid) (PLGA) copolymers have been extensively studied as microparticulate carriers for many bioactive molecules [Anderson and Shive, 1997; Cleek et al., 1997a]. They are biocompatible, biodegradable, and approved for certain human clinical uses [Anderson and Shive, 1997; Holland et al., 1986]. In addition, the degradation rates of PLGA can be altered for periods of days to years by varying the polymer molecular weight, the ratio of lactic to glycolic acid in the copolymer, or the structure of the microparticles [Anderson and Shive, 1997; Hollinger and Leong, 1996].

The drug release rates from the PLGA microparticles are affected by various factors including the structure, size, and solubility of the encapsulated molecules as well as the structure and degradation of the polymer. Previous studies in our laboratory have demonstrated that the drug release profiles can be further modulated by blending poly(ethylene glycol) (PEG), a hydrophilic and water-soluble polymer, with PLGA [Cleek et al., 1997b]. Compounds incorporated into these PLGA/PEG microparticles showed extended linear release profiles. In addition, the release of compounds is also affected by co-encapsulation of other molecules in the microparticle formulations [Krewson et al., 1996].

The drug release profiles are also dependent on the release medium which may have profound effects on the structure, solubility, diffusion rate, and activity of the compound. Most of the in vitro release studies in the literature are
carried out in pH 7.4 phosphate buffered saline (PBS) to simulate body fluids. However, acidic environment can occur naturally in vivo. For example, inflammatory response following implantation often result in decrease in local pH [Anderson and Shive, 1997]. The release of acidic degradation products from PLGA microparticles has also been shown to lower the pH [Lu et al., 1999]. It is therefore of great importance to assess the effects of environmental pH on the degradation of and protein release from biodegradable polymers.

In this study, TGF-β1 was incorporated into PLGA/PEG microparticles. Fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) was co-encapsulated to serve as a porogen to enhance the release of TGF-β1. We examined the effects of PEG content and pH on the release kinetics of FITC-BSA and TGF-β1 and the degradation of PLGA in vitro.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Poly(DL-lactic-co-glycolic acid) (PLGA) of 50:50 lactic to glycolic acid copolymer ratio (Medisorb®, Alkermes, Cincinnati, OH) was used in this study. The polymer weight average molecular weight (Mw) was determined by gel permeation chromatography to be 46,700 ± 500 Da [polydispersity index (PI) = 1.73 ± 0.05; PI is equal to the ratio of the weight average to the number average molecular weight (Mw/Mn)] Poly(ethylene glycol) (PEG) (10,700 Da) and poly(vinyl alcohol) (PVA, 88% mole hydrolyzed, 13,000-23,000 Da) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Recombinant human transforming growth factor β1 (TGF-β1) (25,000 Da) was supplied by R&D
Systems (Minneapolis, MN) as lyophilized powder. Fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) (68,000 Da) was purchased from Sigma Chemical Co. (St. Louis, MO). Phosphate buffered saline (PBS) (pH = 7.4) was acquired from Gibco (Grand Island, NY). Certified pH buffers of pH 3, 5, and 7.4, dichloromethane, and isopropyl alcohol (IPA) were purchased from Fisher Scientific (Pittsburgh, PA).

5.2.2 Microparticle Preparation

Microparticles of PLGA/PEG blends containing 0, 1, or 5% PEG (w/w) were fabricated using a double-emulsion-solvent-extraction technique ((water-in-oil)-in-water) as previously described [Cleek et al., 1997b]. Briefly, PLGA and PEG with a total amount of 247.5 mg were first dissolved in 1 mL dichloromethane. Then, 1 mg FITC-BSA and 1.5 μg TGF-β1 were dissolved in 125 μL distilled deionized water (ddH₂O) and injected into a flint glass tube containing the polymer solution. The entire mixture was emulsified on a vortexer (Vortex Genie 2, Scientific Industries, Bohemia, NY) for 1 min. This solution was then re-emulsified in 100 mL of 0.3% aqueous PVA solution resulting in a double emulsion. The second emulsion was added to 100 mL of 2% aqueous IPA solution and maintained on a magnetic stirrer for 1 h. The extraction of the dichloromethane to the external alcoholic phase resulted in precipitation of the dissolved polymer and subsequent formation of microparticles. The formed microparticles were finally collected, centrifuged, lyophilized to dryness, and stored at -40°C before use.

5.2.3 Microparticle Characterization
Size distribution of the microparticles was measured with a Coulter counter multisizer (Model 0646, Coulter Electronics, Hialeah, FL) after suspending microparticles in an Isoton II solution (Coulter Electronics). The microparticle diameter was reported as a mean ± standard deviation (SD).

The entrapment efficiency of the proteins was determined by normalizing the amount actually entrapped with the starting compounds, using a solvent extraction technique [Cleek et al., 1997b]. Approximately 20 mg of microparticles were allowed to dissolve in 1 mL dichloromethane for 6 h at 37°C. The entrapped FITC-BSA and TGF-β1 were then extracted from the organic phase to the aqueous phase by incubation with 1 mL PBS for an additional 24 h. The concentration of FITC-BSA was determined by absorption at 496 nm in an Amino-Bowman Series 2 Luminescence Spectrometer (SLM-Aminco, Urbana, IL). Each calibration curve was constructed for FITC-BSA using serial dilutions in PBS or the pH buffers.

The concentration of TGF-β1 was analyzed by enzyme-linked immunosorbent assay (ELISA) using a TGF-β1 quantikine kit (R&D Systems). Briefly, 200 µl of the sample solution were added to each well of the kit microtitre plate. After a 3 h incubation, the solution was decanted from the plate, rinsed several times with a buffer solution from the kit, and a secondary antibody solution was added to complex the TGF-β1 to the base of the plate. A colorized reagent was then added and the amount of TGF-β1 present in each sample was quantified using a microplate reader (Model EL310, Bio-Tek Instruments, Burlington, VT) by measuring the absorbance at 450 nm which was corrected using the absorbance at 540 nm. Each calibration curve was generated for each of the buffers from standards ranging from 0 to 2000 ng/mL TGF-β1 that were run with each kit.
5.2.4 FITC-BSA and TGF-β1 Release Kinetics

The release kinetics of proteins from the PLGA/PEG microparticles were studied under six experimental conditions. Microparticles of approximately 20 mg with varied initial PEG contents (0, 1, or 5%) were placed into 1.8 mL microvials containing 1 mL pH 7.4 PBS. Additional microparticles containing 5% PEG were incubated in certified buffers (pH 3, 5, or 7.4). All samples were maintained at 37°C with shaking (~ 100 rpm) for various time periods up to 28 days.

At the end of each time point (in days): 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, the microparticle solution was centrifuged and the supernatant was collected for analysis. The pellet was re-suspended in fresh medium. The samples were assayed for FITC-BSA concentration using spectrophotometry as described above. The amount of TGF-β1 at 0.25, 0.5, 1, 2, 4, 7, 10, 14, 20, and 28 days was quantified by ELISA as previously described. All results were expressed as means ± standard deviation (SD) for n = 4.

5.2.5 In Vitro Degradation of Microparticles

The degradation of PLGA/PEG microparticles (loaded with TGF-β1 and FITC-BSA) were studied under the same conditions as in the protein release experiments. After 0, 1, 4, 7, 10, 14, 18, 22, and 28 days, the microparticle samples were collected, frozen, and vacuum-dried for 24 h before further analysis.

The PLGA molecular weight distribution of microparticles was determined using the gel permeation chromatography (GPC) (Waters, Milford,
MA) equipped with a differential refractometer (Waters, Model 410, Milford, MA) and an absorbance detector (Waters, Model 486). The samples were dissolved in chloroform (Sigma Chemical Co., St. Louis, MO) and eluted through a Phenogel 5 guard column (Model 1063376, 50 x 7.8 mm, 5 μm particle diameter, Phenomenex, Torrance, CA) and a Phenogel 5 linear column (Model 106338, 300 x 7.8 mm, 5 μm particle diameter, Phenomenex) at a flow rate of 1 mL/min. Polystyrene standards (Polysciences, Warrington, PA) were used to obtain a primary calibration curve. The values of the Mark-Houwink constants for PLLA, K = 5.45 x10\(^{-3}\) mL/g and α = 0.73, were utilized to determine the molecular weights of PLGA samples [Mikos et al., 1994b]. The pH of the incubation medium was monitored throughout the experiment using a pH meter (Fisher). All measurements were expressed as means ± SD for n = 4.

