ELECTROSPUN POLYCAPROLACTONE SCAFFOLDS UNDER STRAIN AND THEIR APPLICATION IN CARTILAGE TISSUE ENGINEERING

DISSETRATION

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* * * * *

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ABSTRACT

Electrospinning is a promising fabrication method for three dimensional tissue engineering scaffolds due to its ability to produce a nano-/micro-sized non-woven fibrous structure which resembles the natural extracellular matrix. We investigated the mechanical behavior of two different electrospun microstructures. Polycaprolactone (PCL) fibers with or without “point-bonding” exhibited different deformation behaviors having significant biomedical consequences. While fibers with point-bonded structure failed due to the generation of voids by the fracture of fiber interconnections under strain, fibers without point-bonds produced a ‘bamboo’ structure with fiber joining visible at higher levels of strain. In addition, gelatin and PCL were electrospun and the residual solvent contents were systematically investigated. A simple and effective means of reducing residual solvent content was developed.

The interaction between these electrospun matrices and chondrocytic cells were compared to other topographies having the same chemistry. Electrospun polycaprolactone fibers supported better proliferation and extracellular matrix production than the corresponding semi-porous and dense surfaces and even, at some time points, glass surfaces. The intrinsic capability of electrospinning to produce high porosity appears to offset the relative hydrophobicity of polycaprolactone resulting in a more
uniform cell seeding. Electrospun fibers induced a higher level of glycosaminoglycans (GAG) production by providing a ‘dynamic scaffold’ in which chondrocytes are able to maintain a morphology associated with the appropriate phenotype. Finally, based on this study, a method producing macro-pores within an electrospun scaffold was developed. With this method, not only can cellular infiltration into a thick electrospun scaffold be facilitated, but scaffolds having designed, anisotropic structures can be produced that better approximate the final tissue.
Dedicated to Young and my parents
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1.1. Introduction: Tissue Engineering

Tissue engineering is a cell-based therapy that promises repair or replacement of damaged organs. As a discipline, the field has dramatically evolved from its origins in the late 1980s concurrent with rapid advances in stem cell biology. The term, “tissue engineering” stems from the first symposium under the name of tissue engineering in 1988 [1], where the field was defined as follows:

“The application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissue and the development of biological substitutes to restore, maintain, or improve tissue function.”

As defined above, the underpinnings of tissue engineering are broad and span a wide spectrum of scientific and engineering fundamentals including basic biological sciences (cell biology, physiology and wound healing), engineering fundamentals (materials science, mechanics, chemical kinetics and fluid dynamics) and biotechnologies (cell culture, cell separation and gene transfer).
Conventional clinical approaches for tissue replacement have been non-biological implants such as prosthetic parts or non-patient specific biological implants such as whole organ transplantation. Synthetic or natural materials that lack living cells have been utilized to replace tissue function. Current clinical “biomaterials” almost exclusively provide a mechanical function because they have no metabolic components with which to perform active tissue functions. Two major shortcomings of this approach are the inability to grow with the patient and poor integration with the surrounding tissues. For biological implants, autologous graft, allograft and xenograft approaches have been used. Autologous transplantation that replaces a damaged organ with tissues from patient’s other body parts provides the lowest risk of immune rejection. However, sources of autologous tissue are limited. Allografting, which began about 50 years ago with kidney transplantation between identical twins, has provided an additional option allowing replacement of damaged tissue. It has advantages over autologous graft including better availability and avoids the need for a prior surgical procedure. The use of allogenic tissue, however, may transmit diseases and cause an immune response, which can lead to a high failure rate [2]. In addition, the demand for human donors has already greatly exceeded the supply. Animal tissue transplantation or xenograft has been utilized (for example, porcine heart valve for the mitral valve of patients with valvular heart disease), but it imposes the risk of immune rejection and exposure to pathogens.

On the other hand, tissue engineering can involve patient-specific cells either from a small biopsy or from stem cells. By using the patient’s own cells or genetically treated stem cells with gene therapy, this approach has the advantages of autologous grafts, but without the problems associated with the shortage of supply.
Tissue engineering requires three key components: a suitable scaffold allowing new tissue formation, a biological signal that induces or maintains a cell’s phenotype, and cells capable of differentiation into the targeted tissue phenotype (Figure 1.1). The basic strategy of tissue engineering involves the expansion of cells in temporary three-dimensional scaffolds with an adequate supply of biological substances to form a “neo organ” followed by its implantation after which the body will complete its transformation into a true replacement. From the materials science standpoint, refining the synthesis of materials and fabrication technologies to produce 3D structures having the appropriate pore size and total porosity and its interconnectivity within a scaffold needs to be investigated as well as the optimization of degradation rate and surface roughness. These are parameters that affect the adhesion and proliferation of mammalian cells. Furthermore, biological research involves either on-site recruiting of cells after implantation, or in vitro seeding of cells inside materials followed by long-term in vitro culture without dedifferentiation of committed cells [3].

In this chapter, biodegradable polymers and processing methods to produce three-dimensional scaffolds are reviewed. Among the processing methods, electrospinning of biodegradable polymer and its application in tissue engineering are evaluated in detail.

1.2. Scaffolds for Tissue Engineering

For tissue engineering, a porous three-dimensional scaffold plays an important role by providing a structure to which cells can adhere and proliferate [4]. Like extracellular matrix (ECM), the body’s natural scaffold, it should offer an environment in which cells can maintain their phenotype and facilitate the secretion of their own ECM as
a part of the process of replacing the biodegradable scaffold. Several factors should be considered when the material and its design are chosen: 1) the material should be biocompatible and biodegradable; it should not induce inflammation and produce any toxic by-products; 2) the material should favor cellular attachment and allow the maintenance of differentiated cell function; 3) the scaffold should gradually degrade away as the cells develop into a functional tissue; 4) the pores of scaffold should be interconnected for an improved cellular infiltration and uniform tissue ingrowth.

1.2.1. Biodegradable Polymers for Scaffolds

Traditionally, metals have been widely used for major load-bearing orthopedic applications [5]. There are, however, various problems related to metallic materials in the human body due to corrosion, wear, and/or negative tissue reaction [6]. To avoid these adverse effects of metals, several ceramic materials have been clinically applied [7]. ZrO$_2$ and Al$_2$O$_3$ exhibit high mechanical strength and non-toxicity, but they are bio-inert materials that may induce an encapsulation by fibrous tissue [8]. Recently, hydroxyapatite, one of natural bone’s components, has been plasma-coated to increase the biocompatibility of the orthopaedic devices [9, 10]. However, these traditional biomaterials lack biodegradability, one of the requirement for the scaffold materials.

Biodegradability and easy control over processing and degradation rate have made polymer materials ideal for tissue engineering scaffolds. Polymer materials can be divided into two large categories, natural and synthetic polymers. Natural polymers are the derivations of ECM including polypeptides such as collagen and gelatin, and polysaccharides such as glycosaminoglycans (GAG), alginate and chitosan. Collagen and
its denatured form, gelatin, have been used for tissue engineering scaffolds as a structural component for scaffolds or as a coating of other synthetic polymers to increase biocompatibility and cellular adhesion. They contain RGD (Arg-Gly-Asp) amino acid sequences in their peptide structures and these act as an anchoring site for cellular adhesion by making complexes with cellular integrins [11]. However, collagen and gelatin are water-soluble, which makes them inappropriate for long-term structural scaffolds. To improve the stability of these matrices in aqueous solution, the polymers have been chemically cross-linked with glutaraldehyde [12].

GAG is largely a polysaccharide negatively charged with sulfates. Its unique electro-negativity plays an important role in the ‘Donnan’ effect in cartilaginous tissues by complexing with water molecules [13]. The gel-like nature of this GAG-water molecule complex allows a cartilage tissue to function as both a cushion and a lubricant. Hyaluronic acid, a major GAG component of ECM in cartilage tissue has been used to attempt to manufacture scaffolds for cartilage. It is a very large macromolecule having a molecular weight over a million and it supports structural integrity of cartilage ECM. Like collagen, it contains binding sites for proteoglycan attachment as well as RGD sequences for cellular adhesion.

To overcome the challenge posed by its high molecular weight and high solubility in aqueous solutions, the processibility of hyaluronic acid was improved by esterification of its carboxylic groups using alcohols [14] and this product (HYAFF®) showed an excellent biocompatibility [15]. Chondroitin sulfate, another type of GAG, has been used in hydrogels and cross-linked scaffolds [16-19]. Alginate is a polysaccharide derived from seaweed and is a copolymer of β-D-mannuronic acid and α-L-gluronic acid.
Primary use of alginate for tissue engineering has been as a means of encapsulating living cells for precise delivery. In addition, alginates cross-linked by calcium sulfate have been recently developed and used as scaffolds [20]. Chitosan is a linear polysaccharide derived from chitin which is the structural element in the exoskeleton of crustaceans (crab, shrimp, etc.). It has similar structural characteristics to GAG and has been used in the fabrication of tissue engineering scaffolds [16, 21, 22].

The natural polymers derived from polysaccharides have been extensively used in a gel form. The polymers without crosslink lose their mechanical strength due to its gelation characteristic in an aqueous solution. Therefore, they are considered ineffective to be used as a long-term scaffold for mechanical loading parts. Furthermore, poor mechanical properties and a too-fast degradation rate provide two major obstacles to be solved before the widespread use of naturally derived polysaccharides for tissue engineering scaffolds becomes possible.

Biodegradable synthetic polymers offer a number of advantages over naturally derived polymers for scaffolds. Key advantages include the ability to tailor mechanical properties and degradation kinetics to suit various applications. Synthetic polymers are also attractive because they can be fabricated into various shapes with desired pore morphology conductive to tissue ingrowth. Furthermore, the polymers can be designed with chemical functional groups that can improve cellular attachment and tissue ingrowth. A vast majority of biodegradable polymers studied belongs to polyester family such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and their copolymer, poly(glycolic-co-lactic acid) (PGLA). PGA is a rigid thermoplastic with a relatively high crystallinity (50%). Because of its high crystallinity, PGA is not soluble in most organic
solvents except highly fluorinated organic solvents such as hexafluoro isopropanol (HFP). The attractiveness of PGA as a biodegradable polymer in tissue engineering application is that its degradation product glycolic acid is a natural metabolite. The degradation process occurs in two stages [23-25]; the first involves the diffusion of water into the amorphous regions of the matrix and simple hydrolytic chain scission of the ester groups. The second stage involves largely the crystalline areas of the polymer, which becomes predominant when the majority of the amorphous regions have been eroded. Because of the bulk degradation of PGA, there is a sudden loss of mechanical properties and localized high accumulation of by-product, glycolic acid. In addition, the localized high concentration of glycolic acid may cause tissue damage [26]. PLA has a similar degradation rate to PGA, but it is more hydrophobic than PGA resulting in more resistant to hydrolytic attack. PLA degrades to form lactic acid which is also normally present in the body. The degradation rate of polyester can be tailored by copolymerizing PGA and PLA. Resistance to hydrolysis is more pronounced at either end of the copolymer’s composition range [27, 28]. It is generally accepted that intermediate copolymers are very much more unstable than the homopolymers. Concerns about the biocompatibility of these materials have been raised when PLA and PGA rapidly degrade into acidic by-products that elevate local concentrations of these acids above those normally found in vivo [29]. Another concern is the release of small particles during degradation, which can trigger an inflammatory response.

Another biodegradable synthetic polymer used in tissue engineering scaffold is poly(caprolactone) (PCL). PCL is an ideal candidate of tissue engineering scaffold for load bearing components, i.e. cartilage and bone, due to its slow degradation rate in vivo.
Several scaffolds made of PLA, PGA and their blends showed shrinkage over a short period due to extensive hydrolysis [31]. In contrast, it was shown that phagocytosis of PCL by macrophages and giant cells occurs once molecular weight of the polymer is reduced to 3000 or less by nonenzymatic bulk hydrolysis of the ester linkages, which lasts at least 6 months \textit{in vivo} [32, 33]. The rate of hydrolysis can be altered by copolymerization with other lactones or lactide, for example a copolymer of caprolactone and valerolactone [34], and caprolactone and \(d, l\)-lactide [33].

\textbf{1.2.2. Synthesis Methods for Scaffolding}

Several methods have been developed to synthesize porous three-dimensional scaffolds by using biodegradable natural or synthetic polymers. Those include solvent casting-particulate leaching, gas forming, lyophilization, phase separation, solid free-form fabrication and electrospinning. In this section, these synthetic methods are reviewed; electrospinning is covered in detail in the subsequent section.

Solvent casting-particulate leaching technique is one of the most commonly used methods to prepare tissue engineering scaffolds due to its simplicity and cost-effectiveness. It involves casting a mixture of polymer solution and porogens in a mold and drying the mixture. The porogens in the dried polymer are then leached out with water to generate pores. Water-soluble salts or carbohydrates are usually used as porogens. The pore structure can be manipulated by controlling the variety [35], size [36] and fraction [37] of porogens. Figure 1.2 shows the different microstructures of PLA scaffolds when different porogens were used. However, leaching of porogens, typically sodium chloride, often results in poor interconnectivity among the pores. To resolve this
issue, Draghi et al. [35] employed gelatin microspheres and achieved better pore connectivity and defined pore shape compared to NaCl (Figure 1.2).

Gas forming technique involves the incorporation of high pressure CO\textsubscript{2} gas into polymer solutions. When the polymers are solidified, environmental CO\textsubscript{2} gas pressure is decreased to induce thermodynamic instability that results in clustering of CO\textsubscript{2} gas in the polymers. Dissolved gas molecules diffuse to these nuclei creating the macropores. The porosity and pore structure depend on the amount of gas dissolved, the rate and type of gas nucleation and the diffusion rate of gas molecules through the polymer [38-40]. However, the gas forming technique often results in the formation of closed pores that prevent cellular ingrowth into the scaffold. To improve the pore connectivity, Harris et al. [41] have combined gas forming technique with particulate leaching resulting in significantly greater pore connectivity than when either gas forming or particulate leaching techniques were used alone (Figure 1.3).

Lyophilization or freeze-drying method consists of creating an emulsion by homogenization of a polymer solvent solution and water, rapidly cooling the emulsion to lock in the liquid-state structure, and removing the solvent and water by freeze-drying [42-44] (Figure 1.4). This method generates scaffolds with porosity greater than 90% and the porosity and pore size depend on the fraction of the dispersed water and polymer molecular weight. The shortcomings of these scaffolds are a lack of structural stability due to uneven distribution of pore size.

Woven or knitted meshes of fibers have been produced into three-dimensional patterns with variable pore size using PGA, PLA and PLGA [45-48] (Figure 1.5). The advantages of these fiber meshes are large surface areas and fully interconnected pores in
favor of cell attachment and easy circulation of nutrients and metabolic wastes. To further promote cellular attachment and viability, Ng et al. [46] have developed a hybrid matrix by lyophilizing collagen within PLGA knitted mesh resulting in improved cellular proliferation *in vivo*.

Solid free-form or three-dimensional printing fabrication technique can produce highly complex scaffolds using data generated by CAD systems [49-55] (Figure 1.6). This method involves fabricating three-dimensional objects with layer-by-layer deposition via the processing of solid sheet, liquid or powder material stocks. The advantage of this technique is its customizable scaffold design according to the specific patient. In addition, the use of automated computerized fabrication results in the precise construction of pore size as well as pore interconnectivity. The limitations of scaffolds produced by solid free-form method include the fact that the pore openings in the scaffolds are not consistent in all three dimensions. The pore openings facing the z-direction are formed in between the intercrossing of material and are determined by user-defined parameters. However, for pore openings facing both the x- and y-directions, these openings are formed from voids created by the stacking of material layers and therefore, their sizes are restricted to the thickness of the material layers.

### 1.3. Electrospinning

Electrospinning is a promising technique producing unique interconnected pore structures between non-woven nano/micro fibers. First patented by Formhals in 1934 [56], this technique produces a non-woven fiber mesh that resembles extracellular matrix (ECM) (Figure 1.7). Typical fiber diameters range from 10 nm to 10 µm [57] and provide
large surface area per unit volume, a property promoting cellular adhesion. Electrospinning provides some control of pore size created between inter-fiber contacts by management of fiber diameter [58] via control of spinning parameters such as solution viscosity, distance and voltage.

Electrospinning is a promising method for producing scaffolds for tissue engineering in three-dimensional form partly due to the ease with which it produces nano- to micro-sized fibrous scaffolds having 70 ~ 90% relative porosity. Natural biodegradable materials such as collagen [59-63], gelatin [64, 65], elastin [59, 66], chitosan [67] and hyaluronic acid [68] as well as synthetic biodegradable polymers such as PCL [63, 65, 69-77], PGA [62], PLA [62] and PLGA [78] have been electrospun as scaffolds for cardiovascular, chondral and osseous applications.

