Abstract

Poly(ethylene Oxide)/β-lactoglobulin Electrospun Nanofibers: Chemical Crossliking Assessment and Thymosin-β4 Functionalization

By

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Introduction: Using electrospun nanofiber scaffolds have emerged as a technique for tissue engineering (TE) applications. In 2011, Sullivan *et al.* reported on the process to effectively electrospin and crosslink nanofibers from poly(ethylene oxide) (PEO) and β-lactoglobulin (BLG) aqueous solutions. PEO and BLG are both biodegradable and biocompatible materials. Crosslinking PEO/BLG nanofibers is necessary to improve their aqueous stability for TE applications. However, the heat treatment process suggested by Sullivan *et al.* is time intensive. The purpose of this study was to a) investigate an alternative crosslinking method for electrospun nanofibers made from an aqueous protein solution b) assess the resulting nanofibers for their potential use as scaffolds for TE applications, and c) evaluate the effect of biologically treated nanofiber scaffolds on stem cell proliferation. Chemical crosslinking techniques using Sodium Trimetaphosphate (STMP) combined with sodium hydroxide (NaOH) were evaluated. STMP has been shown to effectively crosslink polysaccharide nanofibers *in situ* during electrospinning. **Methods:** STMP, at various concentrations, was added to PEO/BLG electrospinning solutions. The effects of STMP were characterized by measuring the solution's viscosity, pH and conductivity. Confocal laser scanning microscopy (CLSM) images were acquired to

qualitatively assess electrospun nanofiber morphology and scaffold topography. Human mesenchymal stem cells (hMSC) were grown on PEO/BLG scaffolds under control conditions and when treated with the protein Thymosin-β4 (Tβ4). HMSC proliferation was assessed to evaluate the effects of PEO/BLG nanofiber scaffolds and different Tβ4 treatments at day 2, 4 and 8. **Results:** Using STMP to chemically crosslink PEO/BLG electrospun scaffolds affected solution properties, nanofiber morphology and scaffold topography. PEO/BLG/STMP nanofibers were highly beaded and wavy with little structure relative to PEO/BLG nanofibers. Fibers were not stable in an aqueous solution. Using Tβ4 to treat the PEO/BLG nanofiber scaffolds and/or cell culture media improved hMSC proliferation with increased time in culture. HMSCs remained viable throughout the growth period for all treatments. However, hMSCs did not integrate into PEO/BLG nanofiber scaffolds, but attached to the scaffold surface. **Conclusion:** Using STMP, at the tested concentrations, as an alternative crosslinker for PEO/BLG nanofibers was ineffective and did not result in usable electrospun scaffolds. Chemically crosslinking PEO/BLG nanofibers requires further research in polymer chemistry to identify an alternative *in situ* crosslinking mechanism. Treating the scaffolds and/or media with Tβ4 did result in improved hMSC proliferation. However, while hMSC cultures remained viable and proliferation increased with Tβ4 treatments, further research is necessary to develop protocols that will enable hMSC integration with PEO/BLG nanofiber scaffolds.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA: Analysis of variance

BLG: β-lactoglobulin

CLSM: Confocal laser scanning microscopy

ECM: Extracellular matrix

hMSCs: Human mesenchymal stem cells

NaOH: Sodium hydroxide

PEO: Poly(ethylene oxide)

STMP: Sodium trimetaphosphate

Tβ4: Thymosin-β4

TE: Tissue Engineering

WPI: Whey protein isolate

CHAPTER 1 – Background, Motivation and Goals

1.1. Background

1.1.1. Tissue Engineering

Tissue engineering (TE), a subspecialty of regenerative medicine, is the interdisciplinary field involving knowledge from medicine, biology, engineering and materials science that includes the *in vitro* and *in vivo* development of tissue (Agawal, Wendorff, & Greiner, 2008; Lodono & Badylak, 2015). Due to trauma, organ/tissue failure and congenital defects, and conventional methods to treat and repair tissue defects, TE efforts aim to provide an innovative and promising alternative to treat these deleterious conditions by generating complex tissue and organs in an *in vitro* approach. This approach decreases the need for organ donors. Its novelty arises from utilizing the smallest components of tissue to engineer complex tissue, stem cells and the extracellular matrix (ECM). An approach to develop tissue *in vitro* is to use scaffolds to provide a temporary support where stem cells can be seeded, allowing them to grow and proliferate. Scaffolds are designed to biomimic the ECM by using biocompatible and biodegradable materials. In the process the scaffolds biodegrade and are replaced by ECM produced by stem cells, subsequently forming differentiated and specified tissue. The following sections provide an overview of the natural ECM, and relevance of biodegradable scaffolds to TE.

1.1.2. The Extracellular Matrix

Natural extracellular matrix is a 3-dimensional mesh-network that surrounds cells, providing anchorage and separation between tissues (Agawal, Wendorff, & Greiner, 2008). ECM is composed of various structural and functional proteins, such as collagen, elastin, proteoglycan, fibronectin and laminin, secreted and maintained by resident cells (Stevens & George, 2005).

ECM diversification results from different combinations and interactions of these proteins. Due to evolutionary changes in vertebrates, tissues vary in anatomical complexity as does their native ECM architecture. The ECM has been well characterized for its role in providing structural support for tissues. A 2009 review (Agarwal, Wendorff, & Greiner, 2009) presented research evidence supporting the biological importance of the ECM's protein and mechanical characteristics. Researchers now acknowledge ECM's paramount role in multiple cellular functions that provide chemical and mechanical cues to maintain cellular homeostasis, optimize tissue and organ function, and modify the microenvironment to mediate wound healing and tissue repair (Hynes, 2009; Lodono & Badylak, 2015).

