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#### ABSTRACT

### INTERACTIONS BETWEEN ADULT HUMAN MESENCHYMAL STEM CELLS AND NANOFIBROUS SCAFFOLDS OF DIFFERENT COMPOSITIONS

#### by Lisamarie Moore

Stem cells have become increasingly important in the biomedical field because they have the potential to invade and regenerate damaged tissue. In particular, the bone marrow contains mecsenchymal stem cells (MSCs) that have the capacity to differentiate into many cell lineages. The incorporation of MSCs within a multidimensional biomaterial provides a delivery vehicle to areas of injury. Natural and synthetic biomaterials can be made into scaffolds to represent different types of tissues. The ratio of synthetic to natural biomaterials in the scaffold influences the interaction with MSCs. This thesis created two different nanofibrous scaffolds by a process of electrospinning. The scaffolds consisted of a nanofiber mesh mimicking the extracellular matrix. One is derived solely from Poly-Llactide acid and the other from Poly-L-lactide/Gelatin (50:50). Before and after culturing, measurements of fiber diameter, thermodynamics, and tensile strength were collected. Studies with primary human MSCs were conducted to characterize the *in vitro* behavior of MSCs seeded on and adhered to scaffolds of different compositions.

## INTERACTIONS BETWEEN ADULT HUMAN MESENCHYMAL STEM CELLS AND NANOFIBROUS SCAFFOLDS OF DIFFERENT COMPOSITIONS

by Lisamarie Moore

A Thesis

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Submitted to the Faculty of New Jersey Institute of Technology in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biomedical Engineering

**Department of Biomedical Engineering** 



## **APPROVAL PAGE**

# INTERACTIONS BETWEEN ADULT HUMAN MESENCHYMAL STEM CELLS AND NANOFIBROUS SCAFFOLDS OF DIFFERENT COMPOSITIONS

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To my spunky and always loveable daughter, Wynter, and my parents, Mommy and Papa, whose encouragement and love is endless

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# LIST OF DEFINITIONS

<u>Rheology</u> is the study of the deformation and flow of matter.

<u>Viscoelastic materials</u> can exhibit both viscous and elastic characteristics. *Viscous* materials resist shear flow and strain when stress is applied. *Elastic* materials strain instantly when stretched and return just as quickly to its original state when stress is removed.

<u>Vapor pressure</u> is the given temperature where the pressure, at which the gas of a substance, is in dynamic equilibrium with its liquid or solid forms. The equilibrium vapor pressure is an indication of a liquid's evaporation rate. It relates to the tendency of molecules and atoms to escape from a liquid or a solid.

<u>Electrostatic bonds</u> result from the electrostatic attraction between two ionized groups of opposite charge.

<u>Heat capacity</u> is the amount of heat required to raise the temperature of 1g of polymer  $1^{\circ}$ C.

<u>Enantiomers</u> are stereoisomers that are mirror images of each other, like a left and right hand are the same but opposite. A mixture of equal parts of an optically active isomer and its enantiomer is termed <u>racemic</u>.

#### **CHAPTER 1**

#### **INTRODUCTION**

Biodegradable materials as a platform for the delivery of mesenchymal stem cells have become increasingly important in the treatment of degenerative diseases. Mesenchymal stem cells can differentiate into many lineages while seeded on biomaterials [1-6]. This cell-scaffold construct has the potential for tissue engineered applications that deliver bulks of tissue while functioning as a template for reconstruction.

This thesis investigates the therapeutic potential of constructs made from synthetic, natural, and blended biomaterials. The biomaterials were made into multidimensional nanofibrous scaffolds by an electrospinning process. The nanofiber scaffolds were characterized by fiber diameter, thermal, and tensile properties before and after being submerged in culture medium. Additionally, the effects the scaffolds had on proliferation and viability of adhered MSCs was studied and analyzed.

#### **CHAPTER 2**

#### BACKGROUND

#### **2.1 Electrospinning Process**

Electrospinning is a process that uses electrostatic force to create slender, elongated threadlike structures, known as fibers [Appendix A]. This fairly cheap, simple, and adaptable procedure can create long and continuous fibers from a small amount of polymer solution. Electrospinning produces fibers that have smaller diameters and larger surface areas than that of conventional textile fibers, which enhances cell attachment [7;8]. The fibers formed have diameters in the nanometer range, mimicking many naturally occurring nanofibrous structures found in the extracellular matrix. This makes electrospun nanofibers ideal for developing solid fibrous scaffolds that can be potentially useful in biomedical applications [9].

Electrospinning involves polymer science, applied physics, fluid mechanics, electrical, mechanical, material engineering, and rheology [10]. These disciples will influence the resultant fiber. Fortunately, the electrospinning apparatus [Appendix B] allows parameters to be easily adjusted to control the fiber formation.

There are three distinct and important stages during electrospinning. Firstly, the jet formation, followed by jet instability, elongation, and bending. The final step includes solidified nanofibers that are collected on a grounded plate [10].

The jet is formed by attaching a positive electrode to a metallic needle which is attached to a syringe filled polymer solution. A relatively short distance away, an aluminum collection plate is grounded. The injected charge will induce a potential

2

difference between the solution and the ground plate. An electrostatic charge is formed at the surface of the solution. The electrically charged solution will attempt to propel towards the ground plate because of the potential gradient. However, in order for this to happen the solution must overcome its surface tension and viscoelastic properties.

The solution's viscosity, surface tension, and charge are extremely influential in the beginning stages of the jet formation. At the edge of the needle there is pulling and stretching of the solution caused by the attraction forces from the grounded plate. The surface tension causes the solution to contract in the form of a spherical shape in order to maintain a low surface to volume ratio. The electrostatic force distorts this droplet and forms a Taylor cone [Figure 2.1].

The higher the solution's viscosity, the harder it will be to overcome the surface tension. Therefore, the formation of the jet requires sufficient electrically force to erupt from the Taylor cone [11]. Increasing the electrical charge will increase the flow rate of the jet, allowing the opposing forces to be overcome. Eventually, a steady flow state will occur a short period after the jet is ejected [12]. The jet will extend in a straight line for a certain distance, and then it begins to bend and follow a looping and spiraling path [10;13]. This leads to the second stage of electrospinning.



**Figure 2.1** (1) Electrostatic force and surface tension cause a Taylor cone to form at the edge of the needle. (2) The constant electrostatic force causes the Taylor cone to elongate. (3) Surface tension and viscoelastic properties of the polymer solution are overcome and a small liquid jet is ejected. (4) The jet reaches a steady state of flow and the Taylor cone relaxes.

In the second stage, the jet is propelling in a straight line towards the ground plate. However, at an undefined distance, it becomes unstable. The small diameter of electrically charged liquid becomes smaller, by continuously looping and elongating thousands or millions of times [14;15]. The continuous elongation and growth of each looping segment, is most strongly influenced by the repulsion between the charges carried by adjacent segments [10]. The jet becomes extremely thin and usually no longer visible to the eye.

During the rapid second stage and within a relatively short working distance, the solution will begin drying causing the solvent to evaporate. The many entanglements of the polymer will prevent breakage as the solvent evaporates [7]. Left behind is the original polymer(s) that were dissolved in the solvent. Finally, the attraction from the ground plate will cause the polymer to collide and collect solidified fibers, in a unwoven web like formation, as the third and final step [10].

Optimizing the processing parameters is essential in creating reproducible fiber diameters. Some parameters have more of a significant influence over the fiber diameter than others. The electric charge, working distance, initial jet radius, relaxation time, and solution viscosity are found to be the most influential. Where as, the initial polymer concentration, solution density, electric potential, perturbation frequency, and solvent vapor pressure have moderate effects. Minor effects are associated with the relative humidity, surface tension, and vapor diffusivity [16].

Parameters must be specifically adjusted to meet the needs of varying polymers, solvents, and environments. Most of the equipment used in the electrospinning apparatus is easily manipulated. For example, different polymer solutions, if distance is held constant, reach the collection plate at varying electrically force. In real time, the operator can increase or decrease the applied electrical charge. This will adjust the flow rate necessary for the jet to reach the collection plate. However, if the attraction force is too strong, the jet will not loop as many times, and some solvents may not have enough time to evaporate completely. The operator can also adjust for differences in solvent evaporation rates.

The electrostatic force can also be manipulated to change the resultant fiber diameter. If the applied force is reduced, the jet will have more time to loop and elongate, resulting in a collection of smaller diameter fibers. While a higher electrical force may result in bigger fibers and/or wet droplets collected on the ground plate. Additionally, to further reduce the fiber diameter, the electrostatic force can be slightly lower milliseconds after the jet is ejected. This is due to the opposing forces from the solution having already been overcome. The electrostatic force required to maintain the steady flow state is lower than the force required to eject the jet [Figure 2.1 Step 4].