1.3.1. Fundamental Theory of Electrospinning

A schematic diagram of a typical electrospinning setup is shown in Figure 1.8 (a). Three main components are used in this setup: a capillary tube supplying a polymer solution or melt, a high voltage supplier and a collector. The capillary and the collector are oppositely charged creating an electrical field between them. The electric field is subjected to the end of the capillary tube that contains the polymer solution ‘pendant’ held by its surface tension. This induces a charge on the surface of the solution. Mutual charge repulsion and the attraction of the surface charges to the counter electrode cause a force directly opposite to the surface tension [79, 80]. Therefore, there are two main forces acting on the pendant drop of polymer solution: an upward force (surface tension) is balanced by downward forces (the applied electric field and gravity). As the intensity
of the electric field is increased, the hemispherical surface of the solution at the tip of the capillary tube elongates to form a conical shape known as the Taylor Cone (Figure 1.8 (b)). When the electric field reaches a critical value at which the repulsive electric force overcomes the surface tension force, a charged jet of the solution is ejected from the tip of the Taylor Cone (Figure 1.8 (b)). Since this jet is charged, its trajectory can be controlled by an electric field. The discharged polymer solution jet undergoes an instability and elongation process, which allows the jet to become very long and thin. As the jet travels in air, the solvent evaporates, leaving behind a charged polymer fiber which lays itself randomly on a collector. Thus, continuous fibers are laid to form a non-woven fabric.

Electrospinning of polymer typically involves a single, electrified, continuous and elongated jet shown by experimental and theoretical results [81, 82]. However, branching or splaying of the jet into several smaller jets was also observed under some conditions for a number of polymer solutions including polyethylene oxide (PEO) [79, 81] and PCL [83] (Figure 1.9). It was demonstrated that particular kinds of polymer and solvent affect the occurrence of branching under the usual conditions of electrospinning. Yarin et al. [83] have shown that even secondary and tertiary jet branches exist during electrospinning of PCL dissolved in acetone. In this context, the final fiber size is determined primarily by the number of subsidiary jets formed.

1.3.2. The effect of governing parameters on electrospun fiber morphology

The morphology of electrospun fibers depend on several processing parameters:

1) the intrinsic properties of the polymer solution including the viscosity, electrical
conductivity and surface tension of the solution; 2) the operational conditions including an applied voltage, a flow (feed) rate and the distance between the capillary and the collector. In addition, the humidity and temperature of the environments also play an important role in determining fiber morphology.

**The effect of the intrinsic properties of polymer solution**

The spinnability of different polymer solutions depends on the different ranges of viscosity. For example, PEO dissolved in ethanol-water solution could be only spun in the viscosity range of 1 – 20 poise [84] while cellulose acetate in 2:1 acetone/dimethylacetamide was in the range between 1.2 and 10.2 poise [85]. When the viscosity of the solution was outside of these ranges, both generated beads/droplets (when the viscosity was too low) or only few fibers (when the viscosity was too high).

The morphology of electrospun fiber is dependent on the conformation of polymer chain. Figure 1.10 shows the morphologies of electrospun PCL and gelatin fibers. Although the other parameters (solvent, solution concentration, applied voltage, flow rate and electrospinning distance) are identical the morphology of gelatin shows a ribbon-like morphology of gelatin (Figure 1.10 (b)) compared to a typical circular shape in cross-section of PCL (Figure 1.10 (a)). This ribbon-like shapes of fibers have been observed in other systems such as polystyrene in dimethylformamide (DMF) [86], poly(ether imide) in HFP [86], nylon 6 in HFP [87] and polycarbonate in chloroform [88]. Koombhongse et al. [86] have postulated that the formation of ribbon-like morphology is due to the formation of hollow cylindrical fibers by rapid evaporation of solvent and the subsequent collapse of the shell into flat morphology. However, the interaction between
the polymer chain and the solvent must be an important factor generating this ribbon-like morphology as shown in the cases of PCL and gelatin dissolved in the same solvent, HFP.

The properties of solvent also play a role in determining the fiber morphology. Figure 1.11 shows the fiber morphologies of electrospun PCL dissolve in a series of solvent mixtures of methylene chloride (MC) and DMF. MC only generated larger fiber diameter compared to MC/DMF mixtures. The change in fiber diameter is thought to be due to the decreased surface tension and viscosity with the increased conductivity and dielectric constant when DMF was added to the pure MC [89]. Therefore, it is believed that the dielectric properties of the polymer solution is one of the key factors influencing electrospinning.

Certain polymer/solvent combinations such as PCL/dichloromethane (DCM), polystyrene/tetrahydrofuran (THF) [90], PMMA/chloroform [90], PMMA/THF [91, 92] and PLLA/DCM [93] generate a unique surface feature on the electrospun fibers. The 15 wt. % PCL in acetone generates typical electrospun fiber morphology (Figure 1.12 (a)) while the same concentration of PCL in DCM produces fibers with nano-sized pores on the surface (Figure 1.12 (b)). Megelski et al. [90] explained that the phase separation between the polymer and solvent during the drying process is responsible for the formation of surface pores on the fibers. When a polymer solution is electrospun, evaporation takes place under convective conditions. During solvent evaporation the solution becomes thermodynamically unstable and phase separation occurs into a polymer rich and a polymer lean phase. The concentrated phase solidifies shortly after phase separation and forms the matrix while the polymer lean phase forms the pores.
One of the most significant parameters influencing the fiber diameter is the solution viscosity. A low viscosity generates droplets while a higher viscosity results in a larger fiber diameter [79, 84, 94]. However, the solution viscosity is proportional to the polymer concentration. Therefore, a higher polymer concentration will result in a larger fiber. It was demonstrated by Demir et al. [95] that the fiber diameter was proportional to the cube of the polymer concentration in the electrospinning of polyurethane. Figure 1.13 shows resulting morphologies of styrenated gelatin with a series of different concentrations. It is also recognizable that the density of beads was reduced as the concentration increased. The molecular weight of polymer also affects the solution viscosity. Dong et al. [96] demonstrated a similar pattern of fiber morphology change by electrospinning different molecular weights of polystyrene dissolved in DMF. In addition, temperature influences the viscosity of polymer solution, thus the fiber morphology. A polyurethane solution which was not spinnable at room temperature was successfully spun at an elevated temperature (70°C) [95].

The effect of operational conditions

An applied voltage is an operational parameter that affects the fiber morphology. In general, a higher applied voltage is thought to eject more fluid in a jet, resulting in a larger fiber diameter [95, 97, 98]. However, some studies showed that the diameter is not influenced by the applied voltage [99] or decreased with increasing the voltage [100-102]. Several studies demonstrated that the distribution of fiber diameter gets broader as the applied voltage increased [103, 104]. It is thought that increasing the voltage increases the electrostatic repulsion force on the ejected jet, which favors branching, thus thinner-
fiber formation. Fridrikh et al. [57] have proposed a model that the final diameter of a spinning jet strongly depends on the interplay between surface tension and electrostatic repulsion. The balance point may be related to the strength of electric field, the flow rate and the surface tension of liquid phase.

The flow rate can affect the morphology of electrospun fiber. It is generally thought that a higher feeding rate for the solution leads to the formation of thicker fibers. However, some studies showed that the flow rate affects the bead formation rather than the fiber diameter [103, 105, 106]. When the flow rate exceeds a critical value, the influx rate of the solution to the capillary tip surpasses the removal rate of the solution from the tip by the electric forces. This leads to the ejection of less-charged jets that result in the bead formation. To support this idea, it was shown that the volume charge density of the solution during electrospinning decreases with increasing the flow rate [107].

Increasing the distance between the capillary and the collector is believed to require a longer times for the solvent to evaporate and for the charged jet to form additional branches (if this occurs). However, the effect of the capillary-collector distance must be considered coupled with the reduction of the electric field strength as the distance increases. Sun et al. [108] have recently developed a near-field electrospinning (NFES) in which the collector was placed only 500 μm away from the capillary. This small distance made it possible to use as little as 600 V or a voltage that was 10-fold less than what is required for the usual electrospinning operation (over 10 cm distance). However, one must be cautious when such a small distance is used. He et al. [109] showed in their theoretical work that a minimum distance is required for successful electrospinning. Figure 1.14 shows a critical straight length of a Taylor Cone during
electrospinning. When a polymer pendant at the tip of capillary is subjected to an electric field, the pendant is elongated for a certain length before whipping of jets begins. A critical straight length depends on flow rate, solution properties and applied voltage and the theory is in agreement with experimental results.

1.4. Electrospun scaffolds in tissue engineering

Considerable effort has been directed towards developing suitable scaffolds meeting specific requirements of various tissues. Electrospinning produces a natural ECM-like structure and this matrix promotes cellular adhesion, proliferation and maintenance of cellular function. Cartilage, skin and blood vessel tissue engineering have been the subject of electrospun tissue engineering scaffold research due to their avascular nature which allows complex designs for vasculature to be avoided.

1.4.1. Engineering Cartilage

The main purpose of articular cartilage is to act as a lubricant for the low friction movement of joints and/or a mediator for load transfer to the underlying subchondral bone. Once cartilage is damaged either by trauma or osteoarthritis it is not easily regenerated by itself due to sparse distribution of cartilage cells, chondrocytes in the tissue. The application of the principles of tissue engineering to cartilage repair was first proposed by Green [110] who in 1977 suggested the transplantation of chondrocytes cultured in an \textit{in vitro} environment into a cartilage defect.

Recently, cell-based repair approaches for replacement and regeneration of articular cartilage have been developed using electrospun scaffolds with various
biodegradable polymers and cell sources like chondrocytes and mesenchymal stem cells [70-72, 111-114]. Electrospun PCL scaffolds have demonstrated feasibility for cartilage engineering showing proliferation and maintenance of the chondrocytic phenotype [70, 115]. Furthermore, human mesenchymal stem cells can be successfully differentiated into chondrocytic phenotype in the structure with adequate growth factors [71].

A few studies [113, 114] have shown that collagen type II can be electrospun and utilized for scaffolds. Collagen type II composes 50 to 80% of the dry weight of articular cartilage [116] and it promotes cellular adhesion through its inherent RGD peptide sequence that the integrin, cellular receptor of chondrocyte bind to [117]. The main shortcoming of collagen as a scaffold is its high dissolution rate in aqueous solution, which results in structural instability. However, Shields et al. [113] stabilized the electrospun collagen structure by cross-linking the polymer using glutaraldehyde vapor and the scaffold was biocompatible (Figure 1.15).

Research has shown that mechanical stimulation of chondrocytes improves cellular proliferation and ECM secretion [118-121]. Therefore, incorporation of mechanical stimulation into the in vitro procedures would potentially enhance cellular behavior to more closely mimic natural cartilage. Shin et al. [31] showed the increased proliferation and GAG production of chondrocytes cultured in an electrospun PLGA scaffold.

### 1.4.2. Engineering Skin

In the body, natural dermis is constructed by the interaction between keratinocytes, fibroblasts, endothelial cells and melanocytes, and a soft tissue matrix made up of
collagens (I, IV and VII), elastin and proteoglycans [122]. Rather than closely mimicking this natural but highly complex structure, skin engineering for human wounds like burn damage has been aimed at protection, removal of exudates and improved appearance. Wound dressing with electrospun scaffold can meet requirements such as high oxygen permeability and good barrier properties [61, 123-126].

PCL and PCL-blended collagen electrospun membranes support human dermal fibroblast and keratinocytes [126]. Cells perform amoeboid movement to migrate through the pores and push the surrounding fibers aside to expand the hole as the small fibers offer little resistance to cell movement. Electrospun fibrous membrane also showed good and immediate adherence to wet wound surfaces. Other than PCL-collagen, chitin [124], hyaluronic acid and hyaluronic acid/gelatin blend [123] have been electrospun to examine their feasibility for skin engineering scaffolds.

One interesting experiment showing natural self organizing capability of skin cells was carried out by Sun et al. [125]. In their experiment, low rates of survival and viability of keratinocytes, fibroblasts and endothelial cells were observed when cells were introduced as single populations to an electrospun matrix. However, coculture of the three cell types led to a scaffold populated with viable cells, even under serum-free conditions. In the scaffold, keratinocytes formed a continuous epidermal layer at the upper air-facing surface while fibroblasts and endothelial cells occupied the central and lower regions (Figure 1.16). This shows that a random coculture of different cell types can induce tissue-like constructs by self-organization.
1.4.3. Engineering Blood Vessel

Intimal hyperplasia is a well known clinical phenomenon in which pathological matrix remodeling occurs with excessive ECM accumulation after vascular injury (atherosclerosis) [127]. Small diameter blood vessels, which possess less room for expansion, are much more likely to become occluded during this remodeling process. Traditional prosthetic devices such as polyethylene terephthalic ester (PETE) (better known clinically as DACRON) has been used for vascular grafts but shows a poor patency rate at small vessel diameters [128]. The potential of electrospinning for vascular tissue engineering is enormous since it can not only mimic the natural ECM but also its spatial organization on the mesoscopic scale including control over fiber orientation and spatial placement [129].

In the medial layer present in native blood vessels, the smooth muscle cells and collagen fibrils have a circumferential orientation so as to provide the mechanical strength necessary to withstand the circulatory high pressures [130]. It is also known that the intima layer consists of a layer of endothelial cells lining the vessels internal surface aligned to the blood flow [130]. Between those two layers, an internal elastic lamina exists mainly composed of elastin, conferring elastic properties to the blood vessels [130].

To develop a scaffold architecture mimicking morphological and mechanically that of a blood vessel, several studies have examined the feasibility of electrospun matrices composed of collagen, PCL, PCL-PLA copolymer and poly(ester urethane)urea (PEUU) using endothelial cells [66, 131-133], smooth muscle cells [63, 134] and venous myofibroblasts [135]. Ma et al. [133] surface-grafted gelatin on electrospun PCL by activating –COOH groups using inductively coupled radio-frequency glow discharge
plasma treatment and the grafted scaffold showed the improvement of adhesion and proliferation of endothelial cells. Moreover, aligned fibers showed a strong ability to control the orientation of endothelial cells in the direction of the fibers without flow induced cellular orientation (Figure 1.16). Vaz et al. [135] developed a multi-layered tubular scaffold by electrospinning PCL, followed by PLA onto a rotating mandrel. The results demonstrated that the combination of an outer concentric layer of circumferentially oriented stiff PLA fibers and an inner concentric layer of randomly oriented elastic PCL fibers results in a desirable strong and pliable scaffold.

1.4.4. Engineering Bone

Guided bone regeneration (GBR) is a therapy to repair mandible and alveolar bone defects infected by periodontal diseases. The main purpose of GBR is to prevent an invasion of non-functional scar tissue and to encourage bone growth. Traditionally expanded-poly(tetrafluoroethylene) (ePTFE), also known as Gore-Tex has been used for GBR membranes and it showed excellent clinical results. However, ePTFE membranes have to be removed by a second surgery since the material is not biodegradable. A GBR membrane made of biodegradable materials is necessary.

Several have attempted to produce GBR membranes by electrospinning [76, 136-140]. The biodegradable polymers often have been combined with nano-sized bioceramics such as hydroxyapatite (HA) and calcium carbonate to produce more osteogenic scaffolds [136, 137, 139, 140]. The addition of HA showed significantly improved cellular activity in comparison with pure polymer scaffold and this is thought to be due to its structural similarity to the mineral phase of natural bone. However, bone
tissue engineering using electrospun scaffold is in its infant stage due to the complexity of bone structure including vasculature.

1.5. Conclusions

The degree of biomimicry required in a tissue engineering scaffold is a key element and has yet to be determined in this relatively young field. Exact biomimicry will be too complex and expensive and is unlikely to be necessary. Identification of the key functional requirements of a scaffold is required so that these can be implemented in the design. Furthermore, natural organs do not consist of exclusively one cell type and this makes tackling true tissue engineering operations considerably more complex.