Advancements in health care, medicine, organ-transport modalities and surgery has led to improvements for organ transplantation outcomes. According to the Organ Procurement and Transplant Network, organ transplants have increased 2.2 fold from 1988 to 2012. However, issues stem from the inequality in organ donor donations and number of people on the organ donor waiting list. From 1988 to 2012, organ donors increased by 2.3-fold while the number of patients on the waiting list increased by 7.8 -fold (Hunsberger, Neubert, Wertheim, Allickson, & Atala, 2016). The long term goals of TE are to develop techniques and modalities to diminish the use of donor tissue. Thus, understanding the ECM's biological and structural properties at the micro-level is essential and well established. Research has shown engineered biodegradable scaffolds to have a promising future for developing tissue *in vitro* as an alternative for donor organ/tissue outsourcing (Agarwal, Wendorff, & Greiner, 2009; Chiu & Radisic, 2011; Lodono & Badylak, 2015).

1.1.3. Scaffolds in Tissue Engineering

Biological scaffold architecture plays a critical role in TE, as it must mimic the native ECM's physical dimensions and physiological conditions. Biological scaffolds with nanofiber architecture are ideal for TE applications as they provide larger surface area for absorbing proteins and present more binding sites to cell menbrane receptors compared to micropore and microfiber scaffolds (Agarwal, Wendorff, & Greiner, 2009). Biological scaffolds also provide high porosity for diffusing nutrients, growth factors and other biological elements. These features allow for enhanced ECM biomicry when using nanofiber scaffolds compared to their counterparts. An additional feature intrinsic to some biological scaffold modalities is that porosity can be controlled and modified by making simple modification to the scaffold generating process. Controlling scaffold porosity allows for the optimization of diffusion and cell-to-cell communication for specific cell types.

Material selection is another fundamental factor when creating biological scaffolds for TE. Materials must be biocompatible to prevent an immunoresponse, nontoxic to enhance cell viability, and biodegradible to allow cells to form and replace the scaffolds with native ECM. Furthermore, biodegradation is an important aspect to cell viability, as degradation byproducts must also be nontoxic. As cells grow and proliferate, scaffold materials must biodegrade at a rate similar to that of native ECM formation to further promote cell viability. TE applications using nanofiber scaffolds represent a nontrivial process requiring a fine balance between scaffold biodegradation and ECM formation to promote tissue formation, *i.e.*, as a scaffold biodegrades, it is infiltrated by host cells and eventually replaced with native ECM leading to functional and site-appropriate tissue (Lodono & Badylak, 2015).

1.1.4. Electrospinning

While there are multiple methods to create nanofiber scaffolds, such as template synthesis, phase separation and self-assembly, and electrospinning (Kriegel, Arrechi, Kit, McClements, & Weiss, 2008), electrospinning is one of the most efficient, simple and versatile methods due to its relatively simple approach and cost-effective setup. Electrospun nanofibers are produced by applying a strong electric field between two electrodes in which a polymer solution is continuously pumped through a capillary or a needle (or spinneret) (Figure 1.1B). At the tip of the spinneret, the electrospinning solution forms a jet that travels to a collecting surface (an opposite or grounded electrode) due to electrostatic repulsive forces. The spinning jet is exposed to air and dries as it travels to the collecting surface. The nanofibers randomly accumulate on the collecting surface giving the scaffold its porous architecture. Figure 1.1A provides an illustration of a basic electrospinning setup. Nanofibers are characterized by their average fiber diameter. Factors such as solution properties (viscosity and conductivity), distance between electrodes (tip-to-collector distance), flow rate, electrospinning voltage, relative humidity (partial pressure for solutions made with organic solvents) and temperature play a critical role in producing defect-free nanofibers.

Due to its simplicity, versatility and cost effective set-up, electrospinning in TE applications has gained popularity in the recent decades. The number of scientific publications describing the use of this technique has increased yearly. Electrospinning for TE applications currently emphasizes two applications: i) formation of nonwoven mats of different biomaterials to mimic physical dimensions of native EMCs, that is, geometry and morphology with nanodimensions, and ii) modification of the electrospinning process or electrospun fibers for achieving enhanced biological performance (Agarwal, Wendorff, & Greiner, 2009).