If an electrostatic force is not strong enough to maintain the jet and overcome the surface tension, and viscoelastic properties of the solution, than a mechanical force may be used. This pushing force helps propel the solution out of the needle, requiring less electrostatic force to form the jet. Mechanical forces are useful with an extremely viscous solution.

The working distance can be increased or decreased to alter the distance the jet has to travel to collide with the grounded plate. This parameter can change the resultant fiber diameter, resulting in thicker or thinner fibers [16]. However, it mustn't be too close as to cause sparks between the electrodes or too far so the solidified fibers don't reach the plate [7].

Adjustments can also be made to the solution properties. A reduction in entanglements will lower the surface tension and viscoelastic properties, which decreases the forces needed to form the jet. Additionally, adding salt (NaCl) to the solution, with other parameters held constant, will reduce the diameter of the jet [10]. Salt assists in overcoming the surface tension due to an addition of positive ions in the solution. Overall, lowering the viscosity and creating few entanglements will results in a reduction of fiber diameter. All the parameters mentioned are critical if nanofibers are the desired diameter of the scaffolds.

#### 2.2 Biodegradable Polymers and Trifluoroacetic Acid

There is a growing demand for materials that can be used in biotechnology, tissue regeneration, and implants. Electrospun fibers are attractive platforms because they can be applied to many biological applications while remaining inexpensive and simple to generate. There are many different types of materials that can be electrospun into nanofibers. Many are derived from synthetic polymers, such as nylon [17], chitosan [18], poly(ɛ-caprolactone) [19], and poly(l-lactide-co-glycolide) [20]. Some are natural polymers, such as silk [21], collagen [22], nucleic acids [23], and polysaccharides [24]. The appropriate materials to be electrospun will depend on the intended application. Therefore, it is important to have a basic understanding of the material.

Poly (L-lactide acid) and Gelatin were the materials chosen for this thesis because they can be molded into strong yet biodegradable scaffolds. They have also been successful in pervious experiments using electrospinning, cell cultures, and as copolymer scaffolds [25;25-30]. Scaffolds were created that consisted of either 100% of PLLA or gelatin, or a blend of PLLA\gelatin (PG) (50:50).

#### 2.2.1 Poly-L-lactide acid

Polylactide is biodegradable aliphatic polyester. This polyester can be derived from 1 of the 3 popular lactide monomers. Lactic acid (lactide) is an intermediate in normal human metabolism and is primarily excreted through respiration. It is a water-soluble liquid produced in muscles as a result of anaerobic glucose metabolism. It is also present in sour milk, molasses, various fruits, and wines. Polylactide is prepared from the cyclic diester of lactic acid. A hydrolytic attack of the bonds opens the ring of the diester, and then there is a polymerization of the chains [Figure 2.2.1].



**Figure 2.2.1** Lactide undergoes a hydrolytic attack breaking the ester bond capable of forming a branched polyester.

Lactic acid exists as two optical isomers or enantiomers. The L-enantiomer occurs in nature, a D,L racemic mixture is derived from the synthetic preparation of lactic acid. Fibers spun from "L" polylactide have high crystalline properties. The "DL" polylactide is made of more amorphous material. The crystalline L-lactide is more resistant to hydrolytic degradation than the amorphous DL-lactide form [31].

Polylactide is a commercially available synthetic material that is becoming increasingly important in the biomedical field. Its ability to facilitate tissue growth and repair after damage is very attractive to the medical field. It has been used as absorbable biodegradable sutures for wound healing [32;33], ligating clips [34], and as a matrix and slow release depot for osteoinductive biological agents [35]. PLLA was attached to sheep femoral and showed mechanical deterioration but still evident after three years [36]. Another study observed a decrease in absorption time of PLLA implants with increases in

molecular weight of the polymer [37]. Overall, PLLA is biomaterial with a relatively long absorption and degradation rate.

#### 2.2.2 Gelatin

Gelatin is denatured collagen that is typically isolated from bovine skin, porcine skin, or bone by acid or base extraction. It is widely used in foods as a thickener and in the pharmaceutical industry as an agent in orally delivered drugs. Gelatin consists of a distribution of polypeptide fragments of varying sizes, different isoelectric points, and different gelling properties from lot to lot. The physiochemical differences of lots are based on the method of extraction, amount of thermal denaturation employed, and electrolyte content of the resulting material. This variability will present a challenge in the use of gelatin. However, some secondary preparation processes can alleviate such variability.

Gelatin is an attractive polymer to use because it has been successfully electrospun [38-40] and possesses properties lacking in PLLA. It has a high degradation rate and can therefore be a quick drug delivery system or a temporary scaffold structure. Additionally, since it is derived from natural sources, it can be degraded by natural enzymes, rendering it biocompatible.

Collagen is commonly used in cases when Tissue Engineers seek to incorporate a natural polymer into their applications. It can be electrospun into nanofibers and has favorable results in vivo. However, collagen costs more than gelatin. Therefore, this thesis utilized gelatin as a suitable cheaper alternative for collagen.

#### 2.2.3 Trifluoroacetic Acid

The solvent properties are a vital component of electrospinning. The solvent must be capable of dissolving the polymer and then undergoing the electrospining process. It must diffuse into the polymer bulk and break the bonds, but if the bonds are strong the solvent may not be able to break them. That was evident when solvents such as dimethylchoride and chloroform were used in an attempt to dissolve gelatin.

Trifluoroacetic Acid (TFA) [Figure 2.2.2] is a carboxylic acid that is used in organic chemistry to separate peptides and small proteins. It has a pKa of 0.3 making it very acidic and extremely volatile. As a hydrogen acceptor it can easily break hydrogen bonds that are present in proteins.

Additionally, TFA has a vapor pressure that allows it to evaporate in a short working distance, but not distort the fiber before it reaches the plate. This makes it a good candidate for electrospinning [41].

C<sub>2</sub>HF<sub>3</sub>O<sub>2</sub>

Figure 2.2.3 Chemical and Molecular Formula of Trifluoroacetic acid [42;42].

#### 2.3 Adult Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are present in several fetal and adult tissues, although the predominant site in adults is the vascular region of the bone marrow. They are defined by their intrinsic ability to self-renew, generate numerous cell types, and reconstruct damaged tissues [43]. MSCs have been found to own a broad differentiation capacity, outside of their mesodermal origin [Figure 2.3]. MSCs can generate bone, cartilage, fat, muscle, and neurons [44;45]. In addition, MSCs can be easily isolated, expanded in culture, and have a low incidence of tumor formation [46]. These factors make MSCs attractive for translational science.



Figure 2.3 Mesenchymal stem cells differentiation lineage pathways. Source: Trends of Molecular Medicine

#### **CHAPTER 3**

#### **EXPERIMENTAL DESIGN**

#### **3.1 Solution and Apparatus Preparation**

Initially, dissolving both polymers was challenging. PLLA dissolved in almost all the tested solvents, chloroform, tetrahydrofuran, dimethylformamide, and dimethylchoride. However, Gelatin would not dissolve in any of these solvents. A more acidic solvent was required to break the peptide bonds. Trifluoroacetic acid (TFA) is a solvent that has been used successfully to dissolve other proteins [41;47]. Once Gelatin was introduced, it was visually evident that TFA was able to dissolve the gelatin. TFA was therefore used as the sole solvent in all polymer solutions.

After testing many polymer concentrations a 30 weight % of PLLA, gelatin, and PG faired the best when electrospun. However, in order to obtain nanofibers, variations in the electrospinning parameters had to be optimized. Initially, both were stirred overnight, individually, until completely dissolved and translucent. Each polymer solution was inserted into the electrospinning device. Variations where made in the voltage (10kV - 20kV), syringe pump velocity (0.0206 - 0.0859 ml/min) to create visible fiber formation. The working distance was held constant at 20cm.

The 100% PLLA scaffold, under light microscope, appeared continuous but had large diameter fibers, ranging from 200 - 400µm. The 100% gelatin scaffold had liquid droplets accelerating out from the Taylor Cone. The drops collected on the plate and created "wet spots" that dissolved the fibers. The concentration of gelatin was increased to 40 weight %. After stirring overnight, the solution was more viscous then the previous

mixture. Using the same variations in voltage and syringe pump velocity, the liquid droplets were eliminated. However, under light microscope, beads were observed. Beads tend to appear more frequently and become larger as the viscosity of the solution increases [10]. Since an increase in the concentration created beading, it was lowered back to 30 weight %. Lastly, the PG scaffold was created using a 50:50 blend of PLLA and gelatin totaling 30 weight %.