The morphology of degrading microparticles at 0, 14, and 28 days was assessed by scanning electron microscopy (SEM). Freeze-dried microparticle samples were mounted on a copper tape and gold-coated using a sputter coater (Pelco Sputter Coater 91000, model 3, Ted Pella, Redding, CA) set at 20 mA for a total time of 120 s (coating thickness approximately 40 nm). The samples were then observed with the SEM (JEOL JSM-5300 Scanning Microscope, Boston, MA) operated at 20 kV.

The distribution of FITC-BSA within the microparticles after 0, 14, and 28 days of degradation was examined by fluorescent microscopy. Microparticles were dispersed on glass coverslips and representative fluorescence image sections were taken using a confocal microscope (Zeiss LSM Axiovert, Carl Zeiss, Germany).
5.3 RESULTS

5.3.1 Initial Microparticle Characterization

PLGA/PEG microparticles were fabricated using a double-emulsion-solvent-extraction technique previously described. The average sizes of the microparticles containing 0, 1, and 5 wt% PEG were 20.0 ± 11.9, 18.8 ± 9.9, and 23.3 ± 13.7 μm, respectively. The entrapment efficiencies of FITC-BSA and TGF-β1 in the microparticles varied with the PEG content [Figure 5-1(a,b), respectively]. The reduced entrapment efficiency of both FITC-BSA and TGF-β1 in microparticles with 5% PEG may be due to the leaching of the compound with soluble PEG during microparticle fabrication.

Degradation of PLGA occurred during the microparticle fabrication process due to the contact with aqueous solutions. The degradation resulted in decrease in Mw of PLGA and increase in PI relative to the values of the raw material (Table 5-1).

5.3.2 In Vitro Protein Release Kinetics

The release kinetics of FITC-BSA depended on both PEG content in the PLGA/PEG microparticles (Figure 5-2a) and the pH of the incubation medium (Figure 5-2b). The release profiles were qualitatively similar for all conditions tested and were comprised of four phases: (a) an initial burst effect occurred during the first 24 h, (b) a linear steady release phase that last up to day 8, (c) a temporary plateau phase over the next 6 days, and (d) a second longer lasting linear phase (days 15-28). Quantitative differences in the release profiles were observed indicating that increasing microparticle PEG content or incubation
Figure 5-1. The entrapment efficiencies of (a) FITC-BSA and (b) TGF-β1 in PLGA/PEG microparticles with different PEG content. Error bars represent means ± SD for n = 3.
Table 5-1. Weight average molecular weight (Mw) and polydispersity index (PI) of PLGA after fabrication into microparticles. The Mw and PI of the raw material was 46,700±500 Da and 1.73±0.05, respectively.

<table>
<thead>
<tr>
<th>Initial PEG (wt%)</th>
<th>Mw (Da)</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>42,200±5,500</td>
<td>6.83±2.10</td>
</tr>
<tr>
<td>1</td>
<td>43,100±1,800</td>
<td>6.66±1.71</td>
</tr>
<tr>
<td>5</td>
<td>43,200±3,200</td>
<td>6.41±1.38</td>
</tr>
</tbody>
</table>
Figure 5-2. Dependence of cumulative release of FITC-BSA from PLGA/PEG microparticles on (a) the PEG content and (b) the buffer pH. Error bars represent means ± SD for n = 4.
medium acidity resulted in reduced FITC-BSA release rate. For different PEG contents, the difference occurred during the initial burst (phase a) and the first constant release phase (phase b), whereas medium pH affected more phases a and d. The average release rates for the linear phases (b, c, and d) are given in Table 5-2. The release rates during phase c were small compared to the other phases and therefore this phase may be considered as a temporary plateau.

Both microparticle PEG content (Figure 5-3a) and medium acidity (Figure 5-3b) affected the TGF-β1 release from the microparticles. About 2.9 ± 0.2, 2.7 ± 0.3, and 1.9 ± 0.3 ng of the compound per mg of microparticle were released to PBS after 2 days for initial PEG content of 0, 1, and 5%, respectively. While in buffers of pH 3, 5, and 7.4, about 0.6 ± 0.1, 0.9 ± 0.4, and 1.9 ± 0.2 ng of TGF-β1 per mg of microparticle were released from microparticles containing 5% PEG, respectively. At pH 7.4 [either PBS (Figure 5-3a) or certified buffer (Figure 5-3b)], the initial burst was followed by a linear phase of slow release rate reaching a plateau. In acidic pH however, the initial burst was followed by a fast linear release phase (days 1-8) and then by a slower one (days 9-28). In general, the dependence of the TGF-β1 release profiles on microparticle PEG content and medium pH were qualitatively similar to that of FITC-BSA release profiles: the compound was released faster at higher pH or from microparticles with lower PEG content.

5.3.3 In Vitro Degradation of Microparticles

The dynamics of PLGA Mw loss in pH 7.4 PBS for microparticles with varied PEG content was similar to each other (Figure 5-4a). The Mw decreased continuously throughout the time course, and by day 28, only 35.6 ± 0.5, 34.4 ± 1.3, and 29.5 ± 1.5% of the day 0 Mw remained for microparticles with 0, 1, and
Table 5-2. The release rates of FITC-BSA from PLGA/PEG microparticles at the linear phases of the release profiles for the different experimental conditions (given in mg of FITC-BSA per mg of microparticle per day).

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Phase b (days 2-8)</th>
<th>Phase c (days 9-14)</th>
<th>Phase d (days 15-28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial PEG (wt%)</td>
<td>Buffer pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.4</td>
<td>0.138</td>
<td>0.015</td>
</tr>
<tr>
<td>1</td>
<td>7.4</td>
<td>0.097</td>
<td>0.011</td>
</tr>
<tr>
<td>5</td>
<td>7.4</td>
<td>0.083</td>
<td>0.009</td>
</tr>
<tr>
<td>5</td>
<td>3.0</td>
<td>0.064</td>
<td>0.013</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>0.062</td>
<td>0.012</td>
</tr>
<tr>
<td>5</td>
<td>7.4</td>
<td>0.060</td>
<td>0.017</td>
</tr>
</tbody>
</table>
Figure 5-3. Dependence of cumulative release of TGF-β1 from PLGA/PEG microparticles on (a) the PEG content and (b) the buffer pH. Error bars represent means ± SD for n = 4.
Figure 5-4. Percent of PLGA Mw remaining compared to day 0 values for different (a) initial PEG content and (b) buffer pH as a function of *in vitro* degradation time. Error bars represent means ± SD for n = 4.
5% PEG. Degradation profiles of PLGA microparticles with 5% initial PEG content in buffers with varied pH are shown in Figure 5-4b. Microparticles degraded much faster at lower pH. By day 28, the remaining Mw of PLGA placed in acidic environment was 3.1 ± 0.3% for pH 3 buffer and 14.0 ± 0.8% for pH 5 buffer, which was much less than that in pH 7.4 buffer (25.6 ± 4.6%).

A drop in the pH of the PBS (pH 7.4) was observed during the degradation of microparticles, due to the release of acidic degradation products into the incubation media (Figure 5-5a). By day 28, the pH decreased to 7.12, 7.07, and 6.99 for microparticles with 0, 1, and 5% PEG content, respectively. This was consistent with faster degradation of PLGA with increasing PEG content observed in Figure 5-4a. Little variation of pH (within ± 0.1) was observed for microparticles incubated in various pH buffers (Figure 5-5b) due to their stronger buffering capacities.

In a control experiment, microparticles with 5% PEG were incubated in PBS of pH 7.2 and 4.4. It was interesting to note that after 30 days of continuous incubation at 37°C, the pH dropped in to 4.0 and 3.0, respectively (data not shown). Morphological evaluation at the end of the experiment confirmed that the microparticles degraded faster at acidic pH conditions.

SEM micrographs of PLGA/PEG microparticles with 0 and 5% PEG content after 0, 14, and 28 days of degradation are shown in Figure 5-6. For both formulations, the initial microparticles were spherical with smooth, non-porous surfaces. The integrity of the microparticles was maintained after degradation, however, significant morphological changes were observed. The microparticles became non-spherical and the surfaces were rough. Numerous micropores were revealed after 28 days due to significant degradation. This
Figure 5-5. Variation of buffer pH during a 28-day degradation of PLGA/PEG microparticles: (a) microparticles with varied PEG content incubated in pH 7.4 PBS and (b) microparticles with 5% PEG incubated in different buffers. Error bars represent means ± SD for $n = 4$. 
Figure 5-6. Scanning electron micrographs of PLGA/PEG microparticles with (a,c,e) 0% PEG and (b,d,f) 5% PEG, after (a,b) 0, (c,d) 14, and (e,f) 28 days of in vitro degradation in pH 7.4 PBS.
phenomenon was also observed for microparticles with 5% PEG incubated with various pH buffers (Figure 5-7). The morphology of microparticles in pH 7.4 buffer were similar to that in PBS. However, lower pH resulted in irregularly shaped microparticles due to faster degradation.