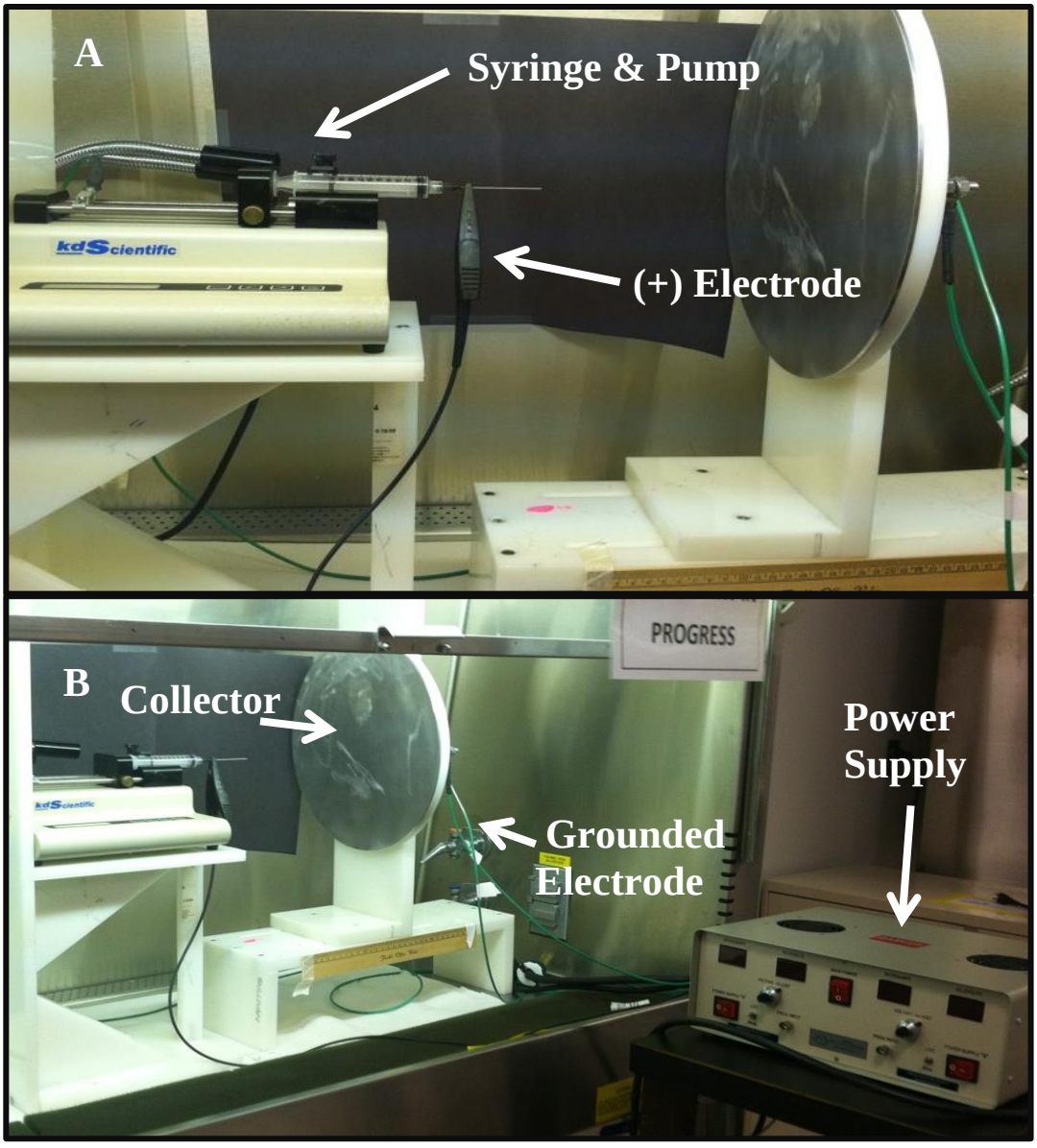

Figure 1.1: Electrospinning equipment at East Carolina University. (A) An electric field is established in the space between the spinneret (positive electrode) and collector plate (grounded) during electrospinning. (B) The circuit is completed when connecting the electrodes to a power supply and applying a voltage. The charges in the solution are repelled from the positive electrode towards the grounded collector plate initiating nanofiber formation.

1.1.5. Materials in Electrospinning

Choosing scaffold materials for TE, depends on the nature of the tissue to be regenerated and the regeneration time (Agarwal, Wendorff, & Greiner, 2009). Thus, a variety of biodegradable synthetic and natural polymers have been studied, as well as combinations of both. Synthetic polymers have been extensively studied and one particular review article reported nearly 50 synthetic polymers have been successfully electrospun with numerous applications (Huang, Zhang, Kotaki, & Ramakrishna, 2003). Electrospun synthetic nanofiber research has been applied to military protective clothing for minimizing air impedance and anti-biochemical gases, thermal and biochemical nanosensors, cosmetic skin masks for skin cleansing and skin healing, life sciences for drug delivery carriers and wound dressings, filter media for gas filtration and molecule filtration, TE scaffolding for porous membrane for skin and 3D scaffolds for bone and cartilage regeneration (Huang, Zhang, Kotaki, & Ramakrishna, 2003). Synthetic polymers such as poly(caprolactone) (PCL), poly(lactide) (PLA), poly(ethylene oxide) (PEO), polyglycolic acid (PGA), and others, have been categorized as biodegradable and identified for TE applications (Huang, Zhang, Kotaki, & Ramakrishna, 2003; Subbiah, Bhat, Tock, Parameswaran, & Ramkumar, 2005). Research suggests that electrospinning biodegradable and biocompatible synthetic polymers is feasible and the ease of availability, understanding of their chemical and mechanical properties, and ease of spinnability makes synthetic polymers suitable candidates for electrospinning.

Electrospinning methods have been used to generate scaffolds for TE using different natural biopolymers including proteins and polysaccharides (Agawal, Wendorff, & Greiner, 2008). Using natural biopolymers can provide instructive cues required for cell attachment and proliferation, thus having a physiologic advantage over biocompatible and biodegradable

synthetic polymers (Agarwal, Wendorff, & Greiner, 2009). However, natural biopolymers display poor electrospinning processability due to lack of entanglement and high surface tension. They often require modifications for better electrospinnability (Jeong, et al., 2011). In addition, natural polymers must be derived and/or isolated from their original source, thus making them scarce (Li, He, Zheng, & Han, 2006). Therefore, a combination of both synthetic and natural polymers have been investigated. Mixtures of both polymer groups result in composite nanofibers that are more suitable for TE scaffolds. These mixtures combine the advantage of property modification of synthetic polymers and the biofunctionality of natural polymers (Agawal, Wendorff, & Greiner, 2008).

1.2. Motivation and Goals

The purpose of this study was to a) investigate an alternative crosslinking method for electrospun nanofibers made from an aqueous protein solution b) assess the resulting nanofibers for their potential use as scaffolds for TE applications, and c) evaluate the effect of biologically treated nanofiber scaffolds on stem cell proliferation. Electrospinning nanofibers from natural materials (biopolymers) can be difficult, and successful defect-free nanofiber generation is limited to certain biopolymers, which may require chemical modification (Ji, et al., 2006). Research has shown combinations of natural polymers and synthetic polymers to improve electrospinning outcomes and nanofiber generation without the need for chemical modification. This prospect suggests a promising future for natural/synthetic electrospun nanofibers.

Whey proteins are naturally occurring and edible, regarded as safe by the US FDA, and used as food and material modifiers, and nutritional supplements (Sullivan S. T., 2011). In addition to their nutritional value as a protein source, studies have investigated the biological activity of whey proteins. Whey proteins possess prophylactic properties and may offer specific health

benefits such as antibacterial, antiviral, anticarcinogenic effects, and synthesis of antioxidant and other bioactive peptides (Chatterton, Smithers, Roupas, & Brodkorb, 2006; Madureira, Pereira, Gomes, Pintado, & Xavier Malcata, 2007; Hernández-ledesma, Recio, & Amigo, 2008). As a result, whey proteins have gained research attention outside the food industry and nutritional field for pharmaceutical and biomedical applications.