The parameters required optimization to address the problems associated with the scaffolds. The fiber diameter needed to be reduced and the wet drops had to be eliminated. After many attempts at spinning fibers from all the solutions, the following parameters were adjusted. These parameters were held constant for all the scaffolds.

The first parameter studied was the solution's viscosity. During the course of the experiments, it was visually obvious that the viscosity decreased as the solution's shelf life increased. If the solution was shelved for over two days it was no longer possible to electrospin fibers because the viscosity was too low. This is caused by TFA constantly breaking the bonds and decomposing the polymer.

The initial concentration largely influenced the polymer solution's shelf life. If a solution was created and spun on the same day a low polymer concentration would yield fibers but if left to stir overnight, the concentration had to be increased. The higher the initial concentration the longer the solution's shelf life would be. Therefore, a higher concentration of 30 weight % was established for all three polymer solutions and left to stir overnight.

The next parameter adjusted was the diameter of the needle. In order to produce smaller fibers and reduce the liquid droplets, the needle diameter was reduced from a 22 gauge to a 24 gauge.

Another parameter changed was the speed of the syringe pump. The speed was reduced from approximately 0.03 ml/min to 0.0206 ml/min. The syringe pump's purpose is to help viscous solutions move easily through the needle without getting lodged. However, the stronger the mechanical force the more liquid will accumulate in the barrel. This will affect the Taylor cone and the jet formation. Also, once the jet reaches a steady state, periodically the syringe pump is shut off.

The working distance was increased from 20cm to 40cm. This allows more space for solvent evaporation, which prevents liquid droplets from reaching the plate. Increasing the distance will yield more time for jet elongation, thereby producing smaller diameter fibers.

The voltage is a very important parameter because it is the primary force. A reduction in the voltage will provide more time for jet elongation. Lower electrical forces typically create smaller diameter fibers. However, with the other parameter changes already established, the voltage had to increase from 10kv -20kV to 20kV- 25kV to optimize the procedure. The additional charge provided the necessary force to push the jet from the viscous solution, through a smaller diameter needle, and a longer working distance to reach the plate.

Approximately 1 mm thick scaffolds were spun, which is relatively dense. The process takes about an hour. Afterwards, the scaffolds are dried in a vacuumed desiccator overnight.

Prior to culturing, the PLLA and PG samples underwent a "Water Soaking Test." This tested whether water would dissolve the scaffolds. If the scaffolds dissolved in water then it would also dissolve in cell culture medium. The PLLA sample didn't visually change when placed in the water. The PG scaffold appeared to absorb the water, similar to how a sheet of paper looks when placed in water, but it remained intact. The Gelatin scaffold wasn't tested at that time. This was unfortunate, because it had to later be removed from the experiment. When it was being prepped for cell attachment, it dissolved immediately when washed with phosphate buffer saline.

#### **3.2 Analysis of Electrospun Nanofibers**

The three electrospun mats, PLLA, gelatin, and PG, underwent several analytical tests to characterize its properties. A tensile test was conducted to analysis stress and strain. Then the thermal properties of the scaffolds, as well as the morphology and fiber diameters, were examined before and after scaffolds were submerged and incubated in medium.

#### **3.2.1 Tensile Analysis**

The tensile strength of a material is classified as the maximum load a material can support without breaking as it is being stretched. The Instron Mechanical analyzer [Appendix C] performs a tensile test that measures the stress and strain of all three scaffolds. This data is important to characterize the behavior of a material that will be under tension *in vivo*.

The ratio of crystalline to amorphous material in the scaffold will have an affect on its mechanical properties. Crystalline polymers are brittle and rigid therefore, if it is stretched the crystal structure will be destroyed [7]. An amorphous polymer that is below its glass transition acts much like a crystalline polymer when it is stretched. However, if it is above its glass transition it can bend to relieve the stress applied by the stretching.

Table 3.2.1 displays the results from the tensile tests. The gelatin scaffold broke at the lowest load and with the lowest percentage of deformation. It was the most brittle and resistance to the stretching force. Whereas, the PLLA scaffold never broke during the course of the experiment. It continued to stretch throughout the duration of the tensile test. It had the highest percentage of deformation. The PLLA scaffold was able to bend to relieve the stress being exerted. As compared to the gelatin scaffold, the PLLA scaffold has a higher ratio of amorphous material above its Tg. The blend, PG, displayed an average of both materials. It wasn't able to stretch as much as the PLLA scaffold but it didn't break at a low load as the gelatin.

Specimen	Max. Load (N)	Stress at Max. Load (MPa)	Strain at Max. Load (%)	Load at Break (Standard) (N)	Stress at Break (Standard) (MPa)	Strain at Break (Standard) (%)
Gelatin	1.14	9.92	-1289.81	0.03	0.23	-1215.60
PLLA	1.47	24.38	-4873.27	-0.01	-0.15	-2580.35
PG	1.96	13.04	-1720.22	0.52	3.47	-1495.94

**Table 3.2.1** Results from Tensile Tests (p < .0001)

#### 3.2.2 Thermal Analysis

Heat response is normally used to classify a polymer's material composition. Contrasting ratios of crystalline to amorphous material will react differently to temperature change. Temperature will cause the material to undergo a thermal transition associated with heat capacitance and molecular mobility. Thermal analysis devices can detect theses transitions. A Differential Scanning Calorimeter (DSC) and a Thermogravimetric Analyzer (TGA) were used to detect differences in heat responses in the PLLA, Gelatin, and PG scaffolds [Appendixes E & F].

The DSC was configured to heat, cool, and then heat all samples at a rate of 10°C/min. The first experiment investigated changes in the polymer due to electrospinning. Figure 3.2.2.A displays the 1<sup>st</sup> heating and cooling cycle of PLLA. The raw PLLA has a very small Tg, no apparent Tc, and a higher Tm as compared to the electrospun PLLA. This implies the electrospun material has a larger amorphous region then the raw material. Also, there was no obvious crystallization of the raw material on the cooling cycle. The raw material must be a densely packed crystalline structure because when the molten was cooled the vast entanglements restricted re-crystallization. The TGA, Figure 3.2.2.B, displays the durability of the electrospun material. It was able to maintain its weight % until 250°C when there was a drastic decline. Whereas, the raw material is able to withstand higher temperatures.



Figure 3.2.2.A DSC (1<sup>st</sup> Heat and Cool cycles) of PLLA raw and electrospun.





An experiment to investigate the changes in gelatin when it is electrospun uncovered some interesting findings. A protein in its native configuration will not be able to undergo the motion associated with the Tg. If a Tg is present the material is behaving like a synthetic polymer that consists of polypeptide chains. Gelatin did have a small Tg therefore, some of it was denatured. However, it was discovered that the lot purchased wasn't completely denatured collagen.

When the raw gelatin material was heated to 300°C, there were three endothermic curves observed [Figure 3.2.2.C]. The first large curve was associated with water evaporating from the material because on the 2<sup>nd</sup> heat the curve was flat. The 2<sup>nd</sup> endothermic curve appears at the known collagen denaturation temperature, approximately 225°C. The third endothermic peak occurs at the know decomposition temperature of collagen, approximately 275°C. Therefore, the extraction procedure for gelatin production doesn't completely denature the collagen but leaves a portion that remains in its natural folded configuration.



Figure 3.2.2.C DSC of raw gelatin heated to 250°C and 300°C.

Since some proteins aren't completely denatured, the gelatin-TFA solution will initially have three components, native collagen, polypeptide chains, and peptide fragments [Figure 3.2.2.D]. TFA will be responsible for continuing to chemically break down the collagen. Unfortunately, a prolonged shelf life will create a homogenous solution of single peptides fragments that will not be vicious enough to electrospin.

There are differences observed in the gelatin molecule after it is electrospun. At lower temperatures there are two glass transitions present in the electrospun material [Figure 3.2.2.E]. This is due to the gelatin-TFA solution possessing small molecular weight peptide fragments and denatured polypeptide chains. The peptides undergo a Tg at a lower temperature then the denatured collagen. The denatured collagen has a slightly higher Tg, but it is still lower than the Tg of the raw material.



Figure 3.2.2.D Extraction and TFA chemical degradation of collagen.

Smaller peptide fragments

In the electrospun gelatin, there is a deviation in the amount of heat required to remove the water. The water is removed at a lower temperature. This may be due to a decrease in water present in the scaffold because it was carried off by the TFA during the electrospining process.