Fluorescent images revealed a fairly uniform distribution of FITC-BSA in the microparticles with 0 or 5% PEG at day 0 [Figure 5-8(a,b)]. After 14 days of in vitro release in PBS, however, aggregation of proteins at the periphery was observed [Figure 5-8(c,d)]. Most of the protein was released by day 28 [Figure 5-8(e,f)]. The microparticles incubated in pH 7.4 buffer followed the same trend [Figure 5-9(a,b)]. However, under acidic conditions such pH 3, aggregation of FITC-BSA occurred gradually which was indicated by intense fluorescence after 14 and 28 days [Figure 5-9(e,f)].

5.4 DISCUSSION

Using a double-emulsion-solvent-extraction technique, PLGA/PEG microparticles with a spherical shape and smooth, non-porous surfaces were fabricated. The average size of the microparticles was about 20 μm. The actual weight percent of PEG incorporated into the microparticles has been previously determined as 0, 0.49, and 1.49% for an initial PEG content of 0, 1, and 5%, respectively [Cleek et al., 1997b]. FITC-BSA and TGF-β1 were encapsulated at high efficiency and dispersed uniformly throughout the microparticles. The effects of PEG content in the PLGA/PEG microparticles and environmental pH on the release kinetics of incorporated proteins and the degradation of PLGA were determined in vitro.
Figure 5-7. Scanning electron micrographs of PLGA/PEG microparticles with 5% PEG after (a,c,e) 14 and (b,d) 28 days of in vitro degradation in (a,b) pH 7.4, (c,d) pH 5, and (e) pH 3 buffers.
Figure 5-8. Fluorescent micrographs showing the distribution of FITC-BSA within PLGA/PEG microparticles with (a,c,e) 0% PEG and (b,d,f) 5% PEG, after (a,b) 0, (c,d), 14, and (e,f) 28 days of in vitro degradation in pH 7.4 PBS.
Figure 5-9. Fluorescent micrographs showing the distribution of FITC-BSA within PLGA/PEG microparticles with 5% PEG after (a,c,e) 14 and (b,d,f) 28 days of *in vitro* degradation in (a,b) pH 7.4, (c,d) pH 5, and (e,f) pH 3 buffers.
Both parameters were found to have significant effect on the protein release processes. Increasing the initial PEG content from 0 to 5% resulted in lower cumulative mass of both proteins released. Although a previous study showed increased drug release rate with increasing PEG content [Cleek et al., 1997b], in the present study, the solubilization of PEG during microparticle fabrication led to the loss of hydrophilic proteins and decreased encapsulation efficiency. In addition, the difference in the release profiles between the present and the previous study was also attributed to the difference in the compounds and loading density used. Increasing PEG content also slightly increased PLGA degradation, probably due to the greater water uptake through micropores created by the dissolution of PEG fraction from the microparticles.

Decreasing the medium pH from 7.4 to 3.0 resulted in decreased protein release. Although PLGA microparticles were almost completely degraded in the pH 3 buffer, the release of both TGF-β1 and FITC-BSA was much slower. FITC was used a fluorescent tagger for the analysis of BSA, however, it had limited solubility at lower pH. This led to the aggregation of FITC-BSA in the polymer matrix, as confirmed by fluorescent images after prolonged incubation. The decreased protein release at lower pH was believed to result from this aggregation of insoluble compounds. This finding is particular interesting since heterogeneous bulk degradation of PLGA often results in accumulation of acidic degradation products in the specimen center (chapter 4). This lower pH may lead to changes in properties of encapsulated proteins such as solubility, aggregation, and activity.

Co-encapsulation of a second molecule such as dextran or BSA in the microparticle systems has been used as a carrier to modulate the characteristics of the compound of interest such as release rate, stability, and
diffusion after release [Krewson et al., 1996]. As discussed above, the release rates of TGF-β1 were decreased by co-encapsulation of a less soluble protein. However, the release profile of TGF-β1 was found to be different than that of the co-encapsulated molecule (FITC-BSA) in the present study. This was probably due to the difference in structure, size, charge, solubility, and loading density of the two proteins. Similar phenomenon was observed showing different release profiles of TGF-β1 and FITC-dextran [Peter, 1998].

The cumulative mass of FITC-BSA released from microparticles with 5% initial PEG at pH 7.4 PBS was 3.8 ± 0.1 μg, very close to the theoretical loading of 4 μg per mg microparticle. However, the entrapment efficiency measurement showed only 52.7 ± 8.1% yield, corresponding to an actual loading of 2.1 ± 0.3 μg per mg microparticle. The discrepancy between these two studies was due to the underestimation of the entrapment yield resulted from protein adsorption at the organic-aqueous interface during the extraction process.

Many factors have been identified as important parameters to affect the protein release kinetics from biodegradable microparticles, including the properties of the protein, co-encapsulated molecules, the polymer matrix, their complex interactions, as well as the microparticle fabrication process and the environmental conditions [Batycky et al., 1997; Blanco and Alonso, 1998; Hollinger and Leong, 1996; Krewson et al., 1996; Yan et al., 1994]. A theoretical model of protein release from biodegradable microparticles has been established [Batycky et al., 1997]. In the present study, FITC-BSA was released in a quadri-phasic fashion. The initial burst was due to the desorption of proteins at the surface. Polymer hydration and protein diffusion led to a linear release phase, followed by a pseudo-steady state. Subsequent solubilization and release of low molecular weight degradation products from the
microparticles resulted in polymer mass loss and a renewed linear protein release phase.

Controlled delivery of various bone growth factors and their effects on cell function and tissue regeneration has been investigated both in vitro and in vivo. TGF-β1 was found to enhance marrow stromal osteoblast proliferation and differentiation in culture [Peter, 1998], and to increase bone formation in critical-sized defect models [McKinney and Hollinger, 1996; Rosier et al., 1998]. Bone morphogenetic proteins (BMPs), which also belong to the TGF-β superfamily, have been shown to induce the expression of osteoblastic phenotype in vitro when released from porous PLGA microparticles [Puleo et al., 1998]. Promotion of new bone formation by BMPs has also been observed in various bone defects [Hollinger and Leong, 1996; Kenley et al., 1994; King et al., 1997; Levine et al., 1997; Marden et al., 1994; Rodgers et al., 1998; Whang et al., 1998; Zeggula et al., 1997]. However, the local needs for TGF-β during bone regeneration at a skeletal defect site are not completely understood. Key questions such as optimal dosages and their temporal and spatial presentation need to be addressed before these delivery systems can be used in clinical applications.

5.5 CONCLUSIONS

TGF-β1 was encapsulated with FITC-BSA into microparticles of blends of PLGA and PEG and released in vitro for up to 28 days. Increasing the initial PEG content resulted in lower protein entrapment efficiency and decreased cumulative mass of proteins released. The degradation of PLGA was slightly increased at higher PEG content. Lowering buffer pH significantly accelerated
PLGA degradation. However, protein released was decreased due to aggregation at acidic environment. These PLGA/PEG microparticles may be potentially useful as delivery vehicles for controlled release of TGF-β1 to induce a desired bone regeneration cascade.
CHAPTER 6
RETINAL PIGMENT EPITHELIUM CELL CULTURE ON PATTERNED SURFACES

6.1 INTRODUCTION

Retinal pigment epithelium (RPE) consists of a continuous monolayer of cuboidal cells located between choroid capillaries and the neurosensory retina. It plays a key role in the maintenance of the normal functions of the photoreceptors [Bok, 1993]. Alteration in RPE structure and function is implicated in a variety of ocular disorders, including age-related macular degeneration (ARMD), Stargardt's disease, and some forms of retinitis pigmentosa. Currently there is no successful treatment for these diseases.

Recent studies have demonstrated that RPE transplantation is a promising therapy for retinal degeneration in humans [Algvere et al., 1994; Peyman et al., 1991]. Healthy RPE grafts have been shown to rescue photoreceptors and prevent neovascularization to some extent in animal models [Gaur et al., 1992; LaVail et al., 1992; Li and Turner, 1988; Little et al., 1996; Lopez et al., 1989; Sheng et al., 1995; Yamamoto et al., 1993]. Most of the existing methods involve the injection of an RPE suspension into the subretinal space [Gaur et al., 1992; LaVail et al., 1992; Li and Turner, 1988; Little et al., 1996; Lopez et al., 1989; Yamamoto et al., 1993]. However, the random placement of these cells does not always result in RPE regeneration, since RPE cells are polar with distinct apical/basal characteristics.