β-lactoglobulin (BLG) is one of the principle components of whey proteins found in the milk of ruminant species and some non-ruminant species such as pigs, horses, dolphins and cats (Hernández-ledesma, Recio, & Amigo, 2008). A study investigated solution electrospinning of whey protein isolate (WPI) and BLG using Poly(ethylene oxide) (PEO), a water-soluble synthetic polymer, as a carrier polymer to enhance spinnability. Different PEO\WPI and PEO/BLG blends were electrospun and evaluated. The study reported fibers with the highest WPI concentration were less uniform in diameter, had a higher mean diameter and wider size distribution, and minor fiber breakage present upon characterization (Sullivan, Tang, Kennedy, & Tawlar, 2014). Nanofibers with the highest BLG concentration did not have these characteristics when compared to their WPI counterparts. In addition, PEO/BLG blends produced fibers with a smaller mean diameter and standard deviation compared to PEO/WPI blends of the same proportion. The researchers also investigated heat treatment for improving nanofiber insolubility. PEO and PEO/BLG nanofibers were heat treated for 24 to 44 hours at 100 ^{o}C. Nanofiber morphology was retained for several days after being immersed in water. This research suggested covalent crosslinking occurred during heat treatment. This result was expected since the treatment temperature was above the gelation temperature of whey protein and the melting point of PEO. Sullivan *et al.* were the first to report on electrospun WPI and BLG nanofibers.

This project using BLG to create electrospun nanofibers originated from the previous work described by Sullivan *et at.* Published research showed PEO/BLG blends produced less variant nanofibers than PEO/WPI blends, and their water insolubility and stability to be improved by heat treatment. Other studies have identified whey proteins to have prophylactic effects that may offer specific health benefits, including antibacterial, antiviral and anticarcinogenic effects (Hernández-ledesma, Recio, & Amigo, 2008). These three attributes formed the basis of this study to determine if PEO/BLG electrospun nanofibers could serve as a TE scaffold and enhance stem cell viability.

The first goal of this study was to investigate an alternative to crosslinking via heat treatment. Based on the methods proposed by Sullivan *et al.*, the time required to produce and crosslink nanofiber scaffolds was determined to be 7 days: 1 day for solution preparation, 2 days for electrospinning and 4 days for crosslinking (unpublished data from Richard Steiner 2014, presented during ECU's Research and Creative Activity Week). Heat treatment accounts for over 50% of the time required to produce nanofiber scaffold. To expedite scaffold generation, *in situ* crosslinking by sodium trimetaphosphate (STMP) was investigated. STMP has been shown to effectively crosslink hyaluronan (Dulong, et al., 2004), xanthan (Bejenariu, Popa, Dulong, Picton, & Le Cerf, 2009) and pullulan hydrogels (Lack, et al., 2004) and pullulan/dextran nanofibers (Shi, Le Visage, & Chew, 2011; Jiang, et al., 2015). Studies using PEO/BLG nanofibers crosslinked with STMP, or other whey proteins, have not been reported. The goal was to establish a critical STMP concentration and electrospinning parameters to produce defect-free and crosslinked PEO/BLG nanofibers. STMP concentrations reported by Shi *et al.* ranged from 4-16 w/v %. STMP requires sodium hydroxide (NaOH) to provide alkaline conditions to activate crosslinking. Two studies using pullulan/dextran nanofibers reported successful

crosslinking with 10 w/v % NaOH aqueous solution at a volume ratio of 1:10 (NaOH:polymer solution) (Shi, Le Visage, & Chew, 2011; Jiang, et al., 2015). The addition of STMP at various concentrations manifests changes in solution viscosity. Solution viscosity plays a major role in electrospinning and has a substantial influence on other electrospinning parameters such as electric field magnitude, solution flow rate and tip-to-collector distance.

The second goal of this study was to determine the effect of PEO/BLG nanofiber scaffolds on stem cell proliferation. This included treating PEO/BLG nanofiber scaffolds with thymosin-β4 (Tβ4). Tβ4 has previously been shown to promote stem cell proliferation and wound healing (Byrum, 2008).

Adult tissues contain stem cell populations capable of regenerating tissue after trauma, disease or aging by differentiating into tissue specific cells. Human mesenchymal stem cells (hMSCs) are multipotent cells that can proliferate in their undifferentiated state, and have the potential to differentiate into multiple cells lines (Pittenger, et al., 1999). HMSCs are autologous cells derived from a donor's or patient's bone-marrow. Using autologous hMSCs for therapy diminishes the likelihood of eliciting an immunoresponse and/or rejection if used in TE applications. These attributes make hMSCs, an attractive candidate for TE research with a promising future for developing clinical applications.

Tβ4 is a polypeptide that participates in various cellular functions, such as migration, adhesion, differentiation, angiogenesis and wound healing (Crockford, Turjman, Allan , & Angel, 2010; Kim & Jung, 2015). Considering Tβ4's physiological properties and its role in cellular functions, studies promoting cell migration, angiogenesis and cardiac wound healing have investigated and demonstrated the efficacy of using Tβ4 as a functionalizing agent for chitosan-collagen hydrogel constructs and poly(ε-caprolactone) electrospun nanofiber scaffolds,

respectively (Chiu & Radisic, 2011; Chiu & Radisic, 2011). To the best of our knowledge, PEO/BLG nanofiber scaffolds for TE have not been studied with or without Tβ4 treatment.

CHAPTER 2 - Materials and Methods

Materials

2.1. Poly(ethylene oxide)

Poly(ethylene oxide) (PEO) is a semicrystalline and biodegradable thermoplastic polymer. PEO is soluble in water and polar organic solvents, and thus, it is a polymer whose solution properties have been extensively studied (Ho, Hammouda, Kline, & Chen, 2006). Due to its biocompatibility and low toxicity, PEO has gained attention for its use in biomedical and food applications (Colín-Orozco, Zapata-Torres, Rodríguez-Gattorno, & Pedroza-Islas, 2015).