In the last few years, electrospinning has attracted a great deal of attention as a way to mimic the structure of natural ECM by means of producing nano-/micro-sized fibers. The process provides an inexpensive and easy way to produce nanofibers from hundreds of types of polymers with a small fiber diameter, variable pore size and high surface area. Control of cellular interaction with bioengineered electrospun scaffolds may allow the generation of a variety of engineered tissues.
Figure 1.1. Schematic illustration of tissue engineering strategy.
Figure 1.2. SEM images of PLA scaffolds after particulate leaching when (a) gelatin and (b) NaCl were used as porogens (scale bar: 1 mm) (taken from [35]).
Figure 1.3. SEM image of PLGA matrix formed by gas forming/particulate leaching combined technique resulting in better pore connectivity (taken from [41]).
Figure 1.4. SEM image of collagen scaffold produced by lyophilization (taken from [42]).
Figure 1.5. SEM image of PLGA knitted mesh (taken from [48]).
Figure 1.6. SEM image of high density polyethylene (HDPE) produced by solid free-form technique (taken from [50]).
Figure 1.7. SEM images showing similarity between (a) ECM of articular cartilage from sheep femur (AC: articular cartilage, CZ: calcified zone) (taken from [141]) and (b) representative electrospun PCL.
Figure 1.8. (a) Schematic diagram of a typical electrospinning setup (taken from [142]) and (b) image of electrospinning jet showing a Taylor cone (arrow) (taken from [81]).
Figure 1.9. Still image of 15% PCL solution dissolved in acetone during electrospinning shows a thick jet with many closely spaced branches and a high taper rate (taken from [83]).
Figure 1.10. SEM images of electrospun (a) PCL and (b) gelatin at same concentration (6.7 wt. %) show different morphologies of fibers; electrospun PCL fiber shows a typical morphology (circular in cross-section) while electrospun gelatin shows a ribbon-like morphology.
Figure 1.11. SEM images of electrospun PCL dissolved in (a) methylene chloride (MC), (b) 85/15 MC/dimethylformamide (DMF), (c) 75/25 MC/DMF and (d) 40/60 MC/DMF showing different fiber diameters (taken from [89]).
Figure 1.12. SEM images of electrospun 15 wt. % PCL in (a) acetone and (b) dichloromethane show different fiber morphologies.
Figure 1.13. SEM images of electrospun styrenated gelatin dissolved in HFP: (a) 2.5 wt.%, (b) 5 wt.%, (c) 7.5 wt.% and (d) 10 wt.% (taken from [95]).
Figure 1.14. Schematic diagram showing a critical straight length (L) (taken from [109]).
Figure 1.15. SEM image of chondrocyte on an electrospun collagen type II scaffold. Cells adhere to the scaffold and maintain their phenotypic morphology (round) (taken from [113]).
Figure 1.16. Immunofluorescence images of human keratinocytes, fibroblasts and endothelial cells in an electrospun scaffold under serum-free conditions. Three-cell cocultures labeled with (a) DAPI, (b) pancytokeratin, (c) DAPI and (d) CD31 showing self-organization of the cells, Keratinocytes (K) at the air-liquid interface and endothelial cells (E) under them (taken from [125]).
Figure 1.17. LSCM images of rhodamine-phalloidin-stained endothelial cells on (a) aligned electrospun PCL and (b) random electrospun PCL cultured for 4 days showing alignment of the cells following electrospun fiber direction (taken from [133]).
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2.1. Abstract

Electrospinning is a broadly useful technology for the generation of fine polymeric fibers. However, our understanding of the mechanical behavior of such micron to submicron diameter fibers is limited. Here we show that a consequence of dividing polymeric matter (polycaprolactone) into nanoscaled fibers is a pronounced tendency toward recrystallization under applied stress. This effect appears to be based on how discrete, strained fibers achieve the familiar alignment of individual polymer chains that lowers the entropic barrier to crystallization. This, along with the possible effects of frictional heating and shrinkage, results in the extensive formation of a ‘bamboo’ structure along the length of adjoining fibers. Recrystallization is observed to sweep through significant amounts of electrospun fiber. The associated heat of recrystallization can be significant enough to destroy the initial fiber-based structure and result in a spherulitic form of PCL.
2.2. Introduction

For the generation of fine fibers, ranging from 15 nm to 10 µm or greater, electrospinning is a broadly useful technology. Electrospinning relies on the application of an electrostatic force to drive fiber formation. Interest in the electrospinning technology was recently revived when Reneker et al. first demonstrated that a multitude of polymers could be electrospun [1] and refined the associated theory [2]. Hundreds of different natural and synthetic compositions have been electrospun into thin fibers since 1990.

In spite of its broad usage, our understanding of the mechanical behavior of electrospun fibers is currently limited. Pioneering research has been conducted on alignment and overall stress-strain behavior [3, 4]. At a much smaller scale, Tan et al. [5] showed a linear stress-strain relationship of a single electrospun polyethylene oxide (PEO) fiber using an innovative form of atomic force microscopy (AFM). Kim et al. [6] demonstrated ‘nanonecking’ or failure of electrospun fiber by shear yielding. Single fiber results are invaluable in understanding the mechanical deformation of individual fibers but cannot capture all the influences inherent to the deformation of electrospun fiber meshes. Such meshes are an assembly of randomly oriented fibers having variable length. Electrospinning inevitably produces imperfections – beading [7, 8], chemical bonds between fibers [4, 9] – that may have effects on how the fiber assembly tolerates stress. Several experiments show a pronounced decrease in the slope of the stress-strain curves [9-15] that do not resemble the behavior of a single fiber [5].

The objective of this study is to establish specifics regarding the mechanical behavior of electrospun polycaprolactone (PCL). As a consequence of the division of
matter into a nanoscaled form, distinctive deformation mechanisms result due to thermodynamically-driven interactions between fibers at both the microstructural and nanostructural level. Familiar concepts involving reduction of the entropic barriers to recrystallization are coupled to the potential effects of frictional heating at the nanoscale. Fiber meshes of electrospun PCL having similar fiber diameter and fiber diameter distribution were produced using two completely different solvents, acetone and hexafluoropropanol (HFP). These different solvent systems appear to lead to subtle differences at the nanostructural level that dictate how these fine polymer structures respond to stress.

2.3. Materials and Methods

2.3.1. Electrospinning

A 12 wt. % solution of polycaprolactone (M₆ 65,000, Aldrich, St. Louis, MO) in acetone (Mallinckroff Chemicals, Phillipsburg, NJ) was prepared and then electrospun using a high voltage DC power supply (Model FC50R2, Glassman high voltage, High Bridge, NJ) operated at 23 kV, a 20 cm tip-to-substrate distance and a flow rate of 20 ml/hr. In addition, 6.7 wt. % solutions of PCL in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (Aldrich, St. Louis, MO) was spun using 26 kV with a 20 cm tip-to-substrate distance and a flow rate of 15 ml/hr. In all the cases above, 4x4” (10.2x10.2 cm) sheets approximately 0.25 mm thick were deposited onto aluminum foil. To produce aligned PCL fibers, the same 12 wt. % solution of PCL in acetone was electrospun onto a rotating mandrel 7 cm in diameter at a speed of 5,000 rpm (corresponding to a linear speed of 18.3 m/s). The thickness of the aligned PCL sample was approximately 70 µm. The
samples were treated at room temperature in vacuum (< 30 mm Hg) for 24 hrs prior to tensile tests. This treatment effectively removes any residual solvent without affecting microstructure [16].

2.3.2. Tensile Test

Tensile dogbones with a gauge length of 20 mm and a gauge width of 2.4 mm were cut from the electrospun sheets using 2-mm thick aluminum templates. A surgical blade (Bard-Parker #15, BD Medical Systems, Franklin Lakes, NJ) was used to carefully cut the straight edges while a 3 mm dermal biopsy punch (Miltex, York, PA) was used to form the radii. Great care was taken in cutting the gauge length to ensure that no tearing or smearing of the electrospun PCL occurred. Tensile sample thickness was measured using a digital micrometer (No. 721, Starrett, Athol, MA) by placing the gauge length of each specimen between two glass microscope slides and subtracting the thickness of the two slides from the total. Tensile properties were determined utilizing a 1 kg load cell (model 31, Honeywell Sensotec, Columbus, OH) and a strain rate of 5 mm/min on load frame (Model 1322, Instron, Norwood, MA) using lightweight carbon fiber grips (A2-166 Fibre Clamp Assembly, Instron, Norwood, MA). Tensile testing utilized five samples for each condition.

2.3.3. SEM Analysis

All the samples were coated with 8 nm thick osmium using an osmium plasma coater (OPC-80T, SPI Supplies, West Chester, PA). The use of osmium plasma instead of sputtered Au or Au-Pd eliminates concerns regarding melting of the PCL during sputter
coating and allows for higher resolution SEM images. A Philips XL-30 ESEM and FEI XL-30 Sirion SEM with a FEG source were used to observe the samples. The diameter of more than 100 randomly chosen fibers was measured and used to determine an average and a distribution.

2.3.4. DSC Analysis

The crystallinity of the samples before or after specific exposures was determined by differential scanning calorimetry (2920 DSC, TA instruments, New Castle, DE) at a heating rate of 10°C/min and a nitrogen flow rate of 60 ml/hr. The DSC curves were analyzed using Universal Thermal Analysis software (Ver. 3.9A, TA instruments, New Castle, DE) and crystallinities calculated using:

\[
Crystallinity(\%) = \frac{\Delta H_f}{\Delta H_f^o} \times 100,
\]

where \(\Delta H_f\) is the measured heat of fusion for the sample and \(\Delta H_f^o\) is the heat of fusion for 100% crystalline PCL. \(\Delta H_f^o\) was 139.5 J/g as reported by Pitt et al.[17].

2.4. Results

The morphologies of PCL electrospun using acetone or HFP as the solvent were compared (Figure 2.1). At a macroscopic level ((a) and (c)) they are largely indistinguishable; both show cylindrical fibers having no obvious beading. The average diameters are approximately 790 and 800 nm for the acetone-derived electrospun PCL fiber (hereafter referred to as “acetone-PCL”) and HFP-derived electrospun PCL fiber (“HFP-PCL”), respectively. Both have a bimodal fiber distribution; most diameters are in
the range of 350 ~ 450 nm with a small quantity (less than 5%) of larger fibers more than 1.5 µm. However, higher resolution images ((b) and (d)) reveal distinct microstructural differences. Inter-fiber (or ‘point’ [9]) bonding was frequently observed in the acetone-PCL while this is far less obvious in the HFP-PCL.

Figure 2.2 shows tensile stress-strain curves for five acetone-PCL dogbones. The average ultimate tensile strength and elongation at failure were 1.29 ± 0.12 MPa and 103 ± 17%, respectively. The stress-strain curves for five HFP-PCL samples are shown in Figure 2.3. The average ultimate tensile strength and elongation at failure were 3.71 ± 0.69 MPa and 46 ± 6%, respectively. The transition between the ‘elastic’ and ‘plastic’ portions of these curves appears relatively smooth for acetone-PCL while that of the HFP-PCL is more evident as a sharper decrease in slope.

These obvious differences in mechanical behavior lead us to suspect that the subtle structural changes (Figure 2.1) must be responsible. Failed specimen and specimens still under strain were observed under SEM (Figures 2.4 and 2.5). For acetone-PCL, alignment of the fibers in the loading direction was observed without obvious morphological changes in the fibers themselves (Figure 2.4). Aligned fibers near the fracture site recoiled slightly due to springback after failure (Figure 2.4 (b)). An ellipsoidal void approximately 20 µm in length and 10 µm wide was observed at 80% elongation (Figure 2.4 (c)). Broken fibers are clearly visible in the vicinity of this defect (Figure 2.4 (d)). Higher resolution images show failure of these fibers by necking followed by shear yielding and the development of bridging crazes [6, 18] (Figures 2.4 (e) and (f)).
In contrast, the microstructure of HFP-PCL exhibits a dramatically different morphology (Figure 2.5) near the failure site. In some areas, aligned fibers exhibiting no obvious change in morphology were observed (Figure 2.5 (b)). However, a ‘melted’ zone was observed at the macroscopic necking point (circled in Figure 2.5 (a)). Closer examination showed that what were formerly individual fibers are now clearly joined together (Figure 2.5 (c)). Higher resolutions showed that a distinctive fiber/solid morphology develops in this ‘melted’ zone (Figure 2.5 (d)). Aperiodic bands perpendicular to the loading direction are consistently observed (Figures 2.5 (e) and (f)). This “bamboo” structure consists of a 1~3 µm bands separated by regions that are approximately 30 nm in thickness protruding from the surface.

To determine if this microstructural change corresponded to significant polymer chain rearrangement, the crystallinities of each condition were determined by DSC. For the baseline, the heat flow profiles of as-spun and heat/vacuum treated acetone-PCL and HFP-PCL were measured (Figures 2.6 and 2.7) and the crystallinity estimated. The approximate crystallinities of as-spun acetone-PCL and HFP-PCL were 46.1 and 45.4%, respectively. This changed little following heat/vacuum treatment: 44.7 and 47.1% for acetone-PCL and HFP-PCL, respectively. An increased melting temperature - from 59 to 61ºC - after heat/vacuum treatment was detected and is likely due to residual solvent removal. The crystallinity of the material contained in the gauge length of the failed acetone-PCL (46.4%) did not change (Figure 2.8). However, that of the HFP-PCL specimen increased substantially to 54.0% (~15% increase).

To verify that a lack of point bonding (and not solvent-based differences) were responsible for this apparent ‘melting’ behavior, aligned acetone-PCL fibers were
produced utilizing the common expedient of a rotating collector (Figure 2.9). As shown in Figure 2.9 (a), the fibers were largely aligned along the direction of rotation. The average fiber diameter was 750 nm with a fiber diameter distribution similar to that of the unaligned acetone-PCL. Figure 2.9 (b) shows the very end of the fracture site following the tensile test; the fibers appear to be joined together. More significantly, the ‘melted’ zone previously seen only in HFP-PCL is now observed (Figure 2.9 (c)). Within this zone, the ‘bamboo’ structure is again observed.

Figure 2.10 shows the stress-elongation curves of 5 aligned acetone-PCL dogbones. The average ultimate tensile strength and elongation at failure were 2.96 ± 0.76 MPa and 33 ± 4%, respectively. More significantly, the decrease in slope previously observed in both the acetone-PCL and the HFP-PCL was eliminated.

2.5. Discussion

Electrospinning has been positioned as a promising, cost-effective method providing scaffolds for tissue engineering. Hundreds of natural and synthetic polymers have been spun for this purpose [3, 10, 11, 19-27]. In this context, however, the mechanical properties of scaffolds, in addition to chemistry and morphology, can conceivably affect cellular behavior [28, 29]. Therefore detailed knowledge of the behavior of individual fibers is needed to achieve the desired biological and mechanical property targets.

With this goal in mind, the mechanical properties of electrospun acetone-PCL, HFP-PCL and aligned acetone-PCL are summarized in Table 1. Although acetone-PCL and HFP-PCL are characterized by similar fiber diameter and diameter distributions, the
ultimate tensile strength of HFP-PCL is nearly three times that of acetone-PCL. This discrepancy in the mechanical property is caused not by direct solvent effects but by how the choice of solvent influences the subsequent fiber structure and its interactions under load. Electrospinning typically requires that a low boiling point solvent suitable for dissolving the polymer be used; beyond that the choice of solvent has been historically arbitrary. The vapor pressure of the solvent should be high enough that it evaporates quickly enough to allow the fiber to maintain its integrity when it reaches the target. However, it should not evaporate so quickly that the fiber hardens before it reaches the nanometer range. The viscosity and surface tension of the solvent must neither be too large (to prevent the jet from forming) nor be so small that the polymer solution drains freely from the pipette [30]. In reality, the removal/evaporation of solvent from an electrospinning jet is more complex. Acetone and HFP have roughly identical boiling points; the vapor pressure of acetone under ambient conditions is actually higher. Boiling point is only somewhat predictive of behavior. For example, N,N-dimethyl formamide (DMF) is a common solvent in electrospinning; toluene is not, even though it has lower boiling point. Even though under ambient conditions DMF is more difficult to evaporate than toluene, the volatility of DMF under high electric voltage is likely higher than that of toluene. Empirically, DMF, as well as HFP, is good electrospinning solvent; the suspected reason is that these solvents are polar and likely to have higher volatility under electrospinning conditions than a solvent that has an equally low boiling point but is non-polar (such as acetone).

Based on observations of the microstructure, HFP evaporates more quickly during spinning and this decreases the amount of fiber-fiber bonding in the resulting mesh. As
shown in Figure 2.1, acetone-PCL fibers contain considerable amounts of bonding at fiber intersections. Point bonds, a concept first introduced by Lee et al. [9], appear to lead to stress concentrations by restricting fiber rearrangement. Thus, either the inter-fiber bond itself and/or the fiber near the bond can be regarded as a localized point of failure under load. Broken inter-fiber bonds represent weak points in the overall structure as they concentrate the applied force along the length of the fiber. This is consistent with the observations of 80% elongated acetone-PCL (Figures 2.4 (c) ~ (f)). The void in the specimen appears to be an aggregation of broken fibers (Figure 2.4 (c)). Such voids act as a stress concentrator and can clearly serve to propagate failure under load. The fibers around the void deform in a ductile manner near “point bonded furcations” as shown in Figures 2.4 (d) ~ (f).

The elongation at failure of acetone-PCL is about twice as large as that of HFP-PCL (Table 1). We believe this to be due to a greater frequency of point bonding as all the other factors are held nearly constant. Figure 2.11 shows schematic diagrams of structural response to load (adapted from [9]) in both the presence and absence of point bonding. Fiber rearrangement along the axis of tensile stress is well-documented [4, 15, 31]. For non-bonded structures (Figure 2.11 (b)), fiber rearrangement begins when fibers aligned parallel to the direction of loading begin to deform. Alignment continues on a relatively global level affecting all fibers (both aligned and, eventually, unaligned). In contrast, fibers having point bonding (Figure 2.11 (a)) can rearrange themselves both by alignment and by breaking both inter-fiber bonds and (potentially) the fibers involved in forming that bond. In essence, when these point bonds break they allow immediate rearrangement by triggering translation of polymer fibers past one another without larger
scale alignment. Therefore, the structure can be deformed further under less load, the stress required to deform a large number of adjacent fibers being greater than that needed to break isolated point bonds.

Although the elongation at failure of the non-point bonded structure is smaller than that of a point bonded structure, the strain experienced by individual fibers is larger. Fiber fracture caused by stress concentrations leads to structural failure.