A more efficient therapy employing tissue engineering strategies has been reported by our laboratory in an attempt to transplant an organized sheet
of RPE cells with appropriate orientation (chapter 3). Thin films made of synthetic biodegradable poly(DL-lactic-co-glycolic acid) (PLGA) have been investigated as temporary substrates for \textit{in vitro} RPE cell culture and carriers for subsequent transplantation. RPE cells were shown to pack together and form a monolayer at confluence. However, their characteristic cuboidal morphology was not preserved throughout the culture period. The RPE cells were more elongated and appeared fibroblast-like prior to confluence, indicating cell dedifferentiation and loss of specific cell functions.

Maintenance of normal RPE cell morphology and function \textit{in vitro} is essential before RPE transplantation could be attempted \textit{in vivo}. Proper epithelial cell shape and function has been modulated using micropatterned substrates with specifically engineered chemical or topological features on the surface [Chen et al., 1998; Curtis and Wilkinson, 1997; Singhvi et al., 1994a; Singhvi et al., 1994b]. These surface micropatterns can affect a range of cellular activities such as cell attachment, spreading, migration, proliferation, as well as cell alignment, orientation, and organization.

Chemical surface modifications are generally made by photolithographic treatment of the test surface [Dontha et al., 1997; Kleinfeld et al., 1988]. An alternative method, microcontact printing (μCP), prints the patterned self-assembled monolayer (SAM) on the material surface, using an elastomeric stamp whose surface has been patterned in an appropriate relief structure [Kumar and Whitesides, 1993]. Compared to photolithography, this technique involves less chemical steps, and multiple patterns can be made from a single stamp. In addition, complex patterns can be created by repeated stamping using different SAMs or stamps with different surface features.
We hypothesized that RPE cells cultured on substrates with desired surface micropatterns would maintain proper cell morphology throughout the cell culture period. In this study, we determined: (1) whether model substrates with defined chemical domains of micrometer scale could be fabricated, (2) whether the micropatterned surfaces would allow the maintenance of normal RPE cell morphology and cytoskeleton organization during in vitro culture, (3) whether cell attachment and proliferation would be affected by surface micropatterning, and (4) whether the dimensions of the patterns would affect cell responses.

6.2 MATERIALS AND METHODS

6.2.1 Stamp Preparation

Silicon wafers (masters) containing a topological representation of a predetermined pattern of a square array of either 10 or 50 μm circles were generated by photolithography as previously described [St. John et al., 1997]. The raised features had a depth of about 5 μm and the distance between circles was about 10-12 μm. Poly(dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning, Midland, MI) mixed with a curing agent in a 10:1 ratio was then poured over the master patterns and cured for 1 h at 60°C. The elastomer stamps with the corresponding relief patterns were peeled away from the masters after cooling. Before each use, the stamps were cleaned by sonication in dichloromethane (Aldrich Chemical Co., Milwaukee, WI) for 15 min, air-dried for 15 min, oven-dried at 120°C for an additional 30 min, and stored in a clean environment for 2 days before each use.
6.2.2 Microcontact Printing

Borosilicate glass coverslips (18-mm diameter, Fisher Scientific, Pittsburgh, PA) were chemically cleaned using established techniques [Puleo et al., 1991]. Briefly, the coverslips were sonicated in acetone (Aldrich) and 70% ethanol (Fisher) in distilled deionized water (ddH₂O) for 10 min each, and etched in 1 N NaOH (Fisher) for 1 h. The coverslips were then thoroughly washed in plenty of ddH₂O, oven-dried at 120°C, and sterilized by autoclave before use.

Cleaned coverslips were patterned with octadecyltrichlorosilane (OTS) using methods adapted from established microcontact printing techniques [Chen et al., 1997a; Kumar and Whitesides, 1993; St. John et al., 1997]. As summarized in Figure 6-1, in an inert environment of dry argon, the patterned side of the elastomer stamps was coated with a solution of 0.4 % OTS in hexadecane (Aldrich) and dried in a stream of argon gas. In this same inert environment, coated stamps (patterned side down) were then placed on top of and in direct contact with the coverslips. A weight of approximately 86 g was placed on top of each stamp, assuring contact between the glass surfaces and the raised features on the stamp. The entire set-up was maintained for 40 min to allow the formation of OTS SAMs. The stamps were then carefully separated from the coverslips, cleaned by rinsing in hexane, dichloromethane, isopropyl alcohol (all Aldrich) and subsequent sonication in dichloromethane, and dried as described above.

The OTS-modified glass substrates were sonicated sequentially in hexane, dichloromethane, acetone (Aldrich), and isopropanol for 15 min each, and then cured at 120°C overnight. Control samples of glass coverslips
Figure 6-1. Schematic of the microcontact printing process used to pattern glass surfaces with SAMs of OTS.
uniformly coated with OTS SAMs were also prepared. These substrates were sterilized in 70% ethanol solution for 15 min before use for cell culture.

6.2.3 DIL Staining of Fabricated Patterns

The glass/OTS surfaces were stained with the cyanine dye, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (dil) (Molecular Probes, Eugene, OR) as previously described [St. John et al., 1997]. The dye contains two long aliphatic hydrocarbon chains (C-18) that promote dye partitioning into hydrophobic domains. Briefly, stock solutions of 250 μg/mL of dil in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO) were first prepared. The patterned surfaces were each incubated with 100 μL of 25 μg/mL of dil solution diluted in phosphate buffered saline (PBS) (Gibco, Grand Island, NY) for 2 h in a 37°C humidified atmosphere of 5% CO₂. The samples were then washed three times with PBS, mounted in Vectashield (Vector Laboratories, Burlingame, CA), and finally examined under a confocal microscope (Zeiss LSM Axiovert, Carl Zeiss, Germany).

6.2.4 RPE cell culture on Patterned Surfaces

A human D407 RPE cell line was kindly provided by Dr. Richard C. Hunt (Department of Ophthalmology, University of South Carolina Medical School, Columbia, SC) [Davis et al., 1995]. The cells were cultured with Dulbecco's modified eagles medium (DMEM) (Gibco) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT), 20 μg/mL fungizone (Gibco), and 20 μg/mL of a penicillin/streptomycin/neomycin (PSN) (Gibco) cocktail. The cells were maintained in a 37°C humidified atmosphere of 5% CO₂. When 90% confluency was reached after about 4 days, the cells were passaged with a solution
containing trypsin and ethylenediaminetetraacetic acid (EDTA) (all Sigma). Cells at passage 57 were then seeded on both glass/OTS substrates (10 and 50 μm patterns) placed in 12-well tissue culture polystyrene (TCPS) plates. Glass coverslips and OTS surfaces served as controls. For all the following experiments, an initial cell seeding density of 30,000 cells/cm² was used and the medium was changed daily when necessary.

6.2.5 RPE Cell Morphology

RPE cells were seeded on patterned glass/OTS substrates and control glass and OTS surfaces as described above. After 4 h in culture, the cells were fixed with 2.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (pH 7.4) (Sigma) for 15 min and then rinsed with 0.1 M cacodylate buffer. The cells were stained with cold 1% osmium tetroxide (Polysciences, Warrington, PA) for 30 min and rinsed with cold ddH₂O. The samples were stored in a deep freezer (-80°C) and freeze-dried for 24 h. The samples were then gold-coated using a sputter coater (Pelco Sputter Coater 91000, model 3, Ted Pella, Redding, CA) set at 20 mA for a total time of 120 s (coating thickness approximately 40 nm) before observation with the scanning electron microscopy (SEM, JEOL JSM-5300 Scanning Microscope, Boston, MA) operated at 20 kV.

Additional cells after 4 h of seeding were fixed with 10% neutral buffered (N.B.) formalin (Sigma) for 15 min at room temperature and rinsed three times with PBS. The cultures were stained with dil and visualized by fluorescent microscopy as previously described.

6.2.6 Cytoskeleton Organization of Cells
The organization of actin microfilaments in RPE cells was assessed after 4 h of seeding and at confluence (day 4). The cells were fixed with 10% N.B. formalin for 15 min at room temperature, rinsed twice with PBS, and then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 4 min. Following two rinses in PBS, the cells were incubated with 200 μL rhodamine phalloidine (Molecular Probes) solution in PBS supplemented with 1% (w/v) bovine serum albumin (BSA) (Sigma) for 20 min at room temperature. The cultures were then washed three times with PBS, mounted, and examined as previously described. Depth projection micrographs were constructed from 16 horizontal image sections through the culture for some samples.