The TGA [Figure 3.2.2.F] clearly displays two different slops after 160°C. Between 160°C to 250°C there is a substantial weight lose. A fraction starts to decompose early. These are the peptides that were created by TFA's chemically degradation, which is consistent with the DSC graph.

Figure 3.2.2.E DSC (1<sup>st</sup> Heat cycle) of gelatin raw and electrospun.


Figure 3.2.2.F TGA of gelatin raw and electrospun.



The PG scaffold was heated to 200°C [Figure 3.2.2.G] and 275°C [Figure 3.2.2.H]. There is a huge endothermic curve up to 160°C, which is consistent with the water evaporation from the gelatin. On the 1<sup>st</sup> heat cycle there appears to be two glass transitions. One represents the Tg of the wet gelatin ( $\sim 37^{\circ}$ C) and the other the PLLA ( $\sim$ 60°C). Then at 80°C the PLLA amorphous region has enough mobility to undergo crystallization. All the crystals melt around 170°C and the polymer becomes a molten. The PG that was heated to 270°C undergoes another endothermic transition at 225°C. This is consistent with the known temperature of collagen denaturation. As the temperature continues to rise gelatin will decompose. This decomposition accounted for the differences in the scaffold heated to 200°C and the scaffold heated to 275°C. As the molten was cooled into a solid, the PLLA in the scaffold heated to 200°C, was able to crystallize. The 275°C heat cycle degraded the gelatin. Therefore, when the molten was cooled it affected the scaffolds ability to undergo a large re-crystallization. However, the amorphous region of the PLLA does undergo a Tg at a lower temperature around 50°C. not seen in the 200°C heat. On the 2<sup>nd</sup> heat cycle we don't see a Tg of the wet gelatin because it isn't wet anymore. There is a Tg and Tc of the PLLA region but it is significantly larger for the scaffold heated to 275°C. This can be explained, because the 275°C heat didn't undergo a cold re-crystallization and therefore has a larger fraction of amorphous material present. Additional, the 275°C shows a double melting point. This is a phenomenon seen in other PG scaffolds and will be addressed later in this section.

Figure 3.2.2.G DSC of PG heated to 200°C.



Figure 3.2.2.H DSC of PG heated to 275°C.



Figure 3.2.2.I represents all three electrospun scaffolds. This illustrates the properties of both the PLLA and gelatin scaffold displayed in the PG blend. The DSC shows the water evaporation, wet gelatin Tg, and collagen degradation of the gelatin. It also displays the Tg, Tc, and Tm of the PLLA. The TGA [Figure 3.2.2.J] also demonstrates the intermediate properties of the PG blend. In the blend, only 50% is attributed to the water evaporation and weight lose while the other 50% will maintain its integrity. Together these graphs confirm both polymers are present in the PG blend.







Figure 3.2.2.J TGA of all three electrospun scaffolds.

As the PLLA scaffold degrades there is a change in the amorphous region. [Figure 3.2.2.K] The Tg and Tc are becoming undetectable when incubated over time in medium. The amorphous region appears to be degrading quicker, while the crystalline region remains in the scaffold. This is also evident by the melting points that are relatively the same. Otherwise, this could be associated with solvent induced crystallization of the amorphous region. This may occur because, as the amorphous material degrades its molecular weight decreases, making it easier for it to form crystals. Whatever event occurred, the only portion of the material that was able to maintain its integrity in medium was the crystalline region.



Figure 3.2.2.K DSC (1<sup>st</sup> Heat cycle) of PLLA in medium.

The degradation of the PG scaffold is more complicated because it is a blend. In this scaffold, the Tg becomes undetectable while the Tc gradually decreases over the duration of the experiment. There is also a double melting point observed [Figure 3.2.2.L].

These transitions can be explained by the interference gelatin must have on the crystallization of the PLLA. The PLLA instead forms varying sizes of less prefect crystalline regions that are more susceptible to degradation. The double melting points imply there is selective degradation. The selective degradation is a reflection of a broad distribution of crystalline sizes and states of perfection. The smaller imperfect regions,

with a lower molecule weight get degraded quicker and at a similar rate as the amorphous materials, which is evident by the Tc.

Electrospinning is a process that is serve and happens extremely fast therefore, it is relatable to an event observed when PLLA was cooled rapidly [48]. The PLLA didn't have enough time to form perfect crystals. Varying sizes of imperfect crystals melted at different temperatures, which are reflected as double melting points.

Finally the TGA [3.2.2.M] demonstrates that both PLLA and PG are present in the medium after 12 days. Although, there is a larger weight percentage lose in the PG (13%) as compared to the PLLA (4%).







Figure 3.2.2.M TGA of PG & PLLA electrospun and PG & PLLA in medium for 12 days.

#### 3.2.3 Fiber Morphology and Diameter

A Scanning Electron Microscope (SEM) [Appendix C] was used to analyze the scaffolds fiber diameter, morphological changes, and observe cellular adherence. All samples were mounted on a metal stub and placed in a sputter coater. The entire sample was coated with gold palladium then securing place in the SEM vacuum chamber.

The first experiment conducted analyzed changes in fiber diameters as a function of shelf life. This was tested to determine optimal stirring time. PLLA and PG solutions were allowed to dissolve in 4, 24, and 48-hour increments. In the 4-hour study, a melt was created by quickly stirring and manually breaking apart clumps. The 24-hour melt was stirred overnight and the 48-hour melt was shelved for one more night.

The 4hour melts were the most viscous melts but fibers were able to be collected and viewed under SEM [Figure 3.2.3.A]. This mixture yielded fibers on the order of 1µm - 2µm. The fiber collected from the 24-hour mixture yielded much smaller fibers, approximately 300nm [Figure3.2.3.B] The 48-hour mixture didn't yield any fibers. Therefore, the 30 weight % solutions had an approximate shelf life of 24 hours. If allowed to exceed this time fibers were usually not producible.







Next, a study was conducted to analyze the fiber diameters of all three scaffolds after they were electrospun and then submerged and incubated in medium for 14 days. The diameters were measured with an image analysis software called analySIS<sup>©</sup>. Figure 3.2.3.B displays the mean diameters for the scaffolds at Day 0, 1, 3, 7, and 14. Gelatin only has one data point because as soon as it was washed with phosphate buffer saline it dissolved. This phenomenon is discussed in a subsequent section.

All statistics in this thesis were performed using SAS<sup> $\circ$ </sup> software. A Kruskal-Wallis test revealed if differences in the mean diameter existed amongst the groups. The p value for Day 0 and Day 1 indicate there were no significant differences between the scaffolds. However, Day 3, 7, and 14 were all significantly different (p <0.001). These differences are associated with water absorption and varying degradation rates of synthetic and natural polymers. The PLLA and PG were adsorbing water at relatively the

same rate until Day 1. Then at Day 3 there was an increase in water absorption in the PG scaffold. Unpublished data in the laboratory has recently discovered the structure these polymers form when electrospun. The polymers undergo a phase separation in the fluid jet and when they solidify the PLLA is wrapped around an inner core of gelatin [49]. Therefore, once the thin sheath of PLLA degrades, and exposes the gelatin to the medium, there is an influx of water and a quicker rate of degradation.

To view morphological differences, SEM images of Day 0, 1, 3, 7, and 14 are outlined in Figures 3.2.3.C - E. Both PLLA and PG maintain their overall integrity but at Day 14 there is an obvious change in the morphology. The fibers appear to be losing their rigidity and have a slumped and "melted" look of degradation.



Figure 3.2.3.B Graph and Table display mean fiber diameters of all scaffolds and standard error.

**Results from Graph:** 

Samples	Day 0	S.E.	Day 1	S.E.	Day 3	S.E	Day 7	S.E.	Day 14	S.E.
Gelatin	214.47	3.94						1		
PLLA	294.77	9.50	618.63	9.97	914.21	16.47	905.39	15.71	1290.95	29.33
PLLA/Gelatin	305.53	5.51	702.47	22.51	1307.08	16.81	1322.44	26.49	855.18	20.32



Figure 3.2.3.C SEM images of PLLA, gelatin, and PG after electrospun.



Figure 3.2.3.D SEM images of PLLA (Day 1, 3, 7, & 14).









Figure 3.2.3.E SEM images of PG (Day 1, 3, 7, & 14).