Individual fibers are clearly joined together in the ‘melted’ zone (Figure 2.5) but this does not infer that melting has truly occurred under load because (1) electrospun polymers are (at best) only semi-crystalline and (2) actual specimen failure does not occur in this zone. Instead, DSC supports the idea that recrystallization has occurred by providing evidence of increased crystallinity in HFP-PCL following straining. In addition, it is important to remember that DSC is a bulk technique: in these samples it analyzes both recrystallized and unaffected fiber. Therefore the true increase in crystallinity associated with the process in Figure 2.5 is greater than what DSC can detect. In spite of this, the observed increase in crystallinity is greater than that resulting from annealing electrospun fibers [32]. This suggests a substantial increase in correspondence with Figure 2.5 (d) in which the crystalline domain visible along the length of the fiber appears to be considerably greater than 54%.

How can we best reconcile the massive transformation of fibrous material (Figure 2.1) into a monolithic structure (Figure 2.5) with the original, completely fibrous nature? We begin by considering the initial nature of electrospun fiber itself. Tan and Lim [33] give special emphasis to “aligned but non-crystalline molecules” present in electrospun
fiber as observed by Cicero and Dorgan [34]. With this as our starting condition, the data show the following:

1. Crystallinity in these systems increases under load.
2. SEM provides substantial evidence for massive recrystallization.

Ignoring the concept of point bonding for the moment, our conjecture is as follows:

1. Under strain, aligned but non-crystalline molecules [34] connecting the crystalline domains bear the majority of the strain experienced by an individual fiber.
2. This creates a strong driving force for crystallization by reducing the entropic barrier [35].
3. If amorphous polymer is converted into crystalline form by either (a) incorporation into neighboring lamella or (b) nucleation and growth processes, in either case the heat of crystallization, $\Delta H_c$, must be liberated.
4. This increase in temperature in a fiber already under considerable strain catalyzes further crystallization.
5. Shrinkage along the length of the fiber associated with localized crystallization likely produces even greater levels of tensile strain. This could extend the phenomenon well beyond the initial event. Such localized heating could explain observations of bamboo running down the length of fibers well outside their points of contact (Figure 2.5 (d)).
6. As the process continues the total $\Delta H_c$ generated increase the temperature enough to result in the destruction of the initial fibrous morphology (Figure 2.5 (c)). Recrystallization sweeps through large swaths of electrospun fiber altering its
morphology, reducing its surface area and eliminating its initially fibrous nature. An additional factor may be that crystalline polymers under strain are believed to experience a suppression in $T_m$ [36] which would further drive recrystallization along the length of the fiber.

Returning to the concept of point bonding, these increases must be subject to the discreteness of the initial fiber. Specific levels of point bonding (Figure 2.1 (b)) result in ‘early’ failure of the mesh before the stress in an individual fiber is high enough to trigger recrystallization. Beyond a certain point, fiber-fiber bonding will form a well-connected network complete enough to eliminate the phenomenon by distributing the load.

An additional factor is that although we would prefer to think of these fiber-based structures as exhibiting only simple physical rearrangement under strain, it is likely that strain causes the fibers to constantly rub against one another. If such contacts lead to even limited frictional heating (an additional parallel with polymer melts), this could be enough to trigger localized recrystallization of the already unstable amorphous polymer leading to recrystallization of fibers starting at the point of contact. As we have observed, a preponderance of the observed bamboo structures involve fiber-fiber contacts.

Under the relatively high values of strain achievable in HFP-PCL, alignment of the individual polymer chains within these aligned fibers [3, 7, 13] is more likely. As a verification of the idea that point bonding affects the mechanical properties of electrospun fibers, aligned fiber was electrospun utilizing the same solvent (acetone) that previously produced the point bonded structure that did not exhibit recrystallization. The increased ultimate tensile strength observed is due to the more efficient transfer of
macroscopic stress to the individual fiber. Decreased elongation to failure is caused by
the relative absence of fiber rearrangement. Figure 2.12 shows multiple necks in a single
as-spun aligned fiber. Zussman et al. [18] observed this intrinsic defect when
poly(ethylene oxide) was electrospun onto a rotating collector at a linear speed of 5.3 m/s
[18]. They also showed that these necks are main failure sites.

This as-spun aligned acetone-PCL has relatively little point bonding and the
microstructure of the failed specimen is now similar to that of HFP-PCL. A recrystallized
zone having the distinctive ‘bamboo’ structure (Figure 2.9) is now visible. The fact that
this is now observed in acetone-PCL fiber proves that the phenomenon is not a
consequence of the use of one solvent versus another but is instead driven by
microstructural alterations in the interaction between fibers produced during the spinning
process.

2.6. Conclusion

The effect of different solvent systems on the microstructure and mechanical
behavior of electrospun PCL was investigated. Point-bonded structure having chemical
bonds at the fiber intersections was produced using acetone. When used as a solvent
during electrospinning of the same polymer HFP did not produce such frequent inter-
fiber bonding. In spite of identical polymer chemistry and fiber diameter the point-
bonded structure exhibited earlier failure prior to individual fiber deformation due to
stress concentrations. In contrast, less bonded electrospun fibers became highly aligned
during straining and developed a distinctive “bamboo” structure that we show is due to
localized recrystallization.
<table>
<thead>
<tr>
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<th>Ultimate Tensile Strength (MPa)</th>
<th>Elongation at Failure (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone-PCL</td>
<td>1.29 (±0.12)</td>
<td>103 (±17)</td>
</tr>
<tr>
<td>HFP-PCL</td>
<td>3.71 (±0.69)</td>
<td>46 (±6)</td>
</tr>
<tr>
<td>Aligned Acetone-PCL</td>
<td>2.96 (±0.76)</td>
<td>33 (±4)</td>
</tr>
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Table 2.1. Summary of mechanical properties of acetone-PCL, HFP-PCL and aligned acetone-PCL.
Figure 2.1. SEM images of electrospun PCL using (a), (b) acetone and (c), (d) HFP as solvents. Higher magnification images ((b) and (d)) reveal larger amounts of inter-fiber (point) bonding in acetone-PCL than in HFP-PCL.
Figure 2.2. Engineering stress vs. elongation curves generated from electrospun acetone-PCL.
Figure 2.3. Engineering stress vs. elongation curves generated from electrospun HFP-PCL.
Figure 2.4. (a) and (b) SEM images of failed dogbones of acetone-PCL showing rearrangement of fibers along the loading direction and brittle fracture. (c), (d), (e) and (f) are SEM images of 80% elongated acetone-PCL showing (c) a void generation and (d), (e) and (f) fractured fiber near the void; images (e) and (f) show ductile fractures near “point bonded furcations”.
Figure 2.5. (a) low magnification SEM image of failed HFP-PCL dogbone. (b) near the fracture surface indicated with an arrow in image (a); (c) and (d) near the neck circled in image (a) showing what appears to be a “melted zone”. (e) and (f) high resolution images of “bamboo” structure in the “melted zone”.

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Figure 2.6. DSC results of as-spun acetone-PCL and HFP-PCL.
Figure 2.7. DSC results of heat/vacuum treated electrospun acetone-PCL and HFP-PCL.
Figure 2.8. DSC results from the gauge length of the failed dogbones of electrospun and heat/vacuum treated acetone-PCL and HFP-PCL.
Figure 2.9. SEM image of aligned acetone-PCL in the gauge length of (a) as-spun and (b), (c) and (d) failed dogbones; (b) fracture surface, (c) “melted zone” and (d) “bamboo” structure visible in the “melted zone”.
Figure 2.10. Engineering stress vs. elongation curves generated from aligned acetone-PCL.
Figure 2.11. Schematic illustrations of the deformation (in the vertical direction) of (a) “point bonded” fiber structure (●: inter-fiber bonding site, ×: broken fiber site) and (b) non-bonding structure.
Figure 2.12. SEM image of as-spun aligned PCL showing intrinsic necking/yielding produced during electrospinning.
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CHAPTER 3

INFLUENCE OF COMPOSITION AND POST-PROCESSING CONDITIONS ON RESIDUAL SOLVENT RETENTION IN BIODEGRADABLE ELECTROSPUN FIBERS

3.1 Abstract

Residual solvent retention in electrospun polycapro lactone (PCL), gelatin and PCL-gelatin blends was investigated. 100% PCL, 25:75 gelatin-PCL, 50:50 gelatin-PCL, 75:25 gelatin-PCL and 100% gelatin dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) were electrospun and the amount of residual HFP determined by electro-spray mass spectroscopy (ESI-MS). PCL dissolved in acetone was also electrospun and the amount of residual solvent established. Immediately after spinning, 100% gelatin fiber could contain as much as 1600 ppm of HFP. Gelatin/denatured collagen clearly has a much greater affinity for this particular solvent than PCL, a widely used synthetic polymer. Very little acetone or HFP was detected in pure electrospun PCL. This supports the idea that PCL is relatively immune to the effects of retained solvent. Simple and effective treatments that reduced the residual solvent of gelatin-containing electrospun fiber were developed. Vacuum combined with heat treatment at 37 and 45°C reduced the HFP content to 10 and 5.6 ppm, respectively. We demonstrate that residual solvent
contents could be reduced to a level that does not appear to influence the viability of adherent mammalian cells.

3.2. Introduction

Tissue engineering is a rapidly expanding technical area that seeks to create specific human tissues and/or organs by combining cells and scaffolds formed typically using either synthetic or naturally-derived polymers. These tissue-engineered products will someday be able to deliver on the promise of functional replacement for diseased or failing organs. In pursuing these goals, our interactions with biologically-based collaborators has inevitably led to an interest in expanding upon or improving the ability of current tissue engineering scaffolds to promote or preserve the appropriate function of adherent mammalian cells. Chief among these is the native chemistry of the scaffold and how it influences cellular development.

A broad variety of techniques have been developed to fabricate such scaffolding. Within this range of options it is generally agreed that electrospinning has the potential to produce scaffolds possessing the appropriate strength, structure, economics, and biocompatibility. The technique enjoys wide usage [1-15]. However, the influence of chemical purity on biological activity is not well described. Electrospinning typically uses a solution of polymer in a solvent [16, 17]. Considerable effort has been directed toward establishing the effects of these solvents on the spinning process and the morphology of the resulting fibers [7, 18-22]. Some of these solvents may be retained and could conceivably affect scaffold biological performance either beneficially or (more likely) adversely. Collagen is favored for use in a number of tissue engineering
applications. Type I collagen can be dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) and this has been used successfully to electrospin scaffolds for smooth muscle cells [23]. Natural collagen is relatively non-immunogenic; however, dissolution of collagen in any organic solvent causes conformational changes and the processed collagen could conceivably retain the solvent to affect both biological and structural properties.

In addition, our knowledge of the microstructural-level behavior of these complex fiber arrangements during exposure to even small amounts of degradation is also limited. Historically, cells are typically cultured on the outside of electrospun fabrics; the viability of adherent mammalian cells could be influenced either beneficially [24-26] or negatively by microstructural changes in the scaffold resulting from environmental exposure.

These scaffolds are currently targeted to provide mechanical support while host-appropriate cells populate the structure and deposit specific extracellular matrix (ECM) components. While some efforts have occasionally been made to minimize the potential presence of residual solvents [14, 15, 18] there has been, to our knowledge, no systematic study of solvent retention or post-processing steps that minimize it. We undertook this investigation to shed light on this potential problem and to understand how materials processing parameters can influence surrounding biological environments.

Immediately after electrospinning from HFP solution gelatin fibers can contain as much as 1600 ppm of residual solvent. The fluorinated analog of isopropanol, a common sterilizing agent, HFP has the potential to negatively influence cellular proliferation both in vitro and in vivo. Subsequent treatment with vacuum and vacuum plus heating reduced HFP levels to below 100 ppm. Composite collagen-PCL fibers that combine the
biological functionality of collagen with the relatively greater dissolution-resistance of PCL, showed that the residual HFP closely scales with collagen content. While much more easily removable solvents like acetone are widely used, the increasing need to electrospin scaffolds from relatively difficult-to-dissolve biological materials suggests that more detailed observations of the retention and biological effects of such residual solvents need to be made.

3.3. Materials and Methods

3.3.1. Electrospinning

A 6.7 wt. % solution of gelatin type A from porcine skin (~300 bloom, Sigma, St. Louis, MO) in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (Aldrich, St. Louis, MO) was prepared and then electrospun using a high voltage DC power supply (Model FC50R2, Glassman high voltage, High Bridge, NJ) operated at 26 kV, a 20 cm tip-to-substrate distance and a flow rate of 15 ml/hr. This 20 cm tip-to-substrate distance and 15 ml/hr flow rate were used in all subsequent depositions. A 12 wt. % solution of polycaprolactone (M_w 65,000, Aldrich, St. Louis, MO) in acetone (Mallinckroff Chemicals, Phillipsburg, NJ) was prepared and then electrospun at 23 kV. In addition, 6.7 wt. % solutions of PCL and gelatin in HFP were spun at 25, 50, and 75% final gelatin contents using 23, 23 and 26 kV, respectively. In all cases a 4x4” (10.2x10.2 cm) sheet approximately 0.5 mm thick was deposited onto aluminum foil.
3.3.2. Post-electrospinning treatment

Electrospun fiber sheets were processed to generate the following categories of solvent content: (1) as-spun (no post-processing); (2) vacuum (< 30 mm Hg) for 24 hours; (3) vacuum (< 30 mm Hg) and 37°C for 24 hours; (4) vacuum (<30 mm Hg) and 45°C for 24 hours. We were mindful of the relatively low melting point of PCL (~60°C).

3.3.3. Electro-spray ionization mass spectroscopy (ESI-MS)

For each of the above samples 0.10 g was submerged in 10.0 g of DI water in separate vials and held at 37°C for 24 hours. For PCL electrospun from either HFP or acetone, 0.10 g was digested in a 15% formic acid (Mallinckrodt Baker, Phillipsburg, NJ) – 85% methanol (EM Science, Gibbstown, NJ) solution using a sonifier (Sonifier 450, Branson, Danbury, CT). The resultant solutions were then analyzed by ESI-MS (Esquire G1979A, Bruker, Billerica, MA). With the ability to achieve mass measurements that are within 0.01% of the calculated values [27], ESI-MS provides accurate analyses of solvent content. For comparison, 10, 50, 100 and 250 ppm standard solutions of HFP in DI water were produced. In addition, 10, 50 and 100 ppm standard solutions of HFP or acetone in the 15% formic acid – 85% methanol solution were also produced to provide quantitative analysis of HFP or acetone content in the digested PCL. All the measurements were calibrated with these known standards to compensate for detector drift.

3.3.4. SEM analysis

All the samples before and after the post-spinning treatments were coated with 8 nm thick osmium using an osmium plasma coater (OPC-80T, SPI Supplies, West Chester,
The use of osmium plasma instead of Au or Au-Pd eliminated concerns regarding PCL melting during gold sputter coating and allowed for higher resolution imaging of the fiber surface.

3.3.5. Toxicity test

The CFK2 cell line (obtained from Dr. Henderson’s lab, Department of Medicine, McGill University, Montreal, Canada) has the phenotypic characteristics of chondrocytes derived from fetal rat calvariae [28] and was used for toxicity testing. Approximately 40,000 CFK2 cells were exposed to 10, 50 and 100 ppm solutions of HFP in media during seeding on 12-well tissue culture plate (Falcon, Franklin Lakes, NJ). Cell culture medium was composed of 10% FBS (Mediatech, Herndon, VA), 1% Penicillin/Streptomycin (Fisher scientific, Fair Lawn, NJ) and 1% L-glutamine (Mediatech, Herndon, VA) in Ham’s F-12 medium (Mediatech, Herndon, VA). HFP-free cell culture medium was used as a control. All samples were processed in triplicate and harvested at day 2.

For the determination of proliferated cell numbers by spectrophotometry, cells were fixed with 10% formalin (Richard-Allen Scientific, Kalamazoo, MI) for 30 minutes followed by four rinses with PBS (Mediatech, Herndon, VA). Two wt. % crystal violet (Sigma, St. Louis, MO) was dissolved in ethanol and the solution was further diluted by mixing 1 ml of the 2 wt. % crystal violet solution with 25 ml of DI water. 300 μl of the crystal violet reagent was added to each well for 10 minutes, and then the wells were washed 4 times with tap water. 250 μl of 1% SDS (National Diagnostics, Atlanta, GA) was added to each well, and the plate was shaken for 30 minutes. 200 μl of the resultant
crystal violet stain-dissolved SDS solutions was put into a 96-well plate (Falcon, Franklin Lakes, NJ) and the absorbance of each well was read at 560 nm using a Wallac Victor\textsuperscript{3TM} plate reader (Perkin Elmer, Wellesley, MA). Approximately 30,000, 60,000, 120,000 and 240,000 cells were stained with the crystal violet reagent using the same procedure and the absorbances read to produce a calibration curve.

### 3.3.6. Cell culture

Approximately 30,000 $4^{\text{th}}$ passage chondrocytes from rat articular cartilage were seeded on the 50:50 PCL-gelatin composite fiber mesh. The mesh was cut with an arch punch generating a round sample with 18 mm in diameter and 0.5 mm in thickness. The sample was treated at 45$^{\circ}$C in vacuum for 24 hours prior to the cell seeding. Adherent cells were then cultured for 5 days before morphological examination with SEM. The cell culture media was the same as that used for the CFK2 cells. These samples were fixed with 10% formalin followed by a graded ethanol series in DI water (50, 70, 85, 90 and 100% ethanol) for dehydration. The dehydration step was finalized using a graded ethanol-HMDS (hexamethyldisilazane, Electron Microscopy Sciences, Hatfield, PA) series (25, 50, 75 and 100% HMDS) followed by drying under a hood overnight. The dried sample was coated with 8 nm of osmium and examined with the SEM as before.