The expression of cytokeratin was evaluated after 4 h of cell seeding, at sub-confluence (day 2), and confluence (day 4). RPE cells were fixed with 10% N.B. formalin for 15 min, rinsed twice with Tris-buffered saline (TBS) (Sigma), and then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 4 min. Following two rinses in TBS, the cells were blocked in 2.5% (v/v) normal goat serum (Boehringer Mannheim, Indianapolis, IN) in TBS-BSA (0.1% BSA in TBS) for 25 min. The excessive serum was drained off, and the cultures were incubated with 25 μg/mL mouse anti-keratin AE1/AE3 antibody (Boehringer Mannheim) diluted in TBS-BSA in a humidified environment at room temperature for 1 h. Following three rinses in TBS, the cells were incubated with 20 μg/mL FITC-conjugated goat anti-mouse IgG antibody (Boehringer Mannheim) in TBS, and finally rinsed three times with TBS. The samples were subsequently mounted and examined.

6.2.7 Cell Attachment and Proliferation Studies
RPE cells were plated as described above for 2, 4, and 8 h in the cell attachment and for 1, 4, and 7 days in the cell proliferation studies. Upon removal, the cultures were washed with Mg\(^{2+}\) and Ca\(^{2+}\)-free PBS and trypsinized. Aliquots of dissociated cell suspensions were analyzed on a Coulter counter multisizer (Model 0646, Coulter Electronics, Hialeah, FL) and only particles between 6.4 and 24.3 µm in diameter were counted.

6.2.8 Statistical analysis

Samples in cell attachment and proliferation study were collected in triplicates and expressed as means ± standard deviations (SD). Single factor analysis of variance (ANOVA) was used to assess statistical significance of results. Scheffé's method was employed for multiple comparison tests at a significance level of 95%.

6.3 RESULTS

6.3.1 Fabrication of Patterned Surfaces

The micropatterned glass/OTS surfaces fabricated using the microcontact printing technique were very reproducible. Dil stained substrates prepared with stamps of 10 and 50 µm circles are shown in Figure 6-2(a,b), respectively. The OTS areas were hydrophobic and stained red, while the circular regions were dark indicating little or no contamination of OTS inside the circles. The contact angles of OTS and glass were about 110° and 20°, respectively [Siedlecki, 1994 #173; chapter 3]. The patterns were very uniform throughout the entire surface. The edges of the circles were very sharp. The
Figure 6-2. Fluorescent micrographs of glass/OTS substrates stained by dil with surfaces having (a) 10 μm and (b) 50 μm patterns fabricated using a microcontact printing technique.
size of the circles was about 10 or 50 µm in diameter defined by the size of the corresponding stamps used. The distance between circles was between 10 and 12 µm, the same features as the stamps.

6.3.2 Morphology of Cells Cultured on Patterned Surfaces

The three-dimensional morphology of RPE cells on both 10 and 12 µm patterns and control glass was investigated by SEM after 4 h of plating (Figure 6-3). Most of the cells that attached to the 10 µm pattern had a rounded morphology, with occasional spread cells [Figure 6-3(a,b)]. The cells existed mostly as single cells. The cell size was approximately 10 µm in diameter. Numerous cellular processes (lamellipodia and filopodia) were developed to facilitate cell-substrate and cell-cell interactions. The cells on the 50 µm pattern were organized differently [Figure 6-3(c,d)]. They existed mainly as groups of cells that were confined within the 50 µm circles. For example, five cells were arranged into a ring structure with a diameter of 50 µm. The cells that attached to control glass were well spread and more elongated than those on the patterned surfaces (Figure 6-3e). The length of a typical cell was about 50 µm. Only a few cells were found on the OTS surface. The ones that attached remained rounded (Figure 6-3f).

Dil staining of seeded cells allowed simultaneous visualization of the underlying pattern. For example, fluorescent micrographs of cells cultured on a 50 µm pattern revealed similar cell morphology as observed by SEM, and further confirmed that the organization of groups of cells into 50 µm circular structures was due to the underlying pattern [Figure 6-4(a,b)]. The cell membranes were stained much brighter by dil than the OTS on the substrates.
Figure 6-3. Scanning electron micrographs of RPE cells cultured on (a,c) 10 μm patterns, (b,d) 50 μm patterns, and controls (e) glass and (f) OTS after 4 h of cell seeding, with an initial density of 30,000 cells/cm².
Figure 6-4. Fluorescent micrographs of RPE cells stained with dil after 4 h of *in vitro* culture on (a,b) 50 μm patterns, and (c) glass and (d) OTS controls. The initial cell seeding density was 30,000 cells/cm².
The cells on control surfaces glass and OTS had similar morphology [Figure 6-4(c,d)] to that described for the corresponding SEM pictures.

6.3.3 Cytoskeleton Organization of Cells

The actin staining of RPE cells after 4 h of seeding also showed single, rounded cells on the 10 μm pattern and groups of cells on the 50 μm pattern [Figure 6-5(a,b)]. The colors in the confocal micrographs indicated the vertical organization of actin microfilaments on the various substrates [Figure 6-5(c-f)]. The cell morphology was different on these substrates reflecting the difference between the surface microstructures of the underlying substrates. In all cases, the actin staining was more intense at the boundary of the cells.

After about 4 days, the cells cultured on both 10 and 50 μm patterned surfaces reached confluence. As an example, the cells on a 50 μm pattern formed a continuous monolayer (Figure 6-6a). In contrast, the cells on control OTS was in a sub-confluent state, indicating they eventually were able to attach, spread and proliferate on OTS (Figure 6-6b). Higher magnification view of the cells at confluence on the 50 μm pattern indicated that the actin was organized into circumferential rings at the apical side, while the actin was arranged mainly as short filaments at the basal side [Figure 6-6(c,d)].

Unlike the expression pattern of actin, the cytokeratin was distributed uniformly in the cytoplasm at different stages of cell growth: 4 h after cell seeding, sub-confluent, and confluent (Figure 6-7). Again the cells were seen to pack tightly together and form a continuous monolayer after 4 days in culture.
Figure 6-5. Rhodamine phalloidin stained RPE cells showing the organization of actin microfilaments after 4 h of *in vitro* culture on (a,c) 10 μm patterns, (b,d) 50 μm patterns, and controls (e) glass and (f) OTS. The initial cell seeding density was 30,000 cells/cm².
Figure 6-6. Rhodamine phalloidine stained RPE cells showing the organization of actin microfilaments after 4 days of *in vitro* culture on (a,c,d) 50 μm patterns and (b) OTS control. For cells on 50 μm patterns, both (c) apical section and (d) basal section were taken. The initial cell seeding density was 30,000 cells/cm².
Figure 6-7. Fluorescent micrographs of RPE cells on (a,c) 10 μm and (b,d) 50 μm patterns after (a,b) 4 h, (c) 2 days, and (d) 4 days of in vitro culture. The cells were immunologically stained for cytokeratin, and the initial cell seeding density was 30,000 cells/cm².
6.3.4 RPE Cell Attachment and Proliferation

The percentage of attached cells increased significantly \((p < 0.05)\) over an 8-h period for all the substrates tested (Figure 6-8). The dynamics of RPE cell attachment on both patterned surfaces was similar to each other \((p > 0.05)\). By 8 h, 71.9 ± 7.6 and 73.9 ± 4.6% of plated cells attached to the 10 and 50 μm patterns, respectively. The cell attachment on these surfaces was significantly lower \((p < 0.05)\) than that on glass control at all time points, and significantly higher \((p < 0.05)\) than OTS control after 2 h.

RPE cell proliferation on the patterned surfaces was studied after 1, 4, and 7 days in culture (Figure 6-9). Cell number increased significantly \((p < 0.05)\) over the 7-day period on all the substrates tested. By day 7, cell numbers had increased to 910,500 ± 63,000 and 923,300 ± 48,300 cells/cm² on the 10 and 50 μm surfaces, respectively, corresponding to about 30-fold increases as compared to the initial cell seeding density. About 30-fold and 15-fold increases were observed for control glass and OTS, respectively. The cell numbers on both patterned surfaces were significantly different \((p < 0.05)\) from the control, except for glass at day 7.