# 3.3 Cell Culture

#### 3.3.1 hMSCs seeded on Nanofibrous scaffolds

# Scaffold:

Analyses were done with the PLLA and PG scaffolds because the Gelatin scaffold dissolved immediately in the presence of Phosphate buffer saline. The remaining scaffolds were cut into circles and placed in 96-well polypropylene plates. The polypropylene plates were used instead of polystyrene because the cells will not stick to the plate therefore, all the data collected is from the adhered cells. Then the scaffolds were sterilized under UV light overnight. Next, they were soaked in 100% ethanol for an hour. The ethanol was aspirated and each scaffold was washed twice with Phosphate

Buffered Saline. Finally, the scaffolds were presoaked overnight in media consisting of DMEM plus sodium pyruvate (Gibco, Carlsbad, CA), 10% fetal bovine serum (Atlantic Biological, Elizabeth City, NC), 1% penicillin/streptomycin (Sigma, St Louis, MO), and 1% glutamate (Gibco, Carlsbad, CA).

# Culture of MSCs:

The use of human BM aspirates followed a protocol approved by the Institutional Review Board of UMDNJ-Newark Campus. Informed consent was obtained from all subjects. Healthy volunteers ranged in age from 18 to 35. Briefly, BM aspirates (3 ml) were cultured with 3 ml DMEM (Invitrogen, Carlsbad, CA) containing 10% FCS (Hyclone, Logan, UT) on Falcon 3003 culture dishes. At Day 3, mononuclear cells were isolated using Ficoll Hypaque (Sigma, St Louis, MO) density gradient and replaced back into the culture dishes. Fifty percent of fresh media was replaced at weekly intervals until MSCs were approximately eighty percent confluence.

## Seeding:

At 80% confluence, MSCs were trypsinized and 2,000 cells were seeded onto each scaffold, placed in wells of 96-well polypropylene plates. Control cells were placed in 96-well polystyrene plates without scaffolds.

### **3.3.2 Cell Adherence**

Cell-Scaffold constructs were fixed prior to imaging by SEM. Media was aspirated and cells were fixed with 4% formaldehyde for 30 minutes. After this, the cells were dehydrated by consecutive incubation with ethanol at 10%, 20%, 40%, 60%, 80%, and 100%. The cells were dried in a sterile tissue culture hood overnight. Prior to imaging the samples were sputter coated with palladium. Figures 3.3.3.A – D displayed adhered MSCs on the PLLA and PG scaffolds. The smaller images are of scaffolds cultured without cells for comparison. At 12 hrs the MSCs appeared as a sheath of cells over the scaffolds. Over 14 days the MSCs appear to have spread or migrated.



Figure 3.3.2.A SEM images of adhered cells on PLLA (Day 1, 3, 7, & 14).





Figure 3.3.2.B SEM images of adhered cells on PG (Day1, 3, 7, & 14).









Figure 3.3.2.C Low magnification of cells on PLLA (Day 14).





#### **3.3.3 Cell Viability Assay**

Cell viability was analyzed using the Cell Titer-Blue Assay (Promega, Madison, WI). The method followed manufacturer's guidelines. Reagent is added to each well and incubated overnight. Viable cells will reduce the reagent from resazurin to resorufin, which is a detectable fluorescence. The fluorescence molecules are excited by a beam of light that causes a photon to be emitted. The intensity is measured with a fluorometer at excitation 579nm and emission 584nm [Appendix G]. Cell viability of PG, PLLA, and controls (Tissue culture plates, TCP) at Day 7 and Day 14 were measured. [Figure 3.3.3.A] Fluorescence was measured by the fluorometer from known cell numbers to determine the approximate cell count in each sample. The TCP had approximately 2,100 stem cells on Day 7 and 5,500 by Day 14. The PLLA and PG scaffolds both had approximately 3,000 stem cells on Day 7 and only 1,000 left by Day 14. There were significant differences (p < 0.0164) in viability among all samples but, there was no significant difference between the PLLA and PG scaffolds [Figure 3.3.3.B]. The viability for both PLLA and PG seeded stem cells decreased as a function of time. Perhaps more experiments will need to be conducted to analysis whether migration of stem cells into the scaffold causes the embedded fluorescence molecules to be undetectable.



Figure 3.3.3.A Viability of PLLA, PG, and TCP (Day 7 & 14).

Figure 3.3.3.B SAS output for Tukey's test comparing differences amongst groups.

The SAS System The ANOVA Procedure

Tukey's Studentized Range (HSD) Test for viability

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha 0.05 Error Degrees of Freedom 12 Error Mean Square 32516714 Critical Value of Studentized Range 3.77278 Minimum Significant Difference 8782.9

Means with the same letter are not significantly different.

Tukey Grouping	Mean	Ν	scaffold
А	28298	6	ТСР
В	18968	6	PLLA
В	18098	6	PLLA_Ge1

## 3.3.4 Cell Proliferation Assay

The incorporation of radiolabeled tritiated thymidine determined the cell proliferation rate while adhered to the scaffolds. Replication of MSCs is indicated by the incorporation of thymidine in its DNA and a release of the tritium. This  $\beta$ -energy is detected and amplified by a Liquid Scintillation Counter. [Appendix H].

Proliferation was assessed on Day 1, 3, 5, 7, 10, 12, and 14 for PLLA, PG, and TCP. Statistically, there is no difference in proliferation among the samples but there is a significant difference associated with days in culture [Figure 3.3.4.A]. Further analyses showed both scaffolds mediated rapid proliferation until Day 5, which was followed by a rapid decline. The cells in the TCP do not display this characteristic; instead proliferation is at relatively the same rate throughout the experiment [Figure 3.3.4.B].

Figure 3.3.4.A SAS output for two-way ANOVA comparing differences amongst groups.

		The SAS System	1		
	т	he GLM Procedur	`e		
Dependent Variable: p	oroliferatio	n			
Source	DF	Type III SS	Mean Square	F Value	Pr > F
scaffold <b>day</b> scaffold*day	2 6 12	106917864.8 <b>485300697.3</b> 475055375.1	53458932.4 <b>80883449.5</b> 39587947.9	1.92 2.90 1.42	0.1546 <b>0.0139</b> 0.1778



Figure 3.3.4.B Graph of proliferation over 14 days for PLLA, PLLA/Gelatin, and TCP.

# **CHAPTER 4**

## CONCLUSION

Electrospinning is a useful technique to create nanofibrous scaffolds of PLLA, gelatin, and PLLA-gelatin that have high surface areas and mimicked the extracellular environment. Its parameters can easily be adjusted create desired fibers that represent, for example, the aligned axons of the spinal cord or the connective tissue of the joints. However, there are limitations to this system. Unlike commercially manufactured fibers that are produced through high throughput devices, electrospinning is a low throughput mechanism. On average, the mat's width only increased 1mm per hour. Although sufficient for cellular infiltration, the next challenge will be to produce fibers with smaller diameters, at a faster rate, without compromising the fiber properties. Additionally, standardizing the parameters for specific biomaterials and tissue engineered applications will make the process more reproducible.

The polymers chosen are optimal candidates for tissue engineered applications because they can be altered to meet specific characteristics of varying tissues. It is evident that polymers derived from natural and synthetic sources can be combined into one material with synergic effects. The PLLA and PG scaffolds met the necessary characteristics for this study. Contrarily, the gelatin scaffold had to be eliminated. Gelatin is unstable in medium and dissolved immediately. In order to maintain a gelatin or collagen scaffold in medium it can be crosslinked [40;43;50;51]. Unfortunately, crosslinking is usually done with chemicals that have adverse cellular effects. Recent laboratory work has discovered that inside the fluid jet, gelatin and PLLA phase separate. Gelatin precipitates first because it is less soluble and then PLLA precipitates around it. There is no chemical bonding between the gelatin and PLLA however the gelatin gets wrapped in a thin sheath of PLLA [49]. Therefore, the PG scaffold will not dissolve instantly when medium is introduced like the gelatin scaffold, because the gelatin isn't initially exposed to the medium. As the thin PLLA sheath degrades gelatin will come into contact with the medium and dissolve rapidly. Due to the phenomenon associated with natural and synthetic blends, electrospinning can be an effective process to create mechanically and thermally durable scaffolds, as an alternative to crosslinking.

The interaction of MSCs and the scaffolds is a direct reflection of the characteristic *in vitro* behavior of the composites. Each composition offers separate advantages and disadvantages. The PLLA has a smaller degradation rate and maintains its overall integrity while the PG had an increased degradation rate. However, during the course of the study the fiber diameters increased for both compositions. This is most likely due to water absorption into the fibers. The PLLA absorbed the water at a continuous rate while the PG had a different reaction. Once the PLLA sheath dissolved and the gelatin was exposed, the fibers degraded quicker, and contact with water simply exasperated the degradation.