### 3.4. Results

Fiber meshes of PCL (G0), 75:25 PCL-gelatin (G25), 50:50 PCL-gelatin (G50), 25:75 PCL-gelatin (G75) and gelatin (G100) could be electrospun as described in the
materials and methods section. As gelatin content increases so does fiber diameter; the presence of gelatin produced a relatively flattened morphology (Figure 3.1).

Exposure of the G100 fibers to 37°C water resulted in their immediate dissolution. In contrast, G0 fibers remained intact. The other fibers appeared to undergo partial dissolution in accordance with their gelatin contents showing that gelatin-containing fibers have a tendency to flatten and break down to produce cell-like structures even when no cells are present (Figure 3.2). The ESI-MS was used to generate standard curve (Figure 3.3) providing a good least-square fit \( R^2 = 0.9642 \) describing HFP content. The solutions resulting from the exposure of the fibers to DI water at 37°C were subjected to ESI-MS analysis and the intensities of the HFP peak \( m/z = 167.2 \) compared to the standard curve (Figure 3.4). As little as 50 ppm was released by the G0 composition; as much as 1660 ppm was released by G100. The amount of HFP detected scales with gelatin content according to \( [HFP(\text{ppm})] = 14.552 \times [\text{gelatin}(\text{wt.\%})] + 23.76 \) \( (R^2 = 0.8496) \).

Next, as-spun G0, G25, G50, G75 and G100 fiber meshes were stored under ambient conditions for 1 week prior to submergence in DI water at 37°C for 24 hours followed by ESI-MS analysis of HFP elution as described above. Figure 3.5 shows the HFP content of the aqueous solution following exposure to these fibers. HFP was not detected in 1 week old PCL (G0), and only 314 ppm of HFP was detected in the 1 week old gelatin (G100). As before, HFP scales with the gelatin content in this case according to \( [HFP(\text{ppm})] = 2.5018 \times [\text{gelatin}(\text{wt.\%})] \) \( (R^2 = 0.8244) \). The effects of several different post-treatments on HFP released from G50 fiber are presented in Figure 3.6. Vacuum for 24 hours reduced the amount of HFP released from 114 ppm (1 week old G50) to 14 ppm.
Vacuum combined with heating at 37 and 45°C further reduced the HFP content to 10 and 5.6 ppm, respectively. The morphologies of treated G50 and other samples were examined by SEM to observe the effects of vacuum-based post-processing and no microstructural changes were observed following treatment (Figure 3.7). Heat treatment at 45°C in vacuum did not lead to any deterioration of surface of the gelatin fibers or change in the morphology of the PCL.

Complete digestion of G0 in 15:85 formic acid-methanol provided a measure of the total amount of HFP, if any, remaining in pure PCL (Figure 3.8). HFP standards (10, 50 and 100 ppm) in 15:85 formic acid-methanol generated an excellent linear fit ($R^2 = 0.9919$). The extrapolated amount of HFP present in 0.1 g of G0 sample was 0.3 ppm. For comparison’s sake, PCL electrospun using acetone was also digested and the resultant solution was analyzed by ESI-MS. No acetone could be detected within the limits of ESI-MS (~1.7 ppm (1 μM)).

The inherent toxicity of HFP was tested by culturing CFK2 cells in 0, 10, 50 and 100 ppm HFP in culture media. The cells were cultured for 2 days before harvesting and spectrophotometric counting (Figure 3.9). The result of univariate analysis of variance (ANOVA) showed no significant statistical difference in the proliferation of the cells among the chosen conditions ($p = 0.158$).

Figure 3.10 shows SEM images of chondrocytes cultured at 45°C on vacuum-treated G50. The chondrocytes exhibit a well-spread morphology in which their filopodia extend across several fibers and also secrete extracellular matrices (likely glycosaminoglycan (GAG)) as evidenced by the crystalline deposits observed near the cells.
3.5. Discussion

Within the field of tissue engineering electrospinning has attracted great interest due to its simplicity and effectiveness in producing fine fibers that closely resemble the extracellular matrix. Hundreds of polymeric compositions have been spun but work involving natural polymers is limited. Biopolymers such as collagen [29], gelatin [15], elastin [30] and hyaluronic acid [31] have been successfully electrospun. Although water can be used to dissolve these biopolymers it is a less than ideal solvent for electrospinning. Its high boiling point relative to standard polymer electrospinning solvent requires forced evaporation such as air blowing at elevated temperatures [31] and less than ideal fiber morphologies result. In addition, blends of natural and synthetic polymers are desirable as they can provide precise control over degradation. The intelligent addition of slower degrading synthetics has been shown to successfully amend the relatively high solubility and rapid degradation characteristic of natural biopolymers [12, 15]. These composites can slow the rapid loss of structural integrity expected from biopolymers in physiological condition. Therefore common solvents for both natural and synthetic polymers are needed to produce fibers that ideally combine the physical stability of synthetic polymers with the biological activity/acceptance of natural polymers [15, 32, 33]. Fluorinated alcohols [34, 35] such as trifluoroethanol (TFE), hexafluoropropanol (HFP) and their variations are logical choices. However, there has been no effort directed toward establishing the existence and influence of residual fluorinated alcohols.

Table 3.1 summarizes the amount of HFP eluted per milligram of electrospun gelatin, PCL and their blends. It is evident that HFP has a much higher affinity for gelatin.
Fluorinated alcohol has been used to denature proteins and stabilize structures in peptides due to its strong interaction with both polar and non-polar amino acid side groups [36]. This interaction with both the hydrophilic and the hydrophobic groups within collagen [37] explains why residual HFP scales directly with gelatin content.

No HFP was detected in the elution experiment involving 1 week old G0 and only a nominal amount (0.003 ppm) was detected in digested G0 (Table 3.1). This implies that most of the HFP is shed either during the electrospinning process or shortly thereafter. The high surface area of the fibers favors this in combination with the relatively lower polarity of the PCL. The fact that no acetone was detected in the PCL when acetone was used as a solvent supports the idea that electrospun PCL is usually relatively free of the effects of retained solvent. This is not meant to suggest that all synthetic polymers are immune to residual solvent retention during electrospinning. Kalayci et al. observed residual dimethylformamide when electrospinning polyacrylonitrile [38].

Several different post-processing treatments were utilized to reduce the amount of residual HFP. All are effective in reducing HFP content but a >99% reduction in the G50 sample could be obtained by treatment at 45°C and vacuum for 24 hours (Table 3.1). Furthermore this method does not compromise microstructure in any visible way (Figure 3.7). The toxicity test (Figure 3.9) suggests that HFP contents of 100 ppm or lower are safe for *in vitro* cell culture. This allows us to estimate that at least 1.7 g of treated G50 could provide a scaffold which does not elute toxic levels of residual solvent. When scaffold volume is considered, this equates to about 5.3 cm$^3$ based on the ~ 80 % relative porosity of the fiber mesh. However, if cells are seeded internally into such scaffolds (either by simple injection or a via flow through configuration) instantaneous localized
HFP concentration could conceivably be higher than those reported here. Cellular growth and proliferation might conceivably be delayed or limited until sufficient outward HFP diffusion has taken place.

Figure 3.2 shows that these blends can break down over a relatively brief time period and produce film-like structures that resemble the morphology of spread cells. We suspect that a gelatin-rich layer forms on specific fiber surfaces and that highly mobile films tend to nucleate and grow at fiber-fiber intersections. Figure 3.7 clearly shows that none of the treatments employed in this investigation resulted in obvious changes to the microstructure of these fibers. This is somewhat anticipated as the uniform removal of a few ppm of a given solvent from an individual fiber is unlikely to cause obvious shrinkage or pitting. Figure 3.10 shows that chondrocytes exhibit a well-spread morphology and other positive indicators on the 45°C vacuum-treated G50 specimens suggesting that this scaffold can successfully promote cell growth. This is in contrast to the results of Kwon et al. [39] who observed rounded or restricted-spread cells on electrospun collagen-PCL fibers fabricated using 30 and 50 wt. % collagen. Highly elongated cells like those seen in Figure 3.10 were observed at lower collagen contents (5 or 10 wt. %) where residual solvent levels might also be expected to be lower. Kwon at al. attributed this effect to the shrinkage of the scaffold during the culture period even though cell motility over many days should have been more than sufficient to allow for cell rearrangement.
3.6. Conclusions

To our knowledge, this study is the first to systematically analyze the residual solvent in fibers produced by electrospinning. Gelatin is clearly more prone to residual solvent retention than PCL. Other biopolymers likely have similar relative tendencies to retain residual solvents. Fortunately, a simple and effective procedure reducing residual solvent content was developed. The majority of the residual solvent could be reduced to a level that had no demonstrated effects on cell interactions with the scaffold.

Electrospinning as a means of investigating tissue engineering needs to include consistent post-processing treatments to avoid possible residual solvent release that could greatly complicate correct interpretation of biological activity on the resulting scaffolds.
<table>
<thead>
<tr>
<th>Condition/Sample</th>
<th>HFP concentration (ppm) per mg of sample</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>As-spun</strong></td>
<td></td>
</tr>
<tr>
<td>G0</td>
<td>0.50</td>
</tr>
<tr>
<td>G25</td>
<td>3.19</td>
</tr>
<tr>
<td>G50</td>
<td>9.91</td>
</tr>
<tr>
<td>G75</td>
<td>7.37</td>
</tr>
<tr>
<td>G100</td>
<td>16.60</td>
</tr>
<tr>
<td><strong>1 week old</strong></td>
<td></td>
</tr>
<tr>
<td>G0</td>
<td>0</td>
</tr>
<tr>
<td>G25</td>
<td>0.55</td>
</tr>
<tr>
<td>G50</td>
<td>1.14</td>
</tr>
<tr>
<td>G75</td>
<td>1.13</td>
</tr>
<tr>
<td>G100</td>
<td>3.14</td>
</tr>
<tr>
<td>Vacuum treated G50</td>
<td>0.14</td>
</tr>
<tr>
<td>37°C with vacuum treated G50</td>
<td>0.10</td>
</tr>
<tr>
<td>45°C with vacuum treated G50</td>
<td>0.06</td>
</tr>
<tr>
<td>Digested 1 week old G0</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Table 3.1. HFP concentration per milligram either eluted (in DI water at 37°C) or digested out of electrospun fibers.
Figure 3.1. SEM images of as-spun (a) G0, (b) G50 and (c) G100.
Figure 3.2. SEM image of G50 dissolved in DI water for 1 day at 37 °C showing partial and non-uniform dissolution. Note that cell-like morphologies are produced by partial dissolution even in the absence of cells.
Figure 3.3. The ESI-MS results of HFP standards (10, 50, 100 and 250 ppm) in DI water.
Figure 3.4. HFP content in as-spun G0 (■), G25(▲), G50(♦), G75(□) and G100(●).
Figure 3.5. HFP content of 1 week old G0 (■), G25(▲), G50(♦), G75(□) and G100(●).
Figure 3.6. HFP content of the G50 composition following various post-processes (♦: 1 week old in an ambient condition, ●: Vacuum, ▲: Vacuum and heat (37°C), ■: Vacuum and heat (45°C)).
Figure 3.7. SEM images of (a) G0, (b) G50 and (c) G100 after exposure to 45°C in vacuum for 24 hours.
Figure 3.8. HFP content of PCL completely dissolved in 15:85 formic acid-methanol (■:PCL, ♦:standards (10, 50 and 100 ppm HFP in 15:85 formic acid-methanol)).
Figure 3.9. The number of CFK2 cells on the 2nd day of culture in cell culture media containing the indicated HFP concentrations. HFP-free cell culture medium was used as a control.
Figure 3.10. SEM images of chondrocytes cultured on post-processed (45 °C in vacuum for 1 day) G50 for 5 days.
REFERENCES


22. Son WK, Youk JH, Lee TS, Park WH. The effects of solution properties and polyelectrolyte on electrospinning of ultrafine poly(ethylene oxide) fibers. Polymer 2004 Apr 27;45(9):2959-2966.


CHAPTER 4

ENHANCED CHONDROCYTIC CELL RESPONSE TO AN ELECTROSPUN TOPOGRAPHY

4.1. Abstract

The effect of topographically different yet chemically identical substrates on cell morphology, proliferation and phenotypic functionality was examined using chondrocyte-phenotypic CFK2 cells. Electrospun polycaprolactone (PCL) encouraged these cells to spread from their initial points of contact and ultimately produced more efficient seeding than the corresponding semi-porous and dense PCL surfaces and more hydrophilic glass surfaces. The topography intrinsic to electrospun surfaces appears to offset the hydrophobicity of PCL resulting in a more uniform cell distribution. On other surfaces, dense PCL in particular, clustering of the initial population of cells occurs. These initial cell seeding characteristics likely affect subsequent cellular behaviors. Proliferation on electrospun topographies was similar to or only slightly better than the other surfaces. However, the glycosaminoglycan (GAG)/cell on electrospun PCL reached levels 2-3 times that of the other PCL surfaces. At day 9 GAG production was greater than on the more hydrophilic glass controls. At day 12 the cells on the glass surfaces experienced a pronounced recovery in GAG production to levels identical to that of
electrospun PCL. GAG levels on semi-porous and dense PCL remained substantially lower. Stress fibers were visible in cells on all surfaces except for those on electrospun PCL suggesting the latter provides a more *in vivo*-like environment. This may be due to the generation of a “dynamic scaffold” due to tensile forces exerted by adherent cells. These results show that a three-dimensional electrospun substrate can enhance growth, proliferation *and* phenotype relative to planar, two-dimensional variations of the same polymer.

### 4.2. Introduction

Tissue engineering is a clinically-driven field that seeks to replace or support damaged organs by culturing target-specific cells *in vitro* on a scaffold followed by implantation. Nearer term approaches involve implanting scaffolds followed by natural in-growth and proliferation of host tissue cells *in vivo*. Both approaches initiate and influence cellular response and subsequent tissue development through the processes of cell adhesion, motility, proliferation, differentiation and functionality at the cell–scaffold material interface. Therefore, understanding how cell–material interactions are influenced by topographic surface characteristics is of fundamental importance for tissue engineering.

Physicochemical scaffold properties influence cellular response. Bulk chemistry can control cytotoxicity because most scaffolds are made of biodegradable materials and must eventually release the by-products of this deliberate degradation. The effect of surface chemistry is often related to the effect of the instantly adsorbed proteins [1] rather than direct material effects on the cells. Adhesion is mediated by proteins such as
fibronectin, collagen, fibrinogen, vitronectin and immunoglobulin [1]. In addition, surface protein-bound integrins activate a signal cascade that controls cell physiologies such as phenotype [2], viability [3], morphology [4], proliferation and differentiation [5]. Considerable research has been conducted on surface chemistry modification using biological cues like RGD and YIGSR peptides to improve adhesion [6-10]. However, hydrophilic and hydrophobic surfaces are known to have dramatically different effects on receptor organization and function in mammalian cells during adhesion [11] proving that surface characteristics remain important in spite of overlying biochemical modifications.

An additional factor affecting cell-material interaction for all two-dimensional surfaces is topography. Numerous studies regarding the effect of surface topography/texture on cellular response have been conducted [12-20]. Topography influences cell adhesion and motility. Moving or extending cells have been observed to localize more frequently at junctions or discontinuities in a surface [12]. Mutually perpendicular actin stress fibers have been observed within the same cell, one aligning along a topographical cue and the other along a chemical cue [21] suggesting that the two factors can have similar influences. More generalized nanoscalar topography exhibiting peaks on the order of 50 nm result in increased chemical sensitivity but had no effects on proliferation or morphology [22]. Increases in nanotopography to 200 nm-long fibrils [23] also produced no effects on proliferation but altered the cytoskeletal response to result in the loss of coarse stress fibers [24] that are the hallmark of cells grown on nominally ‘flat’ surfaces (such as glass coverslips and tissue culture polystyrene) but which are absent in vivo.
In contrast to these impenetrable surfaces heavily interconnected open porosity is one of the few requirements common to all tissue engineering scaffolds. Lee et al. [15] compared the cellular response of human chondrocytes on simple polycarbonate substrates having different pore sizes. Smaller pores (0.2 µm) supported better cell adhesion and proliferation than larger pores (8 µm). However, the larger pores induced more production of chondrocyte-phenotypic extracellular matrix (ECM). Differences in pore shape also affected cellular response to PLLA scaffolds produced by solvent-casting/particulate-leaching [16].

Clearly, both porosity and topography are influential factors that will ultimately govern scaffold design. Electrospinning is an ideal method for achieving this in three-dimensional form partly due to the ease with which it produces non-woven nano- to micro-sized fibrous scaffolds having 70 ~ 90% relative porosity. Natural biodegradable materials such as collagen [25-29], gelatin [30, 31], elastin [25, 32], chitosan [33] and hyaluronic acid [34] as well as synthetic biodegradable polymers such as polycaprolactone (PCL) [29, 31, 35-43], poly(glycolic) acid (PGA) [28], poly(lactic) acid (PLA) [28] and poly(lactic-glycolic) acid (PLGA) [44] have been electrospun as scaffolds for cardiovascular, chondral and osseous applications. In general, however, comparison of cellular response to electrospun topography versus alternate topographies having identical composition has not been carried out.