6.4 DISCUSSION

This study was conducted to answer the following questions: (1) Could model substrates with defined chemical domains of micrometer scale be fabricated? (2) Did RPE cells maintain their normal morphology and cytoskeleton organization during in vitro culture on the micropatterned surfaces? (3) Did the surface micropatterning affect RPE cell attachment and proliferation in vitro? (4) Did the pattern size affect cell responses?
Figure 6-8. Percent of plated RPE cells that attached to 10 and 50 μm patterns and controls OTS and glass, with an initial seeding density of 30,000 cells/cm², as a function of in vitro culture time. Error bars represent means ± SD for n = 3.
Figure 6-9. Proliferation kinetics of RPE cells cultured in vitro, with an initial seeding density of 30,000 cells/cm², on 10 and 50 μm patterns and controls OTS and glass. Error bars represent means ± SD for n = 3.
Model substrates with micropatterned OTS SAMs on glass surfaces were fabricated using the microcontact printing technique. This method has been previously used to pattern hexadecanethiol on gold [Kumar et al., 1994], OTS on silicon [St. John et al., 1997; Xia et al., 1995], and polylysine on glass [James et al., 1998]. The micropatterns obtained from the present study had arrays of circular features in the diameter of 10 or 50 μm, which was precisely controlled by the elastomer stamp. Stamp sagging as a result of capillary forces when thick stamps are used for printing isolated features was not observed. The resulted substrate surface had two distinct chemical regions (OTS and glass) with different affinity for RPE cell attachment.

The micropatterned surfaces promoted the formation of normal RPE cell morphology. The average length (along the long axis) of an RPE cell on the 10 and 50 μm patterns and control glass after 4 h of cell seeding was about 10, 20, and 50 μm, respectively. This was due to the inhibitory effects of OTS on cell attachment between circles. The cells were able to maintain the more rounded morphology throughout a 4-day culture period on both patterned surfaces. This was also preferable to the more elongated shape of cells culture on PLGA films as observed in our previous study (chapter 3).

The cytoskeleton is responsible for the control of cell shape. Prior to confluence, the actin formed linear microfilament bundles and stress fibers, believed to generate forces for cell attachment, spreading, and migration. When the cells reached confluence on micropatterned surfaces, the actin was re-arranged into short filaments at the basal side and compact rings at the apical surface, corresponding to a shift from predominately cell-substrate to cell-cell adhesions. This ring structure circumscribing the cell apices was found in
differentiated cells, and believed to be associated with tight junctions [Chang et al., 1997]. This is crucial in the organization of distinct cell surface domains and thus maintenance of cell polarity [Chang et al., 1997; Gundersen et al., 1991; Huotari et al., 1995; Matsumoto et al., 1990; Opas, 1994].

The RPE cells cultured on the both micropatterns expressed keratin intermediate filaments, which are characteristic of cells of epithelial origin. In contrast, the cells of mesenchymal origin contain vimentin intermediate filaments. When the cells reached confluence, the cytokeratin formed a network throughout the cytoplasm, similar to that reported previously [Davis et al., 1995].

RPE cell attachment on both patterned surfaces was significant different from the controls over an 8-h period due to the difference in surface chemistry. In addition, cell proliferation on the micropatterns was significantly lower than control glass during the initial 4 days. The cells reached confluence on glass at day 4, and continued to grow into multilayers at a slower rate. By day 7, the cell numbers on both patterned surfaces were comparable to that on glass, all exceeding a confluent density of 538,400 cells/cm² as previously determined (chapter 3). The cells cultured on control OTS also proliferated following an initial slow growth, probably due to the absorption of serum proteins onto the OTS region.

The pattern size affected the morphology and distribution of cells on the micropatterned substrates. The circle diameters of 10 and 50 μm were chosen based on the size of an RPE cell at the well-packed or well-spread stages, respectively (chapter 3). The cells were mostly isolated on the 10 μm pattern with a cell size similar to that of the underlying circles. Aggregates of several cells with a larger size were seen within the 50 μm circles. The cells formed
normal monolayers on both micropatterns after 4 days of in vitro culture and no
difference in morphology and cytoskeleton organization was detectable. This
indicated that although the micropatterns affected initial cell attachment and
limited cell spreading during culture, they did not inhibit cell-cell contact and
formation of cell-cell junctions, which is essential for the barrier function of RPE.
The dynamics of cell attachment and proliferation was also not affected by the
pattern size. The confluent monolayers thus obtained may potentially be useful
for RPE transplantation.

Micropatterned surfaces fabricated by microcontact printing have been
used to engineer cell shape and function. Hepatocytes cultured on substrates
with adhesive islands of extracellular matrix protein laminin showed varied cell
shape, size, and albumin secretion depending on the dimensions of the laminin
islands [Singhvi et al., 1994a]. When cultured on substrate surfaces with
alternating micrometer bars of N'[3-(trimethoxysilyl)propyl] diethylenetriamine
(DETA) and OTS, astroglial cells showed preferred cell attachment on DETA to
OTS [St. John et al., 1997]. This result was similar to that observed in the
present study.

Topological modification of the substrate was possible during the
microcontact printing of OTS SAMs. However, the root mean square roughness
of OTS SAM on glass has been determined to be about 0.18 nm [Siedlecki et
al., 1994]. This was much smaller than the dimension of about 500 nm needed
for inducing cell response [Curtis and Wilkinson, 1997]. Therefore, the RPE
cells were considered to react only to the chemical cues presented on the
substrate surfaces.
6.5 CONCLUSIONS

Model substrates with desired chemical patterns were fabricated using the microcontact printing technique. The substrate surface was composed of organized arrays of circular glass domains with a diameter of 10 or 50 μm and OTS SAMs. Both micropatterns affected initial cell attachment and spreading, and allowed the maintenance of differentiated RPE cell morphology and cytoskeletal organization throughout the in vitro culture period. The cells on both substrates also formed confluent monolayers. These results demonstrated the feasibility of using micropatterned surfaces for controlling RPE cell shape, size, and phenotypic expression.
CHAPTER 7

DEVELOPMENT OF NOVEL BIODEGRADABLE POLYMER SUBSTRATES WITH PATTERNED SURFACES

7.1 INTRODUCTION

The major challenge in the development of a successful tissue engineered graft for retinal pigment epithelium (RPE) transplantation has been the maintenance of normal cell morphology and function (chapters 3 and 6). In the previous chapter, we described a contact printing technique for the fabrication of substrate surfaces with specific chemical patterns in the micrometer order. The micropatterns with organized arrays of adhesive domains with 10 or 50 μm diameter were shown to induce the expression of desired cell phenotype. These results demonstrated the feasibility of using micropatterned surfaces for controlling RPE cell shape, size, and ultimately cell function.

The model surfaces developed in the previous study (chapter 6) contained self-assembled monolayers (SAM) of octadecyltrichlorosilane (OTS) patterned on glass. The glass/OTS substrates, however, can not be utilized as scaffolds for RPE transplantation. A desired substrate for RPE tissue engineering would consist of only biocompatible and bioresorbable materials (chapters 1 and 3). This led to the search for the materials of this type that can be fabricated into micropatterned surfaces using the established microcontact printing technique.

Poly(DL-lactic-co-glycolic acid) (PLGA) is among the first synthetic biodegradable polymers approved by FDA for certain human clinical uses. The
biodegradation of PLGA has also been extensively studied (chapter 4). Moreover, thin PLGA films were shown to support RPE cell culture in vitro (chapter 3). Block copolymers of poly(ethylene glycol) (PEG) and poly(DL-lactic acid) (PDLLA) (PEG/PLA), on the other hand, allowed the modulation of protein adsorption and cell adhesion [Göpferich et al., 1999]. By adjusting the length of the PEG segment, the block copolymers can have varied ability for cell adhesion.

We hypothesized that micropatterned surfaces with adhesive PLGA and non-adhesive PEG/PLA domains can be fabricated. In this study, we determined: (1) whether diblock copolymer of PEG/PLA could be fabricated into thin films, (2) whether cell growth on the PEG/PLA films would be diminished, and (3) whether PLGA and PEG/PLA could be fabricated into micropatterns using the microcontact printing technique.

7.2 MATERIALS AND METHODS

7.2.1 Synthesis of Diblock Copolymers

The diblock copolymers were synthesized as previously described [Göpferich et al., 1999]. Briefly, 5 g of monomethyl ether-poly(ethylene glycol) (Me-PEG) with a weight average molecular weight (Mw) of 5,000 (Aldrich Chemical Co, Milwaukee, WI) were dissolved in 50 mL of toluene (Aldrich), and dried by azeotropic distillation of 20 mL of toluene. In addition, 20 g of 1,4-dimethyl-dioxane-dione were dissolved in 50 mL of toluene and similarly dried. Following the combination of the two toluene phases, 0.1 mL of stannous-ethyl hexanoate (Sigma Chemical Co., St. Louis, MO) was added and the mixture
was refluxed for 2 h. During the subsequent ring-opening polymerization, the poly(DL-lactic acid) (PDLLA) block with a Mw of 20,000 was created. After the completion of the reaction, the toluene was removed by distillation. The resulting diblock copolymer PEG/PLA was dissolved in a small volume of dichloromethane (Aldrich), precipitated in ether, filtered, and thoroughly dried.