A PEO/water system has been shown to be a simple model for studying fundamental biomolecular interactions in which hydrogen-bonding and hydrophobic interactions play important roles, such as in protein folding and stabilizations (Ho, Hammouda, Kline, & Chen, 2006). Due to the similarity of the basic interactions involved in PEO/water and protein/water systems (hydrogen bonding and electrostatic interactions), PEO has been studied and used as a visco-modifier to enhance the electrospinnability of biopolymers. Solutions combining PEO with egg albumin (Wongsasulak, Kit, McClements, Yoovidhya, & Weiss, 2007), sodium alginate (Lu, Zhu, Guo, Hu, & Yu, 2006), soy protein isolate (Ramji & Shah, 2014), keratin (Aluigi, et al., 2008), chitosan/alginate (Jeong, et al., 2011) and WPI have been reported to produce electrospun nanofibers (Sullivan, Tang, Kennedy, & Tawlar, 2014).

2.2. β-lactoglobulin

β-lactoglobulin (BLG) is the major whey protein in milk, generally accounting for approximately 50% of the total protein in ruminant milk, and 10% of the total protein in bovine milk (Chatterton, Smithers, Roupas, & Brodkorb, 2006). BLG is a small protein, soluble in dilute salt solutions, with 162 amino acid residues that fold up into 8-stranded, perpendicular β-

barrel with a 3-turn α-helix on the outer surface and a ninth β-strand flanking the first strand (Kontopidis, Holt, & Sawyer, 2004). Its primary sequence reveals two intrachain disulphide bridges (Cys66-Cys160 and Cys109-Cys119) and a free thiol group at Cys121 (Hernández-ledesma, Recio, & Amigo, 2008; Creamer, Parry, & Malcolm, 1983). The reactive thiol has a pH dependent activity. At pH > 6.7, the thiol is susceptible to chemical modification favoring the binding of positively charged reagents to the sulfur atom in Cys121 (Qin, et al., 1998), and thus a potential site for chemical crosslinking.

2.3. Sodium Trimetaphosphate

Sodium trimetaphosphate (STMP), $(NaPO_3)_3$, is a water soluble, crystalline cyclic polyphosphate inorganic salt with a molecular weight of 305.92 Da (Lanigan, 2001). STMP is accepted by the Food and Drug administration and has been used to prepare food-grade phosphorylated starches (Food additives permitted in food for human consumption, 1995). STMP is safe and non-toxic, and has been used to crosslink polysaccharides under alkaline conditions (Dulong, et al., 2004). STMP is used in cosmetics as a buffering agent, chelating agent and pH modifier (Lanigan, 2001).

2.4. Thymosin-β4

Thymosin-β4 (Tβ4) is a major G-actin sequestering protein in all eukaryotic cells and is a potent regulator of actin polymerization in mammals (Kim & Jung, 2015), essential for extracellular matrix assembly and reorganization. This 43-amino acid chain that was first isolated from bovine thymus tissue and has been found ubiquitously in the body (Crockford, Turjman, Allan , & Angel, 2010). Research has shown that Tβ4 plays a major role in different cellular functions including wound healing, angiogenesis, migration, proliferation and suppressing inflammatory response (Ti, et al., 2015).

While studies have demonstrated these cellular functions in *in vivo* models to treat cutaneous wounds and various types of ischemia, these models often use direct injection to deliver treatment which often result in a decrease in efficacy due to inability to control Tβ4 release and concentrations over time. Hence, new models are required to further investigate the effects of prolonged Tβ4 treatment. Current research efforts, specifically in tissue engineering (TE), have approached the issue of *in vitro* applications by functionalizing TE scaffolds. These new *in vitro* models allow for a vehicle where Tβ4 concentration and release are controlled over extended periods of time in laboratory settings. Further understanding the prolonged effects of Tβ4 exposure to cell cultures is necessary for understanding the full effects Tβ4 in physiological systems. This may lead to developing Tβ4 treatments in clinical settings.

Methods

2.5. Solution Preparation

BIOPURE β-lactoglobulin (BLG) was obtained from Davisco Foods Inc. (Eden Prairie, MN). Poly(ethylene oxide) (PEO, MW 600 kDa) and sodium hydroxide (NaOH, MW 40 Da) were obtained from Sigma-Aldrich Corporation (St. Louis, MO). Sodium trimetaphosphate (STMP, MW 305.89 Da) was obtained from Alfa Aesar® (Ward Hill, MA). All materials were used as received. To prepare control solutions, PEO and BLG powder were added in deionized sterile water and mixed for one hour at room temperature (27º C) to make 8 w/v % PEO and 12 w/v % BLG solutions (See Appendix A). The solutions were combined at a 1:1 volume ratio and stirred overnight.

Solutions with STMP were prepared in the same manner as the standard solution; however, 10 w/v % aqueous NaOH was added to 12 w/v% BLG solution at a 1:20 volume ratio prior to combining PEO and BLG solutions. STMP was added to the PEO/BLG solution at 2, 4, 6 and 8

w/v % concentrations. Rhodamine-B and additional deionized sterile water was added to the solution at a concentration of 0.0005 v/v % and at a volume ratio of 1:10 (water/solution) and stirred for one hour before electrospinning.

2.6. Solution Electrospinning

The electrospinning equipment, previously described (Saquing, Manasco, & Khan, 2009), included a Harvard Apparatus precision syringe pump (Holliston, MA) with a flow rate between 0.1-2.0 mL/h, and a Gamma High Voltage Research High-Voltage Power Supply (Model D-ES30 PN/M692) with a positive polarity between 0 and 30 kV. A 10 ml syringe with a stainless steel capillary metal-hub needle attached was filled with the electrospinning solution. When placed on the syringe pump the needle tip-to-collector distance was 15 cm. Solutions were electrospun for approximately 48 hours.