Additionally, a change in fiber diameter and morphology may affect cellular adherence, which will eventually affect cellular proliferation and viability. If the cells are not adhered they can not proliferate and viability will decrease over time. The spike in proliferation at Day 5, for both compositions, and then a rapid decline could be interpreted several ways. Most likely the scaffolds remain relatively intact for five days and then as it starts to degrade the adhered cells are released into suspension. Another possibility is the MSCs have become quiescent, which is their natural *in vivo* behavior in the bone marrow, and this causes the decreases in proliferation and viability. There could be many other factors involved with the change in MSCs behavior but more research will need to be done to determine the exact reason. It is noted that the cells on the PG displayed relatively the same behavior as the PLLA even though it was degrading faster.

Overall, the recent influx of knowledge in stem cell biology has lead to a rapid increase in stem cell based tissue engineered constructs. In particular, MSCs conceivable will have a huge impact on current clinical implications but, in order to translate developmental science into a useful therapy, other technology must be implemented. Combining stems cells within a scaffold not only provides a multidimensional *in vitro* environment, optimizing the data collected, but can be used as a delivery vehicle to implant MSCs *in vivo*. Along with controlling MSCs mobility, proliferation, and viability, the cell-scaffold composite will contain the cells in a localized area and provide a template for regeneration.

In summary, this thesis showed PLLA and gelatin are polymers that can be developed into scaffolds that can maintain viability of MSCs and possibly facilitate implantation. It showed the difference in the behavior of MSCs in a multidimensional environment as compared to standard planar culture conditions. In addition, there was a noted difference in the degradation of the PLLA and PG that can be a useful tool in tissue regeneration. The use the blended polymer solution, PG, produced additive benefits. It was comprised of a natural biocompatible material along with a durable synthetic material. Furthermore, the ratio of the blends can be adjusted and optimized for its intended application. The appropriate ratio will allow control of the integrity and degradation of the scaffolds, along with the interaction with MSCs. For example, if the scaffold required must degrade rapidly, a larger concentration of gelatin can facilitate a speedy degradation, while still being initially protected from the medium. Besides, the reduction in MSCs proliferation and viability could be more beneficial *in vivo*. Controlling and regulating these factors will allow the host to eventually take over repair of the damaged tissue while the composition degrades leaving behind regenerated tissue.

For future studies, the adhered MSCs must be assayed to determine if the scaffold is inducing differentiation of the MSCs. Since the optimal level of differentiated is yet to be determined this cell-scaffold composition may possibly be capable of retaining the "stemness" of MSCs. Other studies need to address the stimulating effects the cellscaffolds may have on peripheral blood. Histological responses to synthetic polymers are generally predictable, whereas reaction to non-synthetic materials is variable and may produce a more intense inflammatory response. Lastly, as a drug delivery device, drugs could possibly be encapsulated in a fiber similar to gelatin in PLLA. The fibers may be a useful mechanism to delivery time released factors to adhered MSCs and induce differentiate and regeneration once implanted.

## **APPENDIX** A

# POLYMERIC FIBERS AND ITS MATERIAL CHARACTERISTICS

A randomly oriented solution is electrospun to form a solid fibrous scaffold. The fibers are composed of repeating units of molecules, synthesized together most likely by covalent bonding, in a chain formation [7]. This long molecular chain consists of repeating monomers, collectively called polymers.

The single solid polymeric fiber has a preferred linear macromolecular orientation along the fiber axis. The stronger bonds form the backbone of the polymer chain while weaker bonds allow the chain to slide over itself. Some crystalline polymers are comprised of two regions, crystalline and amorphous. The ratio of these two regions will determine the material's characteristics. The crystalline region has lamella domains that are oriented in a stacked and aligned formation. This region is hard and brittle with high yield strength. The amorphous region has a non-crystalline arrangement. This region has more freedom of movement making it stretchable [Figure A.1].



Figure A.1 Model of polymer with amorphous and crystalline properties.

## **APPENDIX B**

## **ELECTROSPINNING APPARATUS**

The electrospinning apparatus [Figure B.1] consists of a fairly simple design used for creating fibers. There is an electrical source, which supplies a positive charge to a syringe filled with polymer solution. A syringe pump supplies a continuous mechanical force to the syringe. Finally, an aluminum plate is grounded and produces a negative attraction force. Once the potential difference exceeds the surface tension and viscoelastic properties of the polymer solution, a jet is ejected from the Taylor cone. The solvent evaporates within the working distance and solidified nanofibers are collected on the aluminum plate.





# **APPENDIX C**

# INSTRON MECHANICAL ANALYZER



Figure C.1 Diagram of the Instron Mechanical Analyzer applying a tension test.

The Instron Mechanical Analysis device can conduct stress and strain tests to measure the mechanical properties of a material. Stress (strength) is a measurement in the intensity, of the total internal forces, as a reaction to an externally applied force. Strain (elongation) is the deformation caused by the stress. This experiment was conducted by placing a piece of the polymer mat ends in both vertical clamps. Then the arms are activated causing vertically motion. As the polymer is being pulled, measurements of tension are recorded. Finally, material's properties are graphically displayed on a computer.

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# **APPENDIX D**

### SCANNING ELECTRON MICROSCOPE (SEM)



Figure D.1 Schematic of Scanning Electron Microscope.

The Scanning Electron Microscope [Figure C.1.] can view the topology and morphology of objects with a magnification of 20,000X and a resolution of 10nm. An electron beam is used as the source of illumination. The incident beam is created by applying a voltage to a filament that heats up and functions as a cathode. Then electrons will accelerate

towards an anode due to its positive attraction force. Next, the passing beam is condensed by a condenser lens. Afterwards, the scanning coils are energized and create a magnetic field with a cylindrical force. This will deflect the beam back and forth, scanning in a controlled manner, over a conductive or metallic coated target. Finally the objective lens serves to focus the beam into a fine point.

As the beam scans in a repetitive motion over the conductive surface of the target, the electrons don't project through the sample but instead get emitted from the surface, gathered, and viewed on a computer. The many signals generated from this interaction are Backscattered electrons, Secondary electrons, X-rays, Auger electrons, and Cathodoluminescence [Table C.1]. Although, the only signal used to generate the images where derived from Secondary electrons.

Signals	Signal formed by	Measured by	Image generated because
Backscattered Electrons	incident beam colliding with an atom in the target and is scattered backwards	variations directly dependent on the target's atomic number	higher atomic numbers elements appear brighter then lower atomic number elements
Secondary Electrons	incident beam passing near an atom in the target causing a repulsion force, if repulsion is great enough, energy will be transferred from the beam to the target, causing an ionization of the target electron, which gets pushed out of the atom, and exits at the surface	converting electron to a voltage, and amplifying	changes in intensity account for the differences in the thousands of spots that respond to the topography of the sample
X-rays	after secondary electron produced, the shell that has a vacancy can be filled by a higher electron falling into that shell	the energy emitted by the falling electron	energy detected will have a characteristic energy that is unique to the element from where it came

 Table C.1 Description of Detectable Electron Signals [52]

### **APPENDIX E**

#### DIFFERENTIAL SCANNING CALORIMETER (DSC)



Figure E.1 Sample Chamber in a Quantitative DTA Differential Scanning Calorimeter.

Differential Scanning Calorimeter was used to quantitatively analyze the thermal transitions of the polymers. The DSC provides information on the heat capacity associated with the polymer as a function of temperature and time. The temperature difference between the polymer and an inert reference material (air) are measured, while both are heated and cooled at a controlled rate.

Figure D.1 graphically displays how the sample and reference were enclosed in a single furnace connected by a low resistance heating disk. Both pans were placed on the raised platforms of the heating disk. The sample chamber is heated at a constant rate. The thermocouple, embedded in the platform portion of the heating disk, measures differences in potential between the two pans and converts it to temperature. Finally, an

inert gas is passed through the entire sample chamber, at a constant flow rate, to control oxidation.

As the pans are being heated, at the same rate, the heat capacity changes in the sample and causes a difference in its temperature relative to the reference. When there is a transition, the difference in temperature is detected and measured by the DSC, converted to heat flow by a heat transfer equation, and displayed graphically on a computer.

The detectable transitions are the glass transition, melting, and crystalline phases. The glass transition (Tg), an endothermic transition, only happens in the amorphous region of a polymer. The Tg occurs at a certain temperature unique to each polymer. Below this temperature the material is hard and brittle with little to no movement, but at the Tg the material's heat capacity and movement increase.

Melting (Tm), an endothermic transition, only happens to the crystalline region. The polymer chains will fall out of their crystal structure and become a liquid. In order for this to occur, all the chains in the entire crystalline region must be broken. At this temperature, there is a significant adsorption of heat, but the polymer's temperature will stop rising. The polymer's temperature holds steady until it gets completely melted. Then the temperature of the polymer will start to increase again but at a slower rate. This is because the molten polymer has a higher heat capacity than the solid crystalline polymer, so it can adsorb more heat with a smaller increase in temperature.