### 7.2.2 Fabrication Polymer Films

Poly(DL-lactic-co-glycolic acid) (PLGA) of 50:50 lactic to glycolic acid copolymer ratio was purchased from Alkermes (Medisorb®, Cincinnati, OH). The polymer Mw and polydispersity index (PI) was determined by gel permeation chromatography to be 46,700 ± 500 and 1.73 ± 0.05, respectively. PI is the ratio of the weight average to the number average molecular weight (Mw/Mn). Borosilicate glass coverslips (18-mm diameter, Fisher Scientific, Pittsburgh, PA) were chemically cleaned as described in the previous chapter (section 6.2.2).

PLGA films were manufactured using a solvent casting technique as detailed in chapter 4, section 4.2.2. Briefly, 110 mg/mL of PLGA solution in chloroform (Aldrich) was first prepared. Approximately 260 μL of this solution was then cast onto a clean glass coverslip (diameter 18 mm) (Fisher) placed on a leveled table in the fume hood. The samples were air-dried for 20 h and subsequently vacuum-dried at 10 μm Hg for an additional 24 h to remove any remaining solvent.

PEG/PLA was fabricated into thin films using a spin-coating technique. An 18 mm clean glass coverslip was vacuum-held on the stage of a spinner. Then 100 μL of PEG/PLA solution in chloroform (5% w/w) was added on the
coverslip. The stage was immediately set to spin at 3000 rpm for 50 sec. A very thin film of PEG/PLA was formed on the glass as result of solvent evaporation. The film was then thoroughly dried as described above.

7.2.3 RPE cell Proliferation on Polymer Films

A human D407 RPE cell line was obtained and cultured as discussed in chapter 3, section 3.2.2. PLGA and PEG/PLA films (on glass) were placed in 6-well tissue culture polystyrene (TCPS) plates and sterilized by immersion in 70% ethanol solution for 15 min. Approximately 0.48 and 1.78 mL of an RPE cell suspension were placed on top of each polymer film and control TCPS well (diameter 34.6 mm), respectively, to obtain the same initial cell seeding density of 15,000 cell/cm². An additional 1.5 mL of fresh RPE culture media (DMEM with 5% FBS) were added to each TCPS well. After 8 h of cell seeding, the original media were replaced with 2.5 mL of fresh culture media for each well with polymer films (3 mL for TCPS control). This time scale was determined from the cell attachment study previously described in chapter 3. The media were then changed every day up to a total culture period of 7 days. Cell proliferation after 1, 4, and 7 days in culture was assessed by cell counts as detailed in chapter 3, section 3.2.

7.2.4 Pattern Fabrication by Microcontact Printing

Poly(dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning, Midland, MI) stamps with recessed arrays of 10 or 50 μm circles were prepared as detailed in chapter 6, section 6.2.1. Micropatterned surfaces using biodegradable polymers of PLGA and PEG/PLA were fabricated by the contact printing technique (section 6.2.2), with modifications. In one preparation, a PLGA film
was first placed on a leveled table in the fume hood. The patterned side of a
stamp was then immersed in 1% (w/w) PEG/PLA solution in acetone (Aldrich)
for a few seconds and pressed immediately onto the PLGA film. Special care
was taken to avoid entrapment of air bubbles. A plastic petri dish filled evenly
with sodium chloride crystals (total weight about 120 g) was put on top of the
stamp to ensure direct contact of raised features on the stamp with the PLGA
film. After 1 h, the stamp was carefully separated from the petri dish, and then
from the PLGA film. The stamp was cleaned as previously described (section
6.2.2). The patterned PLGA film was washed in distilled deionized water
(ddH₂O), air- and vacuum-dried before use. Alternative samples were obtained
by patterning PEG/PLA films with 1% (w/w) PLGA solution in acetone. In
addition, control samples were prepared using only the solvent acetone as ink
in the patterning procedure.

7.2.5 Dil Staining of Fabricated Patterns and Cultured RPE cells

The patterned polymer surfaces were stained with dil (Molecular Probes,
Eugene, OR) and examined under a confocal microscope (Zeiss LSM Axiovert,
Carl Zeiss, Germany), as previously described (section 6.2.3). RPE cells were
seeded on PLGA-patterned PEG/PLA surfaces and control PLGA films at a
density of 30,000 cell/cm². After 4 h in culture, the cells were fixed, stained with
dil, and visualized by fluorescent microscopy as described in section 6.2.5.

7.2.6 Statistical analysis

Multiple samples were collected in the cell proliferation study and
expressed as means ± standard deviations (SD). Single factor analysis of
variance (ANOVA) was used to assess statistical significance of results.
Scheffé's method was employed for multiple comparison tests at a significance level of 99%.

7.3 RESULTS

7.3.1 Polymer Films

PLGA 50:50 films of approximately 100 μm in thickness were fabricated by solvent casting as previously described (chapters 3 and 4). Using an alternative spin-coating technique, thin films of PEG/PLA were also fabricated. These films had an estimated thickness of less than 10 μm and appeared transparent and smooth.

7.3.2 RPE Cell Proliferation on Polymer Films

Both PLGA and PEG/PLA thin films were assessed for RPE cell growth after 1, 4, and 7 days in culture (Figure 7-1). Significant increase in cell number ($p < 0.01$) was observed on PLGA films over the 7-day period, similar to TCPS controls. By day 7, the cell density on PLGA was $352,900 \pm 92,000$ cells/cm$^2$, corresponding to a 24-fold increases as compared to the initial cell seeding density. About 19-fold increase was observed for control glass. However, no cell proliferation was seen on the PEG/PLA films. The cell density on these films was less than 10% of the initial number after 7 days.

7.3.3 Micropatterned Surfaces

The previously described microcontact printing technique (chapter 6) was applied to biodegradable polymers for the fabrication of surface
Figure 7-1. Proliferation kinetics of RPE cells cultured *in vitro*, with an initial seeding density of 15,000 cells/cm², on PLGA and PEG/PLA films, and control TCPS. Error bars represent means ± SD for n = 4.
micropatterns. PLGA film were patterned with stamps of 10 or 50 μm circles using the PEG/PLA solution as ink and stained with dil [Figure 7-2(a-d)]. The circular PLGA areas were hydrophobic and stained red, while the rest PEG/PLA regions appeared dark. The patterns were very uniform throughout the entire surface. The edges of the circles were very sharp and the staining inside was fairly homogeneous. The size of the circles was about 10 or 50 μm in diameter which was defined by the size of the corresponding stamps used. The distance between circles was between 10 and 12 μm, similar to that of stamps. In contrast, the control PLGA surfaces prepared by microcontact printing of acetone alone using the 10 or 50 μm stamps were stained bright across most of the area [Figure 7-2(e,f)], respectively]. However, circular structures in the same order of the corresponding features on the stamps were observed due to the physical contact of the stamps with the PLGA films.

7.3.4 RPE Cell Attachment to Micropatterns

RPE cells were seeded onto PEG/PLA films patterned with 50 μm stamps using the PLGA solution as ink. After 4 h of seeding, substantial cell attachment was observed (Figure 7-3a). Most of the attached cells had a rounded morphology with a diameter of about 20 μm. More cells were found on control PLGA films (Figure 7-3b). These cells were mostly elongated with a length of about 50 μm and a width of 15 μm.

7.4 DISCUSSION

This study was intended to answer the following questions: (1) Could the diblock copolymer PEG/PLA be fabricated into thin films? (2) Was RPE cell
Figure 7-2. Fluorescent micrographs of PLGA surfaces patterned with (a,c,e) 10 μm and (b,d,f) 50 μm stamps using (a-d) PEG/PLA solution in acetone or (e-f) aceton only as inks.
Figure 7-3. Fluorescent micrographs of RPE cells stained with dil after 4 h of *in vitro* culture on (a) 50 µm patterns and (b) control PLGA films, with an initial cell seeding density of 30,000 cells/cm². The micropatterns were fabricated by stamping the PLGA solution onto PEG/PLA films.
growth on these films different from the previously used PLGA films? (3) Could biodegradable substrates with chemical micropatterns be fabricated using the PLGA and PEG/PLA by microcontact printing?

In the previous studies, PLGA was fabricated into thin films using a solvent casting technique (chapters 3 and 4). However, the PEG/PLA films fabricated by this method were opaque and the surfaces appeared very rough. An alternative method, spin-coating has long been used in the semiconductive industry for preparation of very flat surfaces. By optimizing the parameters used for spin coating, such as the concentration of the polymer solution, the amount of the solution used for each coverslip, and the spin speed and duration, transparent films with very smooth surfaces were fabricated. The surface roughness of both PLGA and PEG/PLA films was expected to be less than 100 nm (preliminary results obtained from atomic force microscopy not shown). These films were satisfactory for use as substrates by microcontact printing, since the roughness was negligible compared to the depth of the raised features on the elastomer stamps (about 5 μm, section 6.2.1).