2.7. Characterizations of Electrospinning Solutions

Viscosity measurements were collected for PEO/BLG plus NaOH and PEO/BLG/STMP solutions for each STMP concentration. PEO/BLG solutions served as the control. Rheometric measurements were performed using an AR 2000 EX Rheometer from TA Instruments (New Castle, DE) with a 40 mm 2° steel cone geometry. A ramp shear stress in the range of 0.0 to 20.0 Pa was applied at a constant temperature of 25 °C, controlled by a Peltier plate device.

Solution conductivity and pH were recorded with an YSI 3100 conductivity instrument (Yellow Springs, OH) and an Accumet® AB150 pH/mV meter (Rocklin, CA) prior to electrospinning.

2.8. PEO/BLG/STMP Scaffold Characterization

To evaluate nanofiber morphology and scaffold topography, fluorescent confocal laser scanning microscopy (CLSM) was used to acquire 1.0 μm thick images of the electrospun

scaffolds. The fluorescent dye Rhodamine-B (RhB) was obtained from Sigma-Aldrich Corporation and added to the solutions, before electrospinning, to facilitate CLSM imaging. CLSM images were acquired with a Zeiss LSM 700 confocal microscope (Thornwood, NY) equipped with a 20x/0.75NA air objective. Images were acquired with a 488 nm photodiode laser with a 555 nm emission filter.

To evaluate chemical crosslinking and water insolubility, dried scaffolds were immersed in Phosphate Buffered Saline (PBS) for 24 hours and imaged with CLSM.

2.9. PEO/BLG Scaffold Preparation for hMSC Cultures and Thymosin-β4 Treatment

After scaffolds were electrospun, circular sections were cut to a diameter of 14mm, placed on aluminum foil and crosslinked at 100°C for approximately 96 hours (See Appendix A). The scaffolds were sterilized by UV-irradiation for at least 12 hours per side. Scaffolds were placed in multi-well cell culture plates, hydrated in PBS and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 hours. To coat scaffolds with Tβ4 (BACHEM, CA), scaffolds were hydrated in PBS containing 10 μg/mL Tβ4 for 24 hours. After hydration and prior to cell seeding, the media was carefully removed and Tβ4 coated scaffolds were washed once with PBS to remove any excess unbound Tβ4 (See Appendix A).

2.10. Cell Seeding and Cultures

HMSC were acquired from The Texas A&M Science Center (College Station, TX). HMSCs were thawed, pelletized and counted to determine their live-to-dead ratio. Cultures were prepared in two types of media: (1) Dulbecco's Modified Eagle Medium, (DMEM, Gibco) and (2) DMEM containing 1μg/mL Tβ4 (Tβ4-DMEM). HMSCs were seeded in triplicate at a concentration of approximately 500,000 cells/mL/well over four experimental groups shown in Figure 2.1: (1) scaffolds in DMEM (control), (2) Tβ4 coated scaffolds in DMEM, (3) scaffolds

in Tβ4-DMEM, and (4) Tβ4 coated scaffolds in Tβ4-DMEM. HMSCs were allowed to grow for eight days; culture media was exchanged every 4 days.

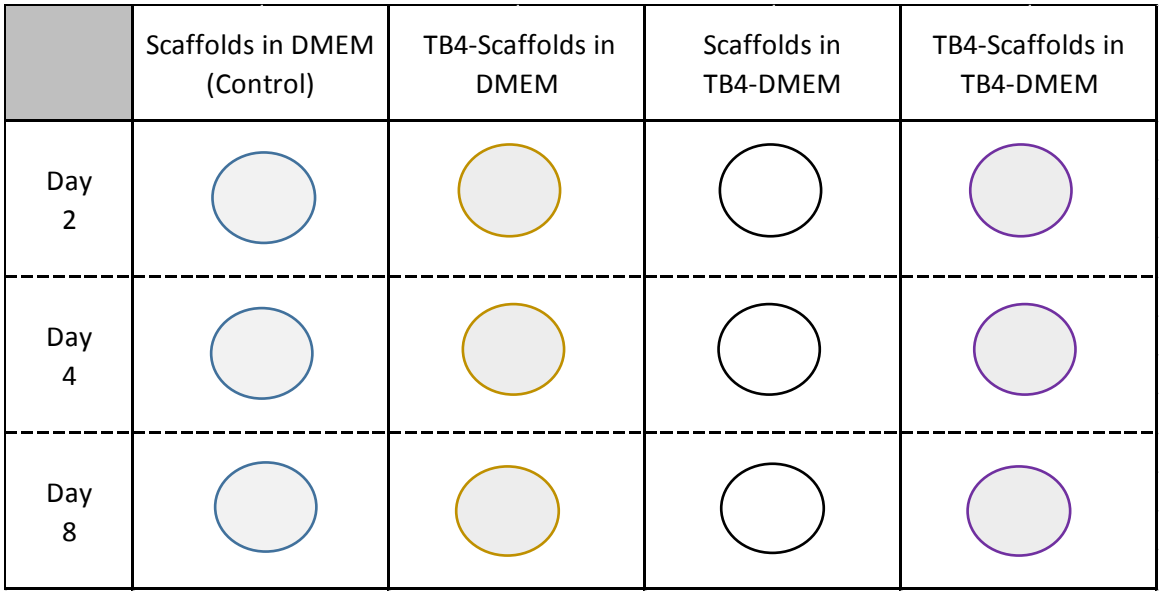

Figure 2.1: Schematic for hMSC proliferation assay. Experimental groups include: Scaffolds; Tβ4-Scaffolds: coated scaffolds with 10μg/mL Tβ4; Tβ4-DMEM: culture medium with 1μg/mL Tβ4, and scaffold with both Tβ4 treatments. Three replicates were evaluated at each time point.