Crystallization (Tc), an exothermic transition, is a phenomenon that occurs in the amorphous region above the Tg. There is a lot of mobility in the amorphous material above Tg, but when a certain temperature is reached, enough free energy is gained to

move the molecules into an ordered arrangements, or crystals. Less heat is required to continue to raise the temperature of the polymer and this will causes a release of heat.

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### **APPENDIX F**

### THERMOGRAVIMETRIC ANALYZER (TGA)



Figure F.1 Diagram of a Thermogravimetric Analyzer.

A Thermogravimetric Analysis (TGA) [Figure E.1] measures a material's weight changes as a function of temperature. A sample is placed in an uncovered aluminum pan and then suspended from a balance. It is lowered inside a chamber filled with an inert gas, nitrogen, and sealed. Then a furnace heats the sample to extremely high temperatures. The balance continuously tracks the weight of the sample as the heat increases, and graphically displays the material's weight changes.

# **APPENDIX G**

# FLUOROMETER



Figure G.1 Schematic of Fluorometer excitation and emission of fluorescence.

A Fluorometer measures parameters of fluorescence, as intensity and wavelength distribution, of emission spectrum, after excitation by a certain spectrum of light. A beam of light supplies a photon of energy that gets passed through a filter then into a sample. The photon excites the electrons in a molecule. The molecule will aggressively vibrate and collide with other molecules. The repeated collisions will cause the excited molecule to lose vibration energy. When it reaches its lowest excited state, a photon will be emitted. This photon passes though a second filter and then to a detector. Frequencies and intensities of the fluorescent are detectable and displayed digitally.

# **APPENDIX H**

# LIQUID SCINTILLATION COUNTER (LSC)



Figure H.1 Liquid Scintillation Counter.

Liquid Scintillation Counters are instruments commonly used to detect radioisotopes that emit low energy  $\beta$ -particles. This is caused by instability due to an imbalance of protons and neutrons in the nucleus of the atom. The atom tries to rearrange the protons and neutrons by ejecting kinetic energy from the nucleus. These secondary particles are emitted during radioactive decay.

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The sample is placed in a scintillation cocktail, which causes the radioisotope to emit small flashes of light. A photomultiplier tube detects the flashes, amplifies it, and converts it to voltages that are proportional to the intensity. Then the voltages are sorted and put into energy ranges. Next, it counts the number of voltages in each energy category and prints out an exact measurement.

### REFERENCES

- H. J. Jin, J. Chen, V. Karageorgiou, G. H. Altman, and D. L. Kaplan, "Human bone marrow stromal cell responses on electrospun silk fibroin mats," *Biomaterials*, vol. 25, no. 6, pp. 1039-1047, 2004.
- [2] W. J. Li, R. Tuli, X. Huang, P. Laquerriere, and R. S. Tuan, "Multilineage differentiation of human mesenchymal stem cells in a three-dimensional nanofibrous scaffold," *Biomaterials*, vol. 26, no. 25, pp. 5158-5166, 2005.
- [3] W. J. Li, R. Tuli, C. Okafor, A. Derfoul, K. G. Danielson, D. J. Hall, and R. S. Tuan, "A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells," *Biomaterials*, vol. 26, no. 6, pp. 599-609, 2005.
- [4] W. J. Li, R. Tuli, X. Huang, P. Laquerriere, and R. S. Tuan, "Multilineage differentiation of human mesenchymal stem cells in a three-dimensional nanofibrous scaffold," *Biomaterials*, vol. 26, no. 25, pp. 5158-5166, 2005.
- [5] J. S. Wayne, C. L. McDowell, K. J. Shields, and R. S. Tuan, "In vivo response of polylactic acid-alginate scaffolds and bone marrow-derived cells for cartilage tissue engineering," *Tissue Eng.*, vol. 11, no. 5-6, pp. 953-963, 2005.
- [6] X. Xin, M. Hussain, and J. J. Mao, "Continuing differentiation of human mesenchymal stem cells and induced chondrogenic and osteogenic lineages in electrospun PLGA nanofiber scaffold," *Biomaterials*, vol. 28, no. 2, pp. 316-325, 2007.
- [7] S. Ramakrishna, T.-C. Lim, K. Fujihara, Z. Ma, and W.-E. Teo, *An Introduction to Electrospinning and Nanofibers* World Scientific, 2005.
- [8] J. S. Kim and D. H. Reneker, "Mechanical properties of composites using ultrafine electrospun fibers," *Polym Compos*, vol. 20, no. 1, pp. 124-131, 1999.
- [9] L. A. Smith and P. X. Ma, "Nano-fibrous scaffolds for tissue engineering," *Colloids and Surfaces B: Biointerfaces*, vol. 39, no. 3, pp. 125-131, 2004.
- [10] H. Fong and D. H. Reneker, "Electrospinning and the Formation of Nanofibers," in *Structure Formation in Polymeric Fibers*. D. R. Salem, Ed. Munich: Carl Hanser Verlag, pp. 225-245, 2001.
- [11] A. L. Yarin, S. Koombhongse, and D. H. Reneker, "Taylor cone and jetting from liquid droplets in electrospinning of nanofibers," *J Appl Phys*, vol. 90, no. 9, pp. 4836-4846, 2001.

- [12] A. F. Spivak, Y. A. Dzenis, and D. H. Reneker, "Model of steady state jet in the electrospinning process," *Mech Res Commun*, vol. 27, no. 1, pp. 37-42, 2000.
- [13] T. Han, A. L. Yarin, and D. H. Reneker, "Viscoelastic electrospun jets: Initial stresses and elongational rheometry."
- [14] A. L. Yarin, S. Koombhongse, and D. H. Reneker, "Bending instability in electrospinning of nanofibers," *J Appl Phys*, vol. 89, no. 5, pp. 3018-3026, 2001.
- [15] D. H. Reneker, A. L. Yarin, H. Fong, and S. Koombhongse, "Bending instability of electrically charged liquid jets of polymer solutions in electrospinning," *J Appl Phys*, vol. 87, no. 9 I, pp. 4531-4547, 2000.
- [16] C. J. Thompson, G. G. Chase, A. L. Yarin, and D. H. Reneker, "Effects of parameters on nanofiber diameter determined from electrospinning model," *Polymer*, vol. 48, no. 23, pp. 6913-6922, 2007.
- [17] C. Huang, S. Chen, C. Lai, D. H. Reneker, H. Qiu, Y. Ye, and H. Hou, "Electrospun polymer nanofibres with small diameters," *Nanotechnology*, vol. 17, no. 6, pp. 1558-1563, 2006.
- [18] K. Ohkawa, D. Cha, H. Kim, A. Nishida, and H. Yamamoto, "Electrospinning of Chitosan," *Macromolecular Rapid Communications*, vol. 25, pp. 1600-1605, July 2004.
- [19] F. Chen, C. N. Lee, and S. H. Teoh, "Nanofibrous modification on ultra-thin poly(ecaprolactone) membrane via electrospinning," *Materials Science and Engineering C*, vol. 27, no. 2, pp. 325-332, 2007.
- [20] T. B. Bini, S. Gao, S. Wang, and S. Ramakrishna, "Poly(1-lactide-co-glycolide) biodegradable microfibers and electrospun nanofibers for nerve tissue engineering: An in vitro study," *J Mater Sci*, vol. 41, no. 19, pp. 6453-6459, 2006.
- [21] S. Zarkoob, R. K. Eby, D. H. Reneker, S. D. Hudson, D. Ertley, and W. W. Adams, "Structure and morphology of electrospun silk nanofibers," *Polymer*, vol. 45, no. 11, pp. 3973-3977, 2004.
- [22] Y. R. V. Shih, C. N. Chen, S. W. Tsai, J. W. Yng, and O. K. Lee, "Growth of mesenchymal stem cells on electrospun type I collagen nanofibers," *Stem Cells*, vol. 24, no. 11, pp. 2391-2397, 2006.
- [23] X. Fang and D. H. Reneker, "DNA fibers by electrospinning," J Macromol Sci Phys, vol. 36, no. 2, pp. 169-173, 1997.
- [24] H. Jiang, D. Fang, B. S. Hsiao, B. Chu, and W. Chen, "Optimization and characterization of dextran membranes prepared by electrospinning," *Biomacromolecules*, vol. 5, no. 2, pp. 326-333, 2004.