In this study, PCL was electrospun to produce a porous topography similar to that utilized in other studies. Electrospun PCL was then also dissolved and this solution used to produce both porous and dense topographies. The effect of these topographically
different yet chemically identical substrates on cell morphology, proliferation and phenotypic functionality was examined using chondrocyte-phenotypic CFK2 cells.

4.3. Materials and Methods

4.3.1. Substrate fabrication

PCL (Mw 65,000, Aldrich, St. Louis, MO) was used as the starting material. A 12 wt% solution of PCL in acetone (Mallinckroff Chemicals, Phillipsburg, NJ) was prepared and electrospun at 23 kV with a 20 cm tip-to-substrate distance and a flow rate of 15 ml/hr onto aluminum foil. Electrospun PCL was then dissolved in acetone to produce a 0.5 wt% solution and this was used to coat 18 mm round glass cover slips (Fisher Scientific, Fair Lawn, NJ) using a spin coater (Model G3P-12, Cookson Electronics, Providence, RI) operated at 2000 rpm for 1 minute. Pre-coating of PCL improved the adhesion of electrospun PCL to the cover slips during cell culture. The PCL-coated cover slips were then placed on aluminum foil and electrospun PCL deposited using the parameters above to produce a deposition thickness of approximately 100 µm (Figure 4.1 (a)).

Previously electrospun PCL was again dissolved in acetone this time producing 5 and 12 wt% solutions. Cover slips were dip-coated using the 5 wt% solution to yield a semi-porous PCL surface (Figure 4.1 (b)) having an average pore size of approximately 0.9 µm. Cover slips dip-coated using the 12 wt% solution and heat-treated at 120ºC for 15 minutes produced a dense PCL surface showing the evidence of spherulitization (Figure 4.1 (c)). The use of previously electrospun PCL ensures that all three samples have the same chemistry.
4.3.2. Cell seeding and culture

CFK2 cells, a chondrocytic cell line derived from fetal rat calvariae [45] (a generous gift from Dr. J.E. Henderson, Department of Medicine, McGill University, Montreal, Canada), were seeded (30,000 cells/scaffold) on the three different topographies (PCL electrospun fiber, semi-porous and dense) in 12-well tissue culture plates (Falcon, Franklin Lakes, NJ). Bare glass cover slips were used as controls. The seeded cells were cultured in Ham’s F-12 medium (Mediatech, Herndon, VA) containing 10 % FBS (Mediatech, Herndon, VA), 1% penicillin/streptomycin (Fisher scientific, Fair Lawn, NJ) and 1 % L-glutamine (Mediatech, Herndon, VA). Samples were harvested at days 3, 6, 9 and 12 for subsequent characterization. The culture medium was changed every other day.

4.3.3. Immunocytochemical staining

Samples from at each time point were fixed using a 10% formalin solution for 20 minutes followed by four rinses with PBS (Mediatech, Herndon, VA). The fixed cells were permeabilized with 0.2 % TritonX-100 (Sigma, St. Louis, MO) for 30 minutes followed by three rinses with PBS. The samples were immunocytochemically stained with fluorescein isothiocyanate (FITC) labeled phalloidin (Sigma, St. Louis, MO) in PBS containing 0.5% bovine serum albumin (BSA, Sigma, St. Louis, MO) for 20 minutes under dark conditions. The stained samples were rinsed three times with PBS before adding nuclear stain, 0.01% 4’,6-diamidino-2-phenyl-indole dihydrochloride (DAPI, Sigma, St. Louis, MO) in PBS containing 0.5% BSA for 5 minutes. The stained samples
were observed under an Axioplan2 epifluorescence microscope (Carl Zeiss, Thornwood, NY).

4.3.4. Histocytochemical staining and Spectrophotometric GAG (glycosaminoglycan) assay

Samples at each condition were harvested at each time point and fixed using a 10% formalin solution for 20 minutes followed by rinsing with PBS. Samples were then stained using a 1.5 % safranin-O (Sigma, St. Louis, MO) reagent for 40 minutes. The stained samples were rinsed quickly in DI water, and then observed under a microscope. For the quantitative analysis of GAG, a spectrophotometric method using safranin-O staining based on the work of Carrino et al. [46] was used. Chondroitin sulfate A sodium salt from bovine trachea (Sigma, St. Louis, MO) used as a standard, was dissolved in DI water to make a 1 mg/ml solution and this solution was further diluted with DI water to make 0.08, 0.2, 0.4 and 0.6 mg/ml solutions. Triplicate solutions were used as standards.

Nitrocellulose membrane was moistened with DI water in a Petri dish and placed into a dot-blot apparatus (Bio-Rad, Hercules, CA). Exactly 250 µl of Safranin-O reagent was put into each well underlaid by the nitrocellulose membrane. Next 25 µl of each standard solution equal to 2, 5, 10, 15 and 25 µg of chondroitin sulfate, was pipetted directly into the safranin-O reagent and vacuum was applied to each well until the samples were completely filtered. The vacuum was removed and the wells rinsed 3 times by filling the wells with DI water and then reapplying vacuum. The dot-blotted nitrocellulose sheet was removed and air-dried. The dots on the dried filter were punched.
using a 4 mm dermal biopsy punch (Miltex GmbH, York, PA) and were placed in a 12-well tissue culture plate.

A 10 wt% cetylpyridinium chloride (CPC, Sigma, St. Louis, MO) solution was prepared. After addition of 1 ml of this CPC reagent to each well, the plate was shaken for 12 hours to completely dissolve the safranin-O. 200 µl of the resultant solutions was put into a 96-well plate (Falcon, Franklin Lakes, NJ) and the absorbance of each well read at 490 nm with Wallac Victor3TM plate reader (Perkin Elmer, Wellesley, MA) to generate a standard curve.

The samples seeded in triplicate for each condition were harvested at each time point and stained with safranin-O in the same way as for the optically observed samples. After rinsing, the samples were air-dried and placed into a 12-well culture plate. One ml of CPC reagent was added to each well and the plate shaken for 12 hours. The resultant solutions were processed in the same way as the standards to spectrophotometrically determine absorbances.

4.3.5. Proliferation

The samples in triplicate for each condition were harvested at each time point, then fixed with 10 % formalin solution for 1 min and rinsed four times with PBS. Crystal violet (Sigma, St. Louis, MO) was dissolved in ethanol to produce a 2 wt% solution and this was further diluted by mixing 1 ml of the 2 wt% crystal violet solution with 25 ml of DI water. 300 µl of the crystal violet reagent was added to each well for 10 minutes and the wells were then washed 4 times with tap water. The stained samples were taken to a separate 12-well plate in order to ensure that we counted only those cells adhering to the
samples. 250 µl of 1 % SDS (National Diagnostics, Atlanta, GA) was added to each well and the plate shaken for 30 minutes. Next, 200 µl of the resultant crystal violet stain-dissolved SDS solutions was put into a 96-well plate (Falcon, Franklin Lakes, NJ) and the absorbance of each well was read at 560 nm with a Wallac Victor3TM plate reader (Perkin Elmer, Wellesley, MA). Approximately 30,000, 60,000, 120,000 and 240,000 cells were stained with crystal violet using the same procedure and the absorbances used to produce a calibration curve.

4.3.6. SEM

All the samples were coated with 8 nm thick osmium using an osmium plasma coater (OPC-80T, SPI Supplies, West Chester, PA). The use of osmium plasma instead of gold eliminated concerns regarding PCL melting during gold or gold palladium sputter coating and allowed for higher resolution imaging of the fiber surface. For the cell cultured samples, the samples were fixed with 10 % formalin (Richard-Allen Scientific, Kalamazoo, MI) followed by a graded ethanol series in DI water (50, 70, 85, 90 and 100% ethanol) for dehydration. The dehydration step was finalized using a graded ethanol-HMDS (hexamethyldisilazane, Electron Microscopy Sciences, Hatfield, PA) series (25, 50, 75 and 100% HMDS) followed by overnight drying under a hood. The dried sample was coated with 8 nm of osmium as described above.

4.3.7. Statistical data analyses

Data were analyzed using univariate analysis of variance (ANOVA) using SPSS software (Ver. 14, Chicago, IL) and the significance among each group was then tested
by Tukey’s Honest Significant Difference (HSD). All the data in this paper are presented as mean ± standard deviation (SD) and the differences were considered statistically significant when p < 0.05.

4.4. Results

The microstructures of the topographies produced by the three different processes with an identical chemistry of PCL are shown in Figure 4.1. The fiber diameter of electrospun PCL ranges from 0.2 to 2.5 µm having an average of ~0.7 µm. The relative porosity calculated by comparison between the measured density of electrospun PCL mesh and the theoretical density of PCL [47] was ~80%. For the semi-porous surface, the pore size ranges from 0.2 to 1.3 µm having an average of 0.9 µm. The SEM image of dense PCL (Figure 4.1 (c)) shows polygonal spherulites with an average size of ~30 µm.

Cellular morphologies on the different topographies on day 3 were examined by SEM (Figure 4.2). The cells on the control surface are generally well-spread and flattened. Even though the seeding methods used were identical, cellular distributions varied markedly and gave typically non-uniform results. On the control surface, round/elongated cells (marked with an arrow in Figure 4.2 (a)) were observed only near the proliferating leading edges of cell clusters (note the empty upper left corner of the image). In contrast to the other samples, electrospun PCL showed a relatively uniform cell distribution and many of the cells were round and elongated, their filopodia extending across several fibers (Figure 4.2 (b)). Secretion of extracellular matrix (ECM) was observed and only a few cells appeared to be underneath the fibers. Figure 4.2 (c) shows the morphology of cells on semi-porous surfaces. In general, the morphologies are represented by two
distinct shapes: spread/flattened or round/elongated. The former is observed on the glass and dense PCL specimens. The latter is extensively observed on the electrospun PCL and the proliferating leading edges on the glass and dense PCL. Globular deposits, likely GAG, were observed typically on top of the rounded cells. The morphology of the cells on dense PCL resembled that observed on the control, but appeared to be thicker.

The morphology of the cells on the glass surface on day 12 is shown in Figure 4.3 (a). Individual cells are often indistinguishable due to the ECM accumulations and cellular overlap. Darker circles (marked with arrows) appear to indicate the presence of cell clusters. The surface of electrospun PCL is nearly obscured by cells and ECM (Figure 4.3 (b)). Unlike glass, some gaps can be observed between the cell- ECM masses likely due to height differences between as-deposited fibers. Figure 4.3 (c) shows the well-spread morphology of the cells on the semi-porous surface. However, the cells appear to be more rounded than those observed on the glass. Flattened morphologies are observed on the dense PCL (Figure 4.3 (d)). On day 12, none of the samples have reached true confluence; local clustering remains evident. As on day 3, electrospun PCL showed the most uniform cell distribution.

The distribution of cellular F-actin and nuclei on day 3 are shown in Figure 4.4. As expected, prominent actin filaments developed in the cells grown on the control (Figure 4.4 (a)). The observable stress fibers had no common direction. Heavy surface coverage under the cell clusters shown in this figure is consistent with the SEM observations (Figure 4.2 (a)).

In comparison to glass, observations of actin distribution in cells on the electrospun substrates are somewhat confounded by the three-dimensional nature of the

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attachment. Cells present (Figure 4.4 (b)) to the viewer from a variety of perspectives – including edge-on – and the nuclei appear relatively smaller at least partially due to the fact that the cells are not flattened against a planar surface. However, we consistently see little evidence for the large stress fibers that characterize cellular response to flat surfaces. A full z-series showed only a few visible actin filaments distributed along the plasma membrane. In addition, F-actin agglomeration in the cytoplasm at points of contact with electrospun fiber is inferred based on the sporadic presence of “bright spots.”

On the semi-porous surface, some cell clusters effectively covered a large area of the surface while others were more restricted (Figure 4.4 (c)). Even though the cells displayed a relatively spread morphology, stress fibers were not as highly developed as those on the control. Clustering of cells also occurred on the dense surface (Figure 4.4 (d)) and lamellipodia extending from the entire circumference of the cell are present along with well-developed stress fibers.

As on day 3, well-developed stress fibers are observed in the cells grown on the control surface at day 12 (Figure 4.5 (a)). On the electrospun surface, the cells were more elongated relative to day 3 but we observed continued stress fiber inhibition along with actin-rich attachment points (Figure 4.5 (b)). The remaining non-electrospun surfaces now exhibit a cellular response similar to that on the glass control. The stress fibers in the cells on the semi-porous surface are highly organized and form a distinctive polygonal pattern (Figure 4.5 (c)). Well-developed stress fibers observed in cells on the dense surface are largely unidirectional (Figure 4.5 (d)) but this varies from cluster to cluster. Larger nuclei were observed on the control and semi-porous surfaces compared to those on the electrospun (as before) and dense surfaces.
To examine the effect of topography on proliferation, cell number was assessed on days 3, 6, 9 and 12 (Figure 4.6). The electrospun surface initially supported superior proliferation relative to both the control and dense surfaces (day 3). The cells on all the topographies exhibited a significant increase in cell number between days 6 and 9. Proliferation was arrested following day 9.

To assess functionality versus topography, GAG content was histochemically imaged using safranin-O (Figure 4.7). On day 12, it is evident that all cells on all surfaces secrete GAG but that the GAG distribution on the electrospun surface was the most uniform and followed the cell distributions shown in Figure 4.3 (b). These images provide additional evidence of cell morphology: a well-spread and flattened shape (control and dense surfaces) versus a round/elongated shape (electrospun) and intermediate shapes (semi-porous). The black spots appear to be deposits of highly concentrated GAG.

For a quantitative analysis of GAG content on each topography, histochemical staining using safranin-O was followed by spectrophotometric analysis [46]. The GAG standards generated a good ($R^2 = 0.9781$) fit in the range of 0 to 25 µg (Figure 4.8). Cell-free samples were first run to test for adsorption of safranin-O to PCL; no significant adsorption was detected.

Finally, the accumulated GAG contents on the different topographies were assessed for days 3, 6, 9 and 12 (Figure 4.9). The cells on the electrospun surface exhibited significantly enhanced GAG production for all time points relative to all other PCL-based surfaces. They also showed the steadiest increase in GAG content with respect to time, consistent with the cell proliferation data. GAG production on the control
surface shows a dramatic, non-linear increase following day 6. However, day 9 GAG production on glass did not mirror that on the electrospun surface while proliferation did. The GAG secretion of the cells on the semi-porous surface was significantly lower than all the other samples on days 9 and 12 even though the cell numbers were similar.

4.5. Discussion

Choice of scaffolding is one of the most important decisions that tissue engineers now face. As one of numerous processes considered for scaffold production, electrospinning has been favored partly due to the easy production of a non-woven nano-macro-fiber mesh that resembles biological ECM. Numerous natural materials have been fabricated into scaffolds to take advantages of their intrinsic biocompatibility but their instability has caused workers to consider, at least for the near-term, suitable synthetic polymers. PCL is a good candidate due to its relatively slow rate of degradation in solution and biocompatibility. Combinations of PCL with natural polymers such as collagen are a more likely long-term solution.

In this study, we examined the effects of three unique PCL surfaces on cell morphology, proliferation and functionality. No data we are aware of suggests that electrospinning alters the structure of PCL. However, Xie and Hseih [48] report that in spinning enzyme-carrying polymers, enzymatic activity was decreased possibly because the “enzyme structure could be affected by the electrospinning.” While the same effects in this study are unlikely we utilized previously electrospun fiber as a starting material to form the range of planar PCL surfaces. This ensures that they were chemically identical to the as-electrospun surfaces.
Electrospun PCL is a three dimensional structure unlike the two dimensional semi-porous and dense surfaces. However, cells appear to reside only on the top of the electrospun surface and the cellular infiltration is minimal in this particular scaffold as-spun. Cells observed underneath the fiber (Figure 4.2 (b)) are thought to have migrated there during cell seeding rather than afterwards. With this reason we can regard the electrospun surface as essentially two-dimensional but possessed of relatively large pores and higher porosity.

The proliferation data (Figure 4.6) shows that these electrospun surfaces support better initial cellular growth than either the semi-porous or the dense PCL surfaces. Surprisingly, the proliferation rate on the electrospun surface is faster on days 3 and 6 than that on glass, a surface known to favor proliferation. The insignificant differences between the final cell numbers combined with the observed differences in the initial cell distributions suggest that the initial cellular affinity for the various scaffolds may be responsible. Electrospun surfaces appear to encourage the cells to spread out from their initial points of contact following seeding and this results in a more distributed, efficient seeding arrangement. On other surfaces, dense PCL in particular, clustering of the initial population of cells is evident.