2.11. HMSC Proliferation

Cell proliferation was evaluated at day 2, 4 and 8 days after hMSC seeding using colorimetric assay Promega CellTiller 96® AQ_{euous} One Solution Cell Proliferation Assay kit (Madison, WI) according to the manufacturer's instructions. Cell culture media was exchanged 24 hours prior to each assessment. Three cell culture plates were evaluated for each experimental group at each time point. After introducing the colorimetric agent, cell cultures were incubated at 37 °C for 2 hours in a humidified 5% CO_2 atmosphere. Absorbance at 490 nm

was recorded using a VersaMax 96-well plate reader (VersaMax, Molecular Devices). The average absorbance for each experimental group was computed (See Appendix C for protocol). In this cell proliferation kit, a tetrazolium compound (MTS) is bioreduced by the cells into a color formazan product that is directly proportional to the number viable cells in the culture (Promega, 2012). At day 2, 4 and 8 after cell plating, absorbance was measured to estimate relative cell proliferation.

2.12. Statistical Analysis

All results are expressed as mean ± SD. A one-way ANOVA and *post hoc* t-test for independent samples were used to evaluate and determine statistical significance for solution conductivity and pH measurements due to changes in STMP concentration. (See Table 2.1). HMSC proliferation was evaluated using two-way ANOVA to demonstrate if there were statistical differences in proliferation due Tβ4 treatment and time. *Post hoc* evaluations were conducted using a one-way ANOVA and t-test for independent samples to evaluate statistical differences for Tβ4 treatments and time independently and to determine the sources of statistical significance for each variable (See Table 2.2). A value of $p < 0.05$ ($n = 3$) was considered statistically significant for all reported results.

Table 2.1: Statistical Analysis of Solution Conductivity and pH

Measurement	Independent Variable	Analysis	Hypotheses	*Post hoc*
Conductivity	STMP concentration	one-way ANOVA	H_o: Mean conductivity for control and PEO/BLG/STMP solutions are equal	If H_o rejected, perform t-test for independent samples to identify groups whose conductivity are statistically significant
pH	STMP concentration after NaOH addition	one-way ANOVA	H_o: Mean pH for PEO/BLG plus NaOH and PEO/BLG/STMP solutions are equal	If H_o rejected, perform t-test for independent samples to identify groups whose pH are statistically significant

Table 2.2: Two-way ANOVA for hMSC Proliferation Assay

Variables		Hypotheses	*Post hoc*
Independent	Tβ4 treatments	H_o: Mean hMSC proliferation for Tβ4 treatments and control are equal	If H_o rejected, perform one-way ANOVA to determine which groups are statistical significant, followed by t-test for independent sample to compare across all assessment days
	Proliferation Assessment Day	H_o: Mean hMSC proliferation for assessment days are equal	If H_o rejected, perform one-way ANOVA to determine which days is statistical significant, followed by t-test for independent samples to compare across all groups
Dependent	Interactions	H_o: No interactions between Tβ4 treatments and assessement days	If H_o rejected, identify sources of variation

CHAPTER 3: Effects of Sodium Trimetaphosphate in Poly(ethylene oxide) and β-Lactoglobulin Electrospinning Solution

3.1. Introduction

A study by Sullivan *et al.* outlined the process to generate electrospun nanofibers using aqueous poly(ethylene oxide)-β-lactoglobulin (PEO/BLG) solutions (Sullivan S. T., 2011). In addition to reporting on the PEO/BLG concentrations that yielded defect-free nanofibers, the group reported their technique to effectively crosslink PEO/BLG nanofibers by heat treatment. This technique rendered nanofibers with structural stability when immersed in aqueous solutions; however, Sullivan's crosslinking technique was time intensive.

Nanofiber structural stability in aqueous solutions is a fundamental property when using scaffolds for tissue engineering (TE) studies. Typically, TE scaffolds are seeded with cells and subjected to cell culturing techniques, which require the use of aqueous based solutions (PBS, culturing media, etc.). The use of materials that produce electrospun nanofibers that are TE ready without requiring additional modifications and/or crosslinking treatments is a focus of much research (Agarwal, Wendorff, & Greiner, 2009).

In light of studies reporting crosslinking during nanofiber electrospinning (Shi, Le Visage, & Chew, 2011; Jiang, et al., 2015), the focus of this investigation was to evaluate an alternative crosslinking method for PEO/BLG electrospun nanofibers. This approach used sodium trimetaphosphate (STMP) as an agent for *in situ* chemical crosslinking. STMP at various concentrations was added to PEO/BLG solutions. STMP effects on electrospun scaffolds, and its effects on solutions viscosity, conductivity and pH are reported (Table 3.1),

Table 3.1: Summary of Solution Characterization

Objective	Experiment		
	Viscosity	Conductivity	pH
To assess the effects of adding NaOH and STMP to PEO/BLG electrospinning solutions	Measured viscosity for PEO/BLG, PEO/BLG plus NaOH and PEO/BLG/STMP solutions	Measured conductivity for PEO/BLG, PEO/BLG plus NaOH and PEO/BLG/STMP solutions	Measured pH PEO/BLG plus NaOH and PEO/BLG/STMP solutions

3.2. Results

3.2.1. Solution Electrospinning

PEO/BLG and PEO/BLG/STMP solutions were electrospun for 48 hours at ambient conditions (22.9±1.4 ˚C and 52.3±4.9 % relative humidity). For all solutions, the tip-to-collector distance was fixed at 15 cm. PEO/BLG solutions were electrospun by extruding the solution at a 0.1 mL/hr flow rate and applying 7.0 kV (Sullivan, Tang, Kennedy, & Tawlar, 2014). As expected, PEO/BLG/STMP solutions required higher voltages to initiate nanofiber electrospinning as a result of adding STMP to PEO/BLG solutions. The electrospinning voltage varied for all STMP concentrations ranging from 15 kV to 22 kV and varied between solution batches of same STMP concentration (Table 3.2).

Table 3.2: Electrospinning Voltages for PEO/BLG/STMP Solutions.