- [25] Y. S. Moon, H. Uyama, S. Inoue, and Y. Tabata, "Nanofibrous scaffold of poly (Llactic acid) with gelatin for bone regeneration," ed 55, p. 2032, 2006.
- [26] J. Lee, G. Tae, Y. H. Kim, I. S. Park, S. H. Kim, and S. H. Kim, "The effect of gelatin incorporation into electrospun poly(l-lactide-co-?-caprolactone) fibers on mechanical properties and cytocompatibility," *Biomaterials*, vol. 29, no. 12, pp. 1872-1879, 2008.
- [27] M. Li, M. J. Mondrinos, X. Chen, M. R. Gandhi, F. K. Ko, and P. I. Lelkes, "Coelectrospun poly(lactide-co-glycolide), gelatin, and elastin blends for tissue engineering scaffolds," *J. Biomed. Mater. Res. Part A*, vol. 79, no. 4, pp. 963-973, 2006.
- [28] Y. S. Moon, H. Uyama, S. Inoue, and Y. Tabata, "Fabrication of non-woven mats of gelatin/poly(L-lactic acid) composites by electrospinning and their application for scaffold of cell proliferation," *Chem. Lett.*, vol. 35, no. 6, pp. 564-565, 2006.
- [29] M. M. Xu, F. Mei, D. Li, X. P. Yang, G. Sui, X. L. Deng, and X. Y. Hu, "Electrospun poly(I-lactic acid)/nano-hydroxyapatite hybrid nanofibers and their potential in dental tissue engineering," *Key Eng Mat*, vol. 330-332 I, pp. 377-380, 2007.
- [30] Y. Zhang, H. Ouyang, T. L., Chwee, S. Ramakrishna, and Z. M. Huang, "Electrospinning of gelatin fibers and gelatin/PCL composite fibrous scaffolds," J. Biomed. Mater. Res. Part B Appl. Biomater., vol. 72, no. 1, pp. 156-165, 2005.
- [31] C. C. Chen, J. Y. Chueh, H. Tseng, H. M. Huang, and S. Y. Lee, "Preparation and characterization of biodegradable PLA polymeric blends," *Biomaterials*, vol. 24, no. 7, pp. 1167-1173, 2003.
- [32] C. M. Bunger, N. Grabow, K. Sternberg, L. Ketner, C. ger, B. Lorenzen, K. Hauenstein, K. P. Schmitz, H. J. Kreutzer, D. Lootz, H. Ince, C. A. Nienaber, E. Klar, and W. Schareck, "Iliac anastomotic stenting with a biodegradable poly-Llactide stent: A preliminary study after 1 and 6 weeks," *J. Endovasc. Ther.*, vol. 13, no. 4, pp. 539-548, 2006.
- [33] A. Viinikainen, H. Ransson, K. Huovinen, M. Ki, P. Tormala and Rokkanen, "Material and knot properties of braided polyester (Ticron) and bioabsorbable poly-L/D-lactide (PLDLA) 96/4 sutures," J. Mater. Sci. Mater. Med., vol. 17, no. 2, pp. 169-177, 2006.
- [34] N. Chegini, D. L. Hay, J. A. Von Fraunhofer, and B. J. Masterson, "A comparative scanning electron microscopic study on degradation of absorbable ligating clips in vivo and in vitro," *J. Biomed. Mater. Res.*, vol. 22, no. 1, pp. 71-79, 1988.

- [35] G. Schmidmaier, M. Lucke, P. Schwabe, M. Raschke, N. P. Haas, and B. Wildemann, "Collective review: Bioactive implants coated with poly(D,L-lactide) and growth factors IGF-I, TGF-?1, or BMP-2 for stimulation of fracture healing," J. Long-Term Eff. Med. Implants, vol. 16, no. 1, pp. 61-69, 2006.
- [36] K. Jukkala-Partio, O. Laitinen, J. Vasenius, E. K. Partio, T. Toivonen, P. Tervahartiala, J. Kinnunen, and P. Rokkanen, "Healing of subcapital femoral osteotomies fixed with self-reinforced poly-L-lactide screws: An experimental long-term study in sheep," *Arch. Orthop. Trauma Surg.*, vol. 122, no. 6, pp. 360-364, 2002.
- [37] S. Gogolewski, M. Jovanovic, S. M. Perren, J. G. Dillon, and M. K. Hughes, "Tissue response and in vivo degradation of selected polyhydroxyacids: Polylactides (PLA), poly(3-hydroxybutyrate) (PHB), and poly(3- hydroxybutyrateco-3-hydroxyvalerate) (PHB/VA)," J. Biomed. Mater. Res., vol. 27, no. 9, pp. 1135-1148, 1993.
- [38] E. J. Chong, T. T. Phan, I. J. Lim, Y. Z. Zhang, B. H. Bay, S. Ramakrishna, and C. T. Lim, "Evaluation of electrospun PCL/gelatin nanofibrous scaffold for wound healing and layered dermal reconstitution," *Acta Biomater.*, vol. 3, no. 3 SPEC. ISS., pp. 321-330, 2007.
- [39] Y. Zhang, H. Ouyang, T. L. Chwee, S. Ramakrishna, and Z. M. Huang, "Electrospinning of gelatin fibers and gelatin/PCL composite fibrous scaffolds," *Journal of Biomedical Materials Research - Part B Applied Biomaterials*, vol. 72, no. 1, pp. 156-165, 2005.
- [40] Y. Z. Zhang, J. Venugopal, Z. M. Huang, C. T. Lim, and S. Ramakrishna, "Crosslinking of the electrospun gelatin nanofibers," *Polymer*, vol. 47, no. 8, pp. 2911-2917, 2006.
- [41] M. Nagata, F. Okano, W. Sakai, and N. Tsutsumi, "Separation and enzymatic degradation of blend films of poly(L-lactic acid) and cellulose," *J Polym Sci Part A*, vol. 36, no. 11, pp. 1861-1864, 1998.
- [42] K. F. Eidman and P. J. Nichols, "Trifluoroacetic Acid," in *Encyclopedia of Reagents for Organic Synthesis*. Paquette L., Ed. New York, NY: J. Wiley & Sons, 2004.
- [43] B. M. Abdallah and M. Kassem, "Human mesenchymal stem cells: From basic biology to clinical applications," *Gene Ther.*, vol. 15, no. 2, pp. 109-116, 2008.
- [44] K. A. Cho, K. A. Trzaska, S. J. Greco, J. McArdle, F. S. Wang, J. H. Ye, and P. Rameshwar, "Neurons derived from human mesenchymal stem cells show synaptic transmission and can be induced to produce the neurotransmitter substance P by interleukin-1a," *Stem Cells*, vol. 23, no. 3, pp. 383-391, 2005.

- [45] S. J. Greco, C. Zhou, J. H. Ye, and P. Rameshwar, "An interdisciplinary approach and characterization of neuronal cells transdifferentiated from human mesenchymal stem cells," *Stem Cells and Development*, vol. 16, no. 5, pp. 811-826, 2007.
- [46] P. Bianco, M. Riminucci, S. Gronthos, and G. Robey, "Bone marrow stromal stem cells: nature, biology, and potential applications," *Stem Cells*, no. 19, pp. 180-192, 2001.
- [47] M.-F. Cote, G. Laroche, E. Gagnon, P. Chevallier, and C. J. Doillon, "Denatured collagen as support for a FGF-2 delivery system: Physicochemical characterizations and in vitro release kinetics and bioactivity," *Biomaterials*, vol. 25, no. 17, pp. 3761-3772, 2004.
- [48] M. Yasuniwa, S. Tsubakihara, Y. Sugimoto, and C. Nakafuku, "Thermal Analysis of the Double-Melting Behavior of Poly(L-lactide acid)," *Journal of Polymer Science, Part B: Polymer Physics*, vol. 44, no. 1, pp. 25-32, July 2003.
- [49] A. Banerjee and G. Collins, "Thermal and Morphological Analysis of Collagen-PLLA Electrospun blends," 2008.
- [50] S. Y. Chew, R. Mi, A. Hoke, and K. W. Leong, "Aligned protein-polymer composite fibers enhance nerve regeneration: A potential tissue-engineering platform," *Adv. Funct. Mater.*, vol. 17, no. 8, pp. 1288-1296, 2007.
- [51] Y. J. Kim and O. H. Kwon, "Crosslinked gelatin nanofibers and their potential for tissue engineering," *Key Eng Mat*, vol. 342-343, pp. 169-172, 2007.
- [52] J. Temple Black, "The Scanning Electron Microscope: Operating Principles," *Principles and techniques of Scanning Electron Microscopy*. I. Litten Educational Publishing, Ed. New York: Van Nostrand Reinhold Co., pp. 1-42, 1974.