Significant RPE cell proliferation on the PLGA films occurred during a 7-day culture period, similar to that observed previously (chapter 3). In contrast, the PEG/PLA surfaces did not support cell attachment and proliferation. Due to the use of Me.PEG, a diblock copolymer of PEG/PLA was synthesized. This allows greater freedom of chain movement as compared to a triblock copolymer that would otherwise form if PEG was used. When placed in aqueous solutions, the PEG chain can stretch at the film surface into the aqueous solution, while the PLA chain remains anchored inside the matrix. The presence of PEG suppressed cell growth throughout the culture period. This was different from the OTS surface discussed in the previous chapter, which eventually allowed
cell attachment and proliferation probably due to the adsorption of serum proteins. Previous studies using marrow stromal osteoblasts also showed significantly less protein adsorption and cell adhesion on these PEG/PLA surfaces than that on the non-PEG coupled polymers [Göpferich et al., 1999].

The cell proliferation study demonstrated the feasibility of using the combination of these two biodegradable polymers, PLGA and PEG/PLA, to fabricate surfaces with distinct chemical domains that would promote or inhibit cell adhesion, respectively. The microcontact printing technique discussed in the previous chapter was successfully applied to these polymers, resulting in patterned surfaces with organized arrays of 10 and 50 μm circles. The micropatterns as assessed by dil staining were uniform and sharp, as observed for the glass/OTS systems. However, the mechanism for pattern transfer was different. For example, PEG/PLA could not spontaneously form a monolayer on the surface of the PLGA film after stamping of its solution in acetone. However, it formed a layer of thin film at the regions where the stamps were in direct contact with the PLGA. This created isolated circular domains of PLGA, which were free of contamination as appeared by dil staining (Figure 7-2).

Acetone was chosen in the present study as the solvent for the preparation of the polymer ink instead of chloroform, which was used as solvent for film fabrication. The lower density of acetone (0.79 g/mL) than chloroform (1.48 g/mL) was thought to help minimizing the stamp sagging which would cause contamination inside the circles. Since acetone is also a solvent of the polymer film, modification of the substrate surface was possible, as evident from the control samples prepared by printing acetone alone. The chemical structures of the micropatterns, especially at the boundaries of PLGA and PEG/PLA are not clear and need to be further characterized.
The preliminary cell culture results indicated that surface micropatterning affected initial cell attachment. The patterns used were prepared by printing PLGA solution onto the PEG/PLA films. The circular domains were expected to contain primarily PEG/PLA and thus non-adhesive for the RPE cells. The cells cultured for 4 h on the patterns exhibited a desirable rounded shape. The cell size was larger than that of non-adherent cells (usually 10 μm). This indicated that the cells were spreading, however, they did not elongate and became fibroblastic as typically observed for spread cells on plain PLGA films. The cells were found to attach preferably to the PLGA regions restricted by four adjacent 50 μm circles of PEG/PLA. Assuming a rounded (or hexagonal) cell shape, the maximal cell radius (or length of each side) confinable within these areas was about 15 μm. The difference in the size of the effective adhesive areas for cell attachment may count for the difference between cell morphology showed in the present study and that on the 50 μm glass/OTS surfaces (chapter 6).

Detailed characterization of the composition and morphology of the micropatterned surfaces and additional experimentation and analysis of their effects on cell growth and function are necessary. Nevertheless, the results obtained from the present study demonstrated the feasibility of producing biodegradable substrates with precisely defined surface patterns using the microcontact printing technique. The methodology thus developed may be applied to other synthetic biodegradable polymers. These micropatterned surfaces hold great promise for the modulation of cell shape and function in a variety of tissue engineering applications, including but not limited to the regeneration of RPE.
7.5 CONCLUSIONS

Micropatterned substrates were fabricated using synthetic biodegradable polymers by the microcontact printing technique. The fabricated surfaces contained organized arrays of isolated circular domains with a diameter of 10 or 50 μm. The two polymers used, PLGA and PEG/PLA, were shown to respectively promote and inhibit RPE cell proliferation during a 7-day culture period. RPE cells cultured on a 50 μm pattern with non-adhesive circles maintained the desired cell morphology. These results demonstrated the feasibility of creating micropatterned surfaces using synthetic biodegradable polymers, which are potentially useful in many tissue engineering applications.
CHAPTER 8

CONCLUSIONS AND FUTURE WORK

This thesis has focused on the development of synthetic biodegradable polymers as cell transplantation scaffolds and controlled-release carriers for the modulation of cell morphology and function. We progressed this work through the following stages: production of thin polymer films as cell culture substrates; investigation of degradation properties of these films; development of polymer microparticles for the controlled delivery of growth factors; fabrication of model substrates with desired surface micropatterns to induce proper cell phenotype; and preparation of micropatterned substrates using synthetic biodegradable polymers.

Poly(DL-lactic-co-glycolic acid) was fabricated into thin films and served as substrates for in vitro culture of a human RPE cell line. RPE cells attached to and proliferated on both 50:50 and 75:25 PLGA films during a 7-day period. Initial cell seeding density affected attachment. At confluence, the cells had a cuboidal morphology and expressed tight junctions apically. The PLGA films were therefore promising substrates for RPE cell culture and transplantation.

The in vitro degradation of PLGA films with varying copolymer ratios and thickness levels was investigated for up to 10 weeks in pH 7.4 PBS. Both parameters had significant effects on the weight loss and molecular weight loss profiles of the films. PLGA 50:50 degraded faster than 75:25 films with similar thickness. Increasing the thickness levels from 10 to 100 μm accelerated degradation. All the PLGA films degraded by heterogeneous bulk degradation,
which was confirmed by the differential morphology of the porous inner layer and non-porous surface layer.

TGF-β1 was encapsulated with FITC-BSA into microparticles of blends of PLGA and PEG and released in vitro for up to 28 days. Increasing the initial PEG content resulted in lower protein entrapment efficiency and decreased cumulative mass of proteins released. The degradation of PLGA was slightly increased at higher PEG content. Lowering buffer pH significantly accelerated PLGA degradation, but decreased the release rates due to protein aggregation. These PLGA/PEG microparticles proved useful for controlled delivery of growth factors.

Model substrates with desired chemical micropatterns were fabricated using the microcontact printing technique. The substrate surfaces had organized arrays of circular adhesive glass domains with a diameter of 10 or 50 μm and non-adhesive OTS SAMs. Surface patterning affected initial RPE cell attachment and spreading, and allowed the maintenance of differentiated cell morphology and cytoskeletal organization throughout a 7-day in vitro culture period. These results demonstrated the feasibility of using micropatterned surfaces for controlling cell shape, size, and phenotypic expression.

The microcontact printing technique was successfully applied to synthetic biodegradable polymers of PLGA and PEG/PLA. These materials were shown to respectively promote and inhibit RPE cell proliferation during a 7-day culture period. The fabricated 10 and 50 μm surfaces contained two distinct chemical regions with different cell affinity, similar to the model glass/OTS system. RPE cells cultured on 50 μm patterns with non-adhesive PEG/PLA circles maintained
the desired cell morphology. These novel biodegradable micropatterned substrates are potentially useful in many tissue engineering applications.

The studies presented in this thesis led to the development of scaffolds for cell transplantation and microparticulate carriers for controlled drug delivery based on synthetic biodegradable polymers. The following studies may be carried out in the future as a continuum of this thesis.

From the materials science point of view, many properties including mechanical stability of these thin biodegradable films can be further characterized. Another aspect would be the development of methods for controlling local pH during film degradation. Modification of these films, such as surface micropatterning introduced here, may render them particularly interesting in certain applications. As briefly discussed in chapter 4, they can serve as barriers for guided tissue regeneration.

RPE transplantation is among the most active, important, and challenging areas of research in ophthalmology. The *in vitro* studies from this thesis suggested the use of thin PLGA films as carriers for RPE cell culture and subsequent implantation. This hypothesis can be further assessed by more detailed functional analysis *in vitro* as well as *in vivo* evaluations including film degradation, host response, and the structural and functional regeneration of RPE and photoreceptors.

The last direction deals with the combination of drug delivery and cell transplantation. The biodegradable microparticles described above can be utilized for controlled release of various bioactive molecules. The delivery of these factors to the cells cultured on biodegradable scaffolds could allow the generation of better functional cellular grafts.
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