STMP (w/v %)	Voltage (kV)	
	Batch 1	Batch 2
2	22	16
4	16	25
6	20	15
8	22	17

3.2.2. Viscosity Measurements for Electrospinning Solutions

STMP was added to PEO/BLG electrospinning solution at 2, 4, 6 and 8 w/v % concentrations. Viscosity measurements were collected using a rheometer, with a cone geometry, by applying constant ramp shear stress. For the control solutions, PEO/BLG and PEO/BLG plus NaOH (PEO/BLG/NaOH) solutions, the viscosity gradually decreased and plateaued along the applied shear stress range. Adding STMP to PEO/BLG electrospinning solutions resulted in S-shape viscosity profiles for all STMP concentrations (Figure 3.1). The S-shape viscosity profile reveals three distinct behaviors along the applied shear stress: (1) high viscosity at low shear stresses, (2) sudden and/or rapid decrease in viscosity and (3) low viscosity at high shear stresses. During low shear stress, viscosity was measured between 100 and 1000 Pa·s for all solutions with STMP. The results show an increase in viscosity up to two orders in magnitude relative to the control group. During high shear stress, viscosities for all STMP concentrations plateaued in the same order of magnitude, between 1 to 10 Pa·s (Figure 3.1).

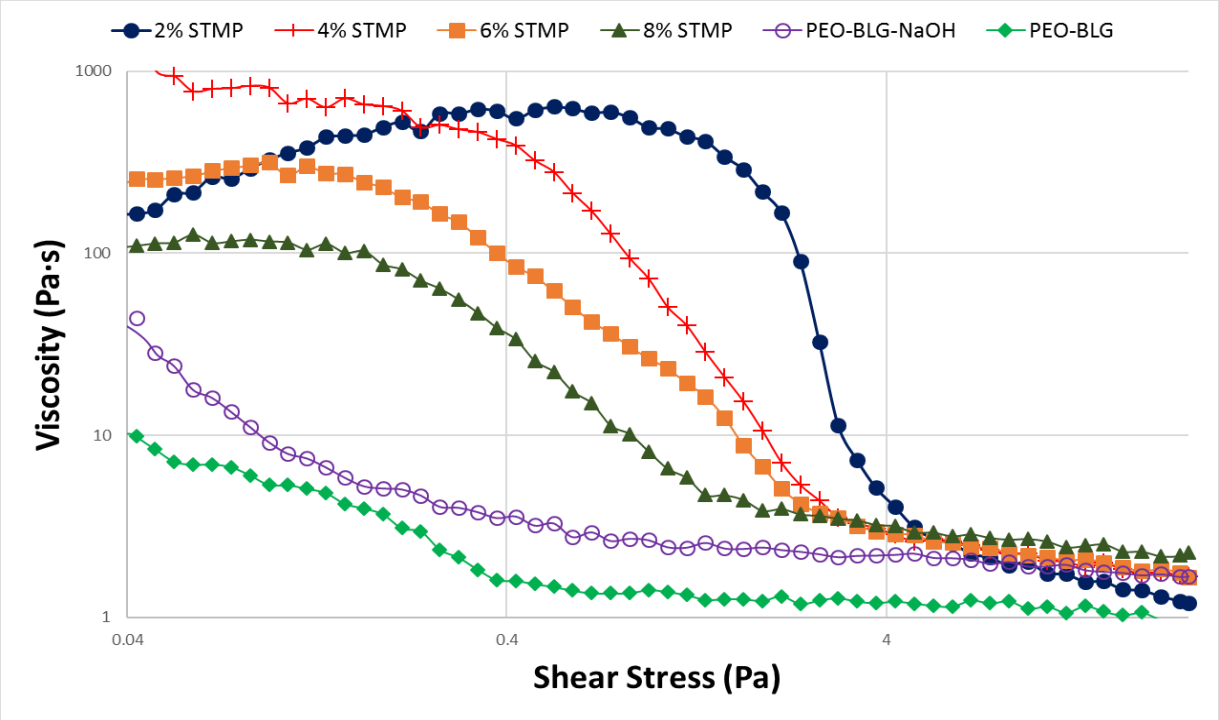

Figure 3.1: Viscosity vs. Applied shear stress for STMP and Control solutions (PEO/BLG/NAOH & PEO/BLG). STMP solutions displayed a pseudo-plastic behavior, i.e., high viscosity at low shear stresses followed by a rapid decrease in viscosity, and plateauing at higher shear stresses. The region showing rapid viscosity decrease is less pronounced as STMP concentration increased. 2 w/v % STMP solutions demonstrated the least pronounced rapid viscosity decrease region. PEO/BLG solutions demonstrated the least variation in viscosity along the applied shear stress range ($n \geq 3$ for STMP solutions and controls).

3.2.3. Conductivity and pH Measurements for PEO/BLG/STMP Solutions

Solution conductivity was recorded at least 3 times for PEO/BLG (control), PEO/BLG plus NaOH and PEO/BLG/STMP solutions. Average conductivity values for the tested solutions are reported in Table 3.3.

Table 3.3: Solution Conductivity for PEO/BLG Solutions with NaOH and STMP (Mean Solution Conductivity ± Standard Deviation (n = 3).

Solution	Mean, mS/cm	S.D., ± mS/cm
Control	0.69	0.06
PEO/BLG/NaOH	5.06	0.38
2 w/v % STMP	11.38	0.10
4 w/v % STMP	17.04	1.24
6 w/v % STMP	22.12	1.06
8 w/v % STMP	27.14	0.85

PEO/BLG plus NaOH solutions were analyzed first to determine changes in conductivity due to NaOH addition since NaOH concentration and volume was constant for all STMP solutions. Conductivity significantly increased by 5-fold for 8 w/v % STMP (27.14±0.85 mS/cm), relative to control. Since the concentration and volume of NaOH added to PEO/BLG/STMP solutions were the same across all STMP concentrations, PEO/BLG/NaOH solutions were used to compare the effects of varying STMP concentrations on solution conductivity (Figure 3.2). Adding STMP to PEO/BLG solutions increased solution conductivity which was directly proportional to the STMP concentration. Statistical significance was determined for solutions at all STMP concentrations relative to PEO/BLG/NaOH solutions.