Interestingly, proliferation is arrested at day 9 even though true confluence has not been reached in any sample; this could be caused by localized confluence. Martin et al. [49] showed that cell-cell contact can signal growth inhibition even at relatively sparse subconfluent densities when chondroprogenitor cells are involved. Based on our observations of more uniform initial seeding, the electrospun surface could conceivably minimize this on a local level to increase the initial proliferation rate.
Much more pronounced are differences in the relative rate of GAG production (Figure 4.9). Mature chondrocytes in their natural form are a rounded cell that secretes ECM components such as GAG attached aggregan, collagen types II and IX. When chondrocytes are isolated from a tissue and cultured in a monolayer, they tend to spread [50]. The cells maintain their native morphology only under certain conditions such as in suspension or within gel-type substrates [50-52]. In our work, cells on electrospun PCL also maintained a rounded or elongated shape (Figures 4.10 (a) and (b)). The cells are forced to adhere on one or more fibers accompanied by ruffling of the cell membrane as they extend along the fiber direction. They can extend over several fibers when in close proximity. In contrast, the cells on the semi-porous surface show a spread shape with many filopodia establishing and maintaining attachment points within individual pores (Figure 4.10 (c)).

GAG production, a well-established indicator of chondrocyte/cartilage maturity either in vivo or in vitro [53], was also monitored. Figure 4.11 shows the accumulated GAG per cell on each surface over the course of the entire culture period. The GAG/cell on the electrospun surface appears to remain at a relatively constant level while other surfaces promote reductions in GAG content followed by a recovery on day 12. The dense surface shows an initially superior level of GAG production followed by a steep decline and rebound similar to the other surfaces. These reductions may be indicative of dedifferentiation of these chondrocytic cells. Previous research has shown that chondrocytes lose their ability to produce GAG when in a spread morphology [50, 54, 55]. We show that CFK2 cells exhibit such spread on control, semi-porous and dense surfaces accompanied by persistent observations of stress fiber formation. Restored levels
of GAG production on day 12 appear to accompany morphological changes of the cells. Figure 4.12 shows cellular agglomerates on control, semi-porous and dense surface on day 12. Rounded cells absent at early time points (except at the proliferating leading edges) are now present and are either surrounded by or reside on top of other of cells and secreted ECM. This appears to resemble the cellular condensation observed during chondrogenesis in the limb bud [56]. Chondrocytes from either epiphyseal growth plate [57] or nasal septum [55] have been observed to form cellular aggregates and regain a rounded shape on tissue culture dishes after they reach confluence. None of our samples reached this overall level of confluence but it was evident that localized confluence that separates cells from the substrate affects cellular behavior. This coincides with the result from Li et al. [36] in which chondrocytes regained levels of protein synthesis consistent to phenotype following prolonged culture on TCPS.

We can attribute the absence of stress fibers on the electrospun surface to both (1) surface dimensionality and (2) the pliability of individual electrospun fibers. The common thread of stress fiber observations in vitro is that they involve two-dimensional substrates. Although the three-dimensional nature of the cells makes the absence of visible stress fibers less obvious than in previous investigations involving nanostructured planar surfaces [24], we can still invoke that integrin-ligand binding model for focal adhesion formation to explain the current observations. Even though electrospun fibers are relatively small, contact along the length of even a 500 nm diameter fiber provides substantial, contiguous space upon which to establish focal adhesions; the scattered “bright spots” of F-actin observed in these experiments supports this. Cellular contact with electrospun fiber provides the only opportunity for integrin accumulation normally
leading to the stress fiber formation widely observed on planar surfaces. However, the preferred morphology of prominent stress fibers (as straight lines see Figures 4.4 and 4.5) could be more difficult to achieve if cells on electrospun fiber meshes connect to multiple attachment points using complex, three dimensional polyhedral morphologies.

Fiber pliability provides an additional explanation if cellular locomotion causes fiber movement at a critical point during actin polymerization; this could conceivably prevent visible stress fiber formation. A simple beam calculation [58] (Figure 4.13) using 75 nN [59] of applied force (as an example) shows that cellular forces could substantially deform electrospun nanofibers. Fibers less than 0.5 microns can be moved over distances greater than a cell diameter suggesting a fully pliable scaffold at 75 nN of tensile force. The loose, continuous nature of electrospun structures allows dimensional compliance greater than that of a relatively rigid two-dimensional substrate or even an array of relatively rigid posts [59] and could conceivably frustrate the development of constant isometric tension [60, 61]. Additionally, any given cell is surrounded by a population of neighboring cells each of which exerts a constantly shifting array of similar mechanical forces leading to a “dynamic scaffold” in which an actively changing electrospun structure prevents sustained isometric tension and stress fiber development.

In vivo, the absence of stress fibers in chondrocytes in mature articular cartilage [62] is well documented. The distance between focal adhesions at the ends of stress fibers is typically constant in vitro but is not constant in vivo as the tissues of the body undergo motion. In the absence of large stress fibers experiencing sustained levels of isometric tension, our data suggests that cellular functions other than adhesion could become more dominant. Here GAG production, an activity associated with chondrocytic functionality,
is one possible example. In contrast with observations for purely two-dimensional substrates [63], these results show that a flexible three-dimensional electrospun substrate can enhance growth, proliferation and phenotype relative to planar, two-dimensional versions of the same substrate. As Figure 4.13 illustrates, decreased fiber diameter increases scaffold flexibility suggesting that smaller electrospun fibers might lead to greater benefits.

4.6. Conclusions

We demonstrate that electrospun fiber surfaces support better proliferation and chondrocyte-specific ECM production than corresponding semi-porous and dense surfaces and even, at some time points, glass surfaces. We also show that the behavior during initial cell seeding likely affects subsequent cellular behaviors. The intrinsic topography of electrospun surfaces appears to offset the relative hydrophobicity of PCL resulting in a more uniform cell seeding. Electrospun fibers induced a higher level of GAG production suggesting that in these structures chondrocytic cells can more easily maintain the appropriate phenotype.
Figure 4.1. SEM images of the three different topographies: (a) electrospun (b) semi-porous and (c) dense PCL (scale bar: 20 μm).
Figure 4.2. Day 3 SEM images of cells grown on (a) control (glass) (b) electrospun (c) semi-porous and (d) dense PCL surfaces (scale bar: 50 μm).
Figure 4.3. Day 12 SEM images of cells grown on (a) control (glass) (b) electrospun (c) semi-porous and (d) dense PCL surfaces (scale bar: 50 μm). ECM accumulation is visible in all images.
Figure 4.4. Day 3 fluorescence microscopy images of cellular actin (green) and nuclear (blue) distribution on (a) control (glass) (b) electrospun (c) semi-porous and (d) dense PCL surfaces (scale bar: 20 μm).
Figure 4.5. Day 12 fluorescence microscopy of cellular actin (green) and nuclear (blue) distribution on (a) control (glass) (b) electrospun (c) semi-porous and (d) dense PCL surfaces (scale bar: 20 μm).
Figure 4.6. Proliferation of CFK2 cells on the different topographies at days 3, 6, 9 and 12. Each condition exhibiting a statistical difference from electrospun PCL within given time point is marked with an *.
Figure 4.7. Day 12 optical microscopy results of safranin-O stained samples: (a) control (glass) (b) electrospun (c) semi-porous and (d) dense PCL surfaces. The non-uniform cellular distribution on both the control and semi-porous surfaces is evident. Black spotting is caused by concentrated GAG deposits that saturate the detector elements.
Figure 4.8. Spectrophotometric standard curve ($R^2 = 0.9781$) of GAG content using 2, 5, 10, 15 and 25 μg chondroitin sulfate solutions.
Figure 4.9. Total GAG production on the control (glass), electrospun, semi-porous and dense PCL surfaces at days 3, 6, 9 and 12. Statistical differences from electrospun PCL are marked with an *.
Figure 4.10. Characteristic effects of topography on cell morphology on day 3: (a) rounded cells present on electrospun PCL; (b) elongated cells on electrospun PCL; (c) a spread morphology and interaction with individual pores on semi-porous PCL (scale bar: 10 μm).
Figure 4.11. GAG production on a per cell basis on the control (glass), electrospun, semi-porous and dense PCL surfaces at days 3, 6, 9 and 12. Statistical differences from electrospun PCL are marked with an *.

Figure 4.11. GAG production on a per cell basis on the control (glass), electrospun, semi-porous and dense PCL surfaces at days 3, 6, 9 and 12. Statistical differences from electrospun PCL are marked with an *.
Figure 4.12. Cellular clustering on (a) control (glass) (b) semi porous and (c) dense PCL surfaces on day 12 (scale bar: 20 μm). Rounded cells are marked with white arrows.
Figure 4.13. Deflection (under 75 nN of force) of an individual electrospun PCL fiber versus (a) fiber diameter (the distance between contact points being fixed at 20 μm) and (b) distance between contact points (at a fixed fiber diameter of 0.7 μm). The size distribution (inset) shows that the majority of the fibers within this electrospun array can be moved significant distances by adherent cells.
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CHAPTER 5

IMPROVED CELLULAR INFILTRATION IN ELECTROSPUN FIBER
VIA ENGINEERED POROSITY

5.1. Abstract

Small pore sizes inherent to electrospun matrices can hinder efficient cellular ingrowth. To facilitate infiltration while retaining their extracellular matrix-like character, electrospinning was combined with salt leaching to produce a scaffold having deliberate, engineered delaminations. We made elegant use of a specific randomizing component of the electrospinning process, the Taylor Cone, to produce a uniform, well-spread distribution of salt particles. After 3 weeks of culture up to 4 mm of cellular infiltration was observed along with cellular coverage of up to 70% within the delaminations. To our knowledge, this represents the first observation of extensive cellular infiltration of electrospun matrices. Infiltration appears to be primarily driven by localized proliferation rather than coordinated cellular locomotion. Cells also moved from the salt-generated porosity into the surrounding electrospun fiber matrix. Given that the details of salt deposition (amount, size, and number density) are far from optimized the result provides a convincing illustration of the ability of mammalian cells to interact with appropriately tailored electrospun matrices. These layered structures can be precisely fabricated by
varying the deposition interval and particle size to conceivably produce *in vivo*-like gradients in porosity such that the resulting scaffolds better resemble the desired final structure.

5.2. Introduction

Tissue engineering has emerged as a promising means of replacing damaged organ. Cells are seeded *in vitro* in/on a scaffold and supplied with adequate nutrients and removal of waste products. Scaffolds should promote appropriate cellular adhesion, proliferation and function. Materials used are typically bioresorbable and are resorbed following/during successful tissue regeneration. In addition, a high level of porosity is desired to enable both the efficient influx of anabolic nutrients and outflow of catabolic wastes.

Methods such as solvent casting/particulate leaching [1], gas forming [2], emulsion lyophilization [3] and phase separation [4] have generated three-dimensional scaffolds from natural and synthetic biodegradable polymers including collagen, poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone (PCL). These scaffolds show promise in promoting overall levels of cellular proliferation.

Another method producing interconnected pore structures is electrospinning. First patented by Formhals in 1934 [5], this technique produces a non-woven fiber mesh that greatly resembles extracellular matrix (ECM). Typical fiber diameters range from 10 nm to 10 µm [6] providing a substantial surface area per unit volume, a property promoting cellular adhesion. Electrospinning allows limited control of the pore sizes found between
inter-fiber contacts by selecting an average diameter [7] via control of spinning parameters such as solution viscosity, distance and voltage.

PCL is a good candidate material for scaffolds requiring short-term load bearing capability due to its relatively slow degradation rate in vivo [8]. Scaffolds made of PLA, PGA or PLGA exhibit shrinkage and substantial chemical degradation shortly after biological exposures involving hydrolysis [9]. PCL, in contrast, is relatively inert; phagocytosis of PCL by macrophages and giant cells only occurs once molecular weight of the polymer is reduced to 3000 or less by nonenzymatic bulk hydrolysis of the ester linkages [10, 11]. Electrospun PCL has, logically, been selected as a scaffolding for bone [12-15] and cartilage [16-18] to support cell proliferation and ECM deposition.

Paradoxically, however, the small fiber size intrinsic to electrospinning can hinder efficient cellular infiltration. Eichhorn et al. [7] have shown that the mean pore radius of electrospun matrices varies with fiber diameter. For example, a 100 nm fiber diameter yields a mean pore radius < 10 nm at a relative density of 80%. The size of a rounded cell - ranging from 5 to 20 µm - shows that such small pore sizes will obstruct cellular migration. For a scaffold that requires minimal cellular infiltration (a vascular graft for example) proliferation limited to the surface may be acceptable or even desirable. However, the thickness of human articular cartilage in the knee has been observed to range from 0.5 to 7.1 mm [19]. To achieve uniform cellular proliferation throughout such a thickness large porosity and an extended culture period are necessary. Furthermore, articular cartilage can be naturally anisotropic being composed of superficial, middle, deep and calcified zones [20] having variable porosity and architecture.
In this study, we engineered a delaminated PCL scaffold designed to provide localized, controllable macroscopic porosity by combining electrospinning with the well-established technique of salt leaching [1, 21, 22]. Specific characteristics of the two processes form a useful synergy producing a more uniform scaffold than would normally be expected from a salt-based technique. The result facilitates anisotropic cellular infiltration while retaining the highly porous, ECM-like nature of electrospun scaffolds.

5.3. Materials and Methods

5.3.1. Electrospinning

A 12 wt. % solution of PCL (M_w 65,000, Aldrich, St. Louis, MO) in acetone (Mallinckroff Chemicals, Phillipsburg, NJ) was prepared and electrospun at 23 kV using a high voltage DC power supply (Model FC50R2, Glassman high voltage, High Bridge, NJ) with a 20 cm tip-to-substrate distance and a flow rate of 18 ml/hr onto aluminum foil wrapped on a 4x4” steel plate. Salt crystals previously sieved to sizes between 90 and 106 µm were introduced into the Taylor Cone as shown in Figure 5.1. The amount of NaCl in each layer was approximately 0.75 g and covered approximately 10% of the surface area of the aluminum foil. Deposition of each allotment of NaCl required 30 ~ 60 seconds during electrospinning; the interval between each allotment was either 5 or 10 minutes. Electrospinning under these conditions required ~ 90 minutes to make ~ 5 mm thick sheets.

In specific cases, fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO) was added (0.1 mg/ml) to the polymer-acetone solution prior to electrospinning to
fluorescently label PCL fibers. This enabled us to observe both the electrospun fiber and the infiltrating cells in sectioned samples using fluorescent microscopy.

The deposited sheet was carefully punched with a dermal biopsy punch (Miltex, York, PA) to generate cylindrical ‘plugs’, 3 or 6 mm in diameter and ~ 5 mm in height. The plugs were then placed in a plastic bag and treated at 45ºC in a water bath to achieve the partial sintering needed to prevent the extensive delamination otherwise observed upon exposure to aqueous solution. The embedded salt crystals were then leached out by exposure to DI water at 37ºC for 3 days in which the DI water was replaced daily.

5.3.2. Cell culture

The CFK2 cell line (obtained from Dr. Henderson’s lab, Department of Medicine, McGill University, Montreal, Canada) having the phenotypic characteristics of chondrocytes derived from fetal rat calvariae [23] was used. Approximately 650,000 cells per sample placed in a 12-well tissue culture plate (Falcon, Franklin Lakes, NJ) were gravitationally seeded onto salt-leached plugs lying on their sides in each well. The seeded cells were cultured in Ham’s F-12 medium (Mediatech, Herndon, VA) containing 10% FBS (Mediatech, Herndon, VA), 1% Penicillin/Streptomycin (Fisher scientific, Fair Lawn, NJ) and 1% L-glutamine (Mediatech, Herndon, VA). The culture medium was changed every other day. The samples were harvested at day 3 and week 3 prior to subsequent characterization.
5.3.3. SEM

Samples were coated with an 8 nm thick layer of osmium (OPC-80T, SPI Supplies, West Chester, PA). Samples emerging from cell culture were fixed with 10% formalin (Richard-Allen Scientific, Kalamazoo, MI) and exposed to a graded ethanol series in DI water (50, 70, 85, 90 and 100% ethanol) to achieve dehydration that was then finalized using a graded ethanol-HMDS (hexamethyldisilazane, Electron Microscopy Sciences, Hatfield, PA) series (25, 50, 75 and 100% HMDS) followed by drying under a hood overnight. The dried sample was either coated directly with osmium or gently parted along the salt-generated gaps to observe cell migration prior to osmium coating.

5.3.4. Cryosection

Samples cultured for 3 weeks were fixed with 10% formalin followed by three 10 minute rinses in PBS (Mediatech, Herndon, VA). The fixed samples were embedded in OCT compound (Sakura Finetek, Torrance, CA) and then frozen at –80°C. The frozen samples were cut to 12 µm sections using a cryostat (CM3050S, Leica Microsystems, Bannockburn, IL) and placed onto Super Frost Plus glass slides (Fisher Scientific, Fair Lawn, NJ). The samples were stored at –80°C until staining.

5.3.5. Immunohistochemical staining

The cryosectioned samples were rinsed four times with PBS to remove residual OCT. The cells were then permeablized with 0.2% TritonX-100 (Sigma, St. Louis, MO) for 30 minutes followed by three rinses with PBS. The samples were stained with 0.01% 4’,6-diamidino-2-phenyl-indole dihydrochloride (DAPI, Sigma, St. Louis, MO) in PBS
containing 0.5% BSA for 5 minutes in the dark. The stained samples were observed using an Axioplan2 microscope (Carl Zeiss, Thornwood, NY).

5.3.6. Histochemical staining

The cryosectioned samples were rinsed four times with PBS. The samples were then stained using a 1.5% safranin-O (Sigma, St. Louis, MO) reagent for 40 minutes. The safranin-O solution was filtered through a 0.45 µm pore size nitrocellulose membrane (Bio-Rad, Hercules, CA) before use. The stained samples were quickly rinsed several times in DI water and observed under a microscope.

5.4. Results

The interaction between the dispensed salt crystals and the fibers during electrospinning is shown in Figure 5.2 (a). Under normal circumstances the falling salt would deposit directly below the exit of the sheath in Figure 5.1. Interaction with the Taylor Cone carried falling salt out beyond the circumference of the sheath via spirally moving fibers resulting in a uniform distribution within the deposited layer. Uniformity was dependent on the weight, and hence the size, of the crystals. Previous use of salt particles larger than 150 µm produced visible non-uniform salt distributions concentrated directly below the sheath. Figure 5.2 (b) shows electrospun fibers wrapped around an incorporated NaCl crystal. A normal electrospun morphology (rounded fibers) is observed in spite of the presence of the salt crystal.

A low magnification SEM image of a cylindrical plug containing incorporated salt crystals is shown in Figure 5.3 (a). A partially delaminated layered structure is
evident due to the volume of incorporated particles. Gap sizes appear to range from 100 to 200 µm. Salt distribution within each delamination is relatively uniform. The side of the plug was slightly smeared due to shear forces applied during punching. Much more shallow (less than 10 µm in depth) delaminations were also produced within each layer and these are likely a characteristic of the electrospinning process itself since no salt was being deposited at those points during the spinning. A salt crystal entrapped by fibers marked by an arrow is shown (Figure 5.3 (b)). Each delaminated layer is held together by fibers deposited during salt incorporation. These fibers were strong enough (thanks in part to limited sintering) to maintain an overall monolithic structure during salt-leaching and subsequent cell culture. The structure of PCL fibrous plug after salt-leaching is shown in Figure 5.4 (a). As expected, leaching did not visibly degrade the fibers but some slight swelling of the overall structure was observed. This expansion seemed to be largely springback resulting from the release of compressive forces applied during punching. Figure 5.4 (b) shows partially delaminated layers joined by ‘vertically’ (in this image) oriented fibers.

The distance between each delamination can be controlled by the intervals between additions of salt crystals. A 10 minute interval resulted in approximately 450 µm thick layers (Figure 5.5 (a)) while 5 minute intervals resulted in approximately 230 µm thick layers (Figure 5.5 (b)). The shorter interval also resulted in less isolated delaminations as is evident from the less distinctive layers seen in Figure 5.5 (b).

Figure 5.6 documents the various morphologies adopted by CFK2 cells seeded on the salt-leached plug at day 3. Some cells are rounded (Figure 5.6 (a)) while others
display an elongated morphology (Figure 5.6 (b) and (c)). ECM accumulation on the plasma membrane is apparent in Figures 5.6 (a) and (b).

Horizontal cross-sections of the cell-seeded plug cultured for 3 weeks were observed (Figure 5.7). Even though the samples were 6 mm in diameter these cross-sections were approximately 35 ~ 70% infiltrated by cells (Figures 5.6 (a) and (b)). The average (from 18 layers) cell coverage was determined to be approximately 60%. Lighter colored regions (marked as regions 1 and 3 in the picture) contain cells while no cells were present in the darker colored region (marked as region 2). High resolution images of regions 1 and 3 reveal infiltrating cells along with the presence of accumulated ECM. Cellular penetration began at the edge of the salt-generated porosity and progressed inwards (in some cases substantially).

Figure 5.8 provides an image taken from a stained plug cryosection following 3 weeks of culture. Cellular nuclei are blue and the FITC-labeled PCL fibers are green. The presence of cells along the deposition plane shows that they clearly infiltrated along the pores formed by the salt crystals. A few cells infiltrate ‘vertically’ into the electrospun mesh (see the circle in Figure 5.8 (a)) but the majority appears to be enclosed within the cavities formed by salt dissolution. Figure 5.8 (b) shows a magnified image of the cells (marked with a circle in Figure 5.8 (a)) infiltrating both horizontally and vertically.

Finally, Figure 5.9 shows glycosaminoglycan (GAG) distribution in the cryosectioned sample following 3 weeks of culture using a safranin-O (red) stain. The majority of the GAG content is observed in the gaps generated by salt-leaching consistent with the cell distributions shown in Figures 5.7 and 5.8. In this figure, the vertically
aligned fibers that hold the delaminated layers together, and the associated adherent cells, can be clearly observed.

5.5. Discussion

Electrospinning is a promising technique allowing efficient, economical production of tissue engineering scaffolds. The process produces a unique non woven nano- and/or micro-fibrous structure that resembles natural ECM. The influence of nano- or micro-structures on cellular migration, orientation and cytoskeletal organization has been demonstrated [24-27] and we have previously shown that electrospun topographies significantly enhance cellular behavior [28].

However, as has been pointed out pore size exponentially decreases with fiber size [7]. Zhang et al. reported cellular infiltration of only 48 µm using bone-marrow stromal cells [29]. Van Lieshout et al. also showed poor penetration of human myofibroblasts into electrospun PCL compared to a knitted equivalent [30]. Li et al. postulate that cells on an electrospun ‘surface’ could penetrate by enzymatic degradation of individual fibers [31] but this mechanism is improbable for relatively resistant synthetics like PCL. Faster ‘degraders’ such as PGA, PLA and gelatin can be used but at the cost of poorer initial chemical and mechanical stability.

Within this framework it seems clear that efficient initial seeding is critical for tailored cellular ingrowth into electrospun scaffolds to produce in vivo-like cellular distributions. Dynamic depth-filtration has achieved effective seeding in other fibrous scaffolds [32, 33] but would not be as successful in standard electrospun matrices due to the small pore sizes. Stankus et al.[34] developed a method that simultaneously
introduces cells into a scaffold simultaneous with electrospinning providing concurrent deposition of cells and fibers throughout the scaffold.

The inherent appeal of marrying salt deposition with electrospinning is that it utilizes the inherent randomness of the Taylor Cone to achieve uniform results that guide subsequent cell proliferation. Simple vertical additions of salt to the surface of the collector plate would be biased based on their method of introduction. The result could not be uniform unless considerable effort went into altering the normal gravity-driven trajectory of the introduced salt particles. With the current technique a much simpler approach succeeds in providing a uniform distribution because it interacts with the elongating, whipping fiber formed by the balance between electrostatic repulsion and solution viscosity to result in a relatively random, uniform arrangement of salt particles in the as-deposited mass. Neither solvent evaporation nor the formation of nanometer-scale fiber diameters appears to be affected by the presence of the adhering salt particles. In other contexts involving the use of salt as a porogens for tissue engineering scaffolds a lack of control of salt placement can result in distinct gradients in porosity and pore size. An additional consequence, a lack of interconnected porosity, is avoided here because electrospinning is characterized by highly interconnected porosity.

Figure 5.6 shows cells in various stages of their cell cycle in which they exhibit different levels of shape and hence mobility [35-37]. Most surprising, given the relative inefficiency of the simple gravity-based seeding employed here, was that the nearly complete (in some cases) penetration of the salt-generated gap observed (Figures 5.7 and 5.8). Figure 5.9 validates the observations of both horizontal and vertical penetration while showing that the cells have apparently retained an appropriate phenotype capable
of producing glycosaminoglycan. Given that the details of salt deposition (amount size, number density) are far from optimized the result provides a clear illustration of the ability of mammalian cells to infiltrate appropriately engineered electrospun matrices.

To our knowledge this represents the first observation of extensive cellular infiltration of electrospun matrices. Thus it is important to examine and understand the nature of infiltration. Figure 5.10 shows that cell clustering can take several forms. Relatively ‘thick’ cell populations are observed at points of initial cell seeding (Figure 5.10 (a)) at the edge of the cylinders. These represent the initial gravity-driven cellular deposition into the edge of the gap (Figure 5.4 (b)) followed by proliferation leading to the observed mass of cells. Internal to the plug is evidence for both large numbers of cells (Figure 5.10 (b)) as well as more scattered, isolated cell populations (Figure 5.10 (c)). Infiltration was highly variable; Figure 5.7 (a) shows nearly complete penetration of a delaminated cross-section; Figure 5.7 (b) shows cell populations scattered in between areas in which fiber density remains high enough such that no cells are present. The origin of these populations is worthy of discussion. Distances of 3000 to 6000 µm were clearly spanned by these cells. Given the apparent good adhesion of the cells to the electrospun fiber it seems likely that infiltration is primarily driven by localized proliferation rather than cellular locomotion. The latter, at roughly 100 µm/day [38], would not be sufficient to allow for the cross-sectional coverage observed following only 3 weeks of culture. It would be interesting to determine how much culture time or initial porosity would be needed to cause physical delamination of these structures.

The method developed in this study provides large pores into which cells can infiltrate without first requiring fiber degradation. We showed that cells can migrate up to
~4 mm and can cover ~70% of the cross-sectional area of these 6 mm diameter plugs using simple gravitational seeding. If a dynamic seeding - like the depth-infiltration seeding developed by Li et al. [33] - is applied to this scaffold infiltration would likely be even more effective. This technique clearly provides considerable flexibility in scaffold design and in promoting cellular ingrowth along specific directions. Both the amount of salt and the particle size will doubtless be important. Furthermore, the layered structure can be precisely tailored by varying the deposition interval to produce controlled gradients in pore distribution. This gradient can lead to cellular density gradient in a scaffold that better resembles the in vivo equivalent, for example, the zonal anisotropic structure of cartilage.

5.6. Conclusions

We have demonstrated a method that produces a delaminated structure in an electrospun scaffold by combining salt-leaching technique with electrospinning. With this method, not only can cellular infiltration into a thick electrospun scaffold be facilitated, but scaffolds having designed, anisotropic structures can be produced to guide cellular proliferation to better approximate targeted tissues.
Figure 5.1. Schematic illustration of an electrospinning setup showing the mechanical introduction of salt crystals (gray particles): (a) syringe pump, (b) extension tubing, (c) sheath surrounding the needle into which the crystals are added, (d) needle experiencing the applied voltage, (e) electrospun fiber interacting with falling NaCl crystals and (f) grounded collector.
Figure 5.2. (a) Image of salt particles interacting with fibers during electrospinning (b) SEM image of a salt particle entrapped by fibers.
Figure 5.3. (a) Side view of an as-punched 3 mm diameter PCL plug containing salt crystals, (b) a salt particle (arrow) entrapped by fibers within a delamination.
Figure 5.4. SEM images of (a) PCL plug after NaCl leaching showing ~200 µm size pores and (b) fibers holding two partially delaminated layers together.
Figure 5.5. SEM images of different NaCl crystal dispense intervals; (a) 10 minutes and (b) 5 minutes resulting in 450 and 230 µm thick layers, respectively.
Figure 5.6. SEM images of the seeded cells showing either rounded or elongated shapes on the salt leached PCL plug at day 3.
Figure 5.7. SEM images of the delaminated layers showing cellular infiltration following 3 weeks of culture; cells are shown covering (a) ~70% and (b) ~35% of the 6 mm cross-sections in the same plug; regions 1 and 3 show cellular infiltrated areas; region 2 - no cellular coverage, (c) high resolution image of region 1 and (d) high resolution image of region 3.
Figure 5.8. Immunostained (DAPI; blue stains) sample cryosectioned to 12 µm in thickness following 3 weeks of culture. (a) cellular infiltration through salt-generated pores (50X) and (b) cellular infiltration into the PCL fiber meshes in some area (magnified image of the circled area in Figure 5.8 (a)) (100X).
Figure 5.9. Safranin-O stained sample cryosectioned in 12 µm after 3 week culture shows GAG (red stains) accumulation in the NaCl crystal-generated pores. Vertical fibers that hold partially delaminated electrospun layers are also observed (100X).
Figure 5.10. SEM images of (a) dense cellular population near the edge of the plug, (b) dense cellular population and (c) sparse population in the middle of the plug.
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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Tissue engineering is a rapidly expanding area that seeks to create specific human tissues and/or organs by combining cells and scaffolds formed typically using either synthetic or naturally-derived polymers. These tissue-engineered products will someday be able to deliver on the promise of functional replacement for diseased or failing organs. In pursuing these goals, dealing with biological systems has inevitably led to an interest in expanding upon or improving the ability of current tissue engineering scaffolds to promote or preserve the appropriate function of adherent mammalian cells. Among numerous scaffold fabrication processes, electrospinning is a promising method for achieving this in three-dimensional form partly due to the ease with which it produces non-woven nano- to micro-sized fibrous scaffolds having 70 – 90 % relative porosity. In this context, the mechanical behavior and the residual solvents of electrospun scaffolds were investigated. In addition, the effects of electrospun topographies on the morphology, proliferation and functionality of chondrocytic cells were studied. Finally, salt-leaching was combined with electrospinning to generate engineered porosity within electrospun scaffolds to improve cellular infiltration. Important findings from these studies are summarized below.
6.1. Fracture and Recrystallization of Electrospun Fibers under Strain

The effect of different solvents on the microstructure and the mechanical behavior of electrospun PCL was investigated. Point-bonded structures having physical bonds at the fiber intersections were produced using acetone while HFP did not result in these inter-fiber bonds. The point-bonded structure led to premature failure prior to individual fiber deformation due to stress concentration along the length of the fibers. In contrast, relatively bond-free fibers were more highly aligned by the straining process and this resulted in a distinctive “bamboo” structure caused by fiber recrystallization.

6.2. Residual Solvent Retention in Electrospun Fibers

The residual solvent in fibers produced by electrospinning was systematically analyzed using gelatin/PCL-HFP and PCL-acetone systems. Gelatin is clearly more prone to residual solvent retention than PCL. PCL is clearly less likely to retain either of the solvents used. A simple and effective procedure reducing residual solvent content of PCL-gelatin blends was developed. The majority of the residual solvent could be reduced to a level that had no demonstrated effects on cell interactions with the scaffold. Electrospinning as a means of investigating tissue engineering needs to include consistent post-processing treatments to avoid possible residual solvent release that could greatly complicate correct interpretation of biological activity on the resulting scaffolds.

6.3. The Effects of Electrospun Topography on Cellular Behavior

Electrospun fiber supported better proliferation and ECM production than the corresponding semi-porous and dense surfaces and even, at some time points, glass
surfaces. The behavior during initial cell seeding likely affects subsequent cellular behaviors. The intrinsic capability of electrospinning to produce high porosity appears to offset the relative hydrophobicity of PCL resulting in more uniform seeding. Electrospun fibers induced a higher level of GAG production by providing a structure in which chondrocytes are able to maintain a morphology associated with the appropriate phenotype.

6.4. Improved Cellular Infiltration in Electrospun Fiber via Engineered Porosity

A method that can produce a delaminated structure in an electrospun scaffold by combining a salt-leaching technique with electrospinning was developed. The random nature of the whipping polymer jets in the electrospinning process resulted in a uniform, well-spread distribution of salt particles and, therefore, of the subsequent porosity. It seems likely that cellular infiltration into this scaffold is primarily driven by localized proliferation rather than coordinated cellular locomotion. With this method, not only can cellular infiltration into a thick electrospun scaffold be facilitated, but scaffolds having designed, anisotropic structures can be produced to better approximate the final tissue.

6.5. Recommendations

Tissue engineering is a rapidly evolving discipline with continuous improvements in scaffold fabrication and cell culture technology. Basic knowledge obtained from this research should facilitate the development of artificial organs. Several recommendations for further studies are suggested.
• The recrystallization behavior of electrospun matrices needs to be further studied. To investigate the onset of structural changes in situ observation of deformation under ESEM can be used. In addition, temperature changes during this process would be best investigated by combining a load frame and a DSC.

• Incorporation of naturally derived polymers such as collagen, fibronectin and hyaluronan into the electrospun scaffold made of synthetic biodegradable polymers by means of blending or coating is recommended. This will improve cellular adhesion upon cell seeding and induce more appropriate cellular responses.

• Dynamic cell seeding needs to be developed to further improve seeding uniformity. The initial distribution of the cells in a scaffold clearly influences cellular response. Uniform cellular distribution in a scaffold will induce faster and more suitable tissue generation. If this technique can be applied to scaffolds having salt-generated porosity further improvements in cellular penetration and biological function are anticipated.

• Mechanical conditioning of cell/scaffold complex, for example dynamic compressive straining for cartilage engineering and shear flow for vascular engineering, is suggested. This will encourage faster proliferation and induce more appropriate cellular function.

• Electrospun matrices can be used in a variety of biologically-based studies. One example is a cellular signal pathway study. These scaffolds provide more physiologically relevant 3-D structures compared to 2-D surfaces. The high open porosity of the structure also assists in collecting signal molecules in time-
dependent studies such as real time-PCR. In addition, the feasibility of stem cell differentiation into appropriate phenotypes by mechanical stimuli without the use of serum can be studied.

- The signaling pathways can be applied to improve engineered tissues. For example, molecules such as growth factors that induce the expression of specific genes can be introduced during *in vitro* culture for tailored production of proteins. This will help to eliminate the use of serum, which is a cocktail of unknown factors.

- Ultimately, *in vivo* studies need to be done to evaluate the feasibility of these tissue engineered products under truly physiological environments.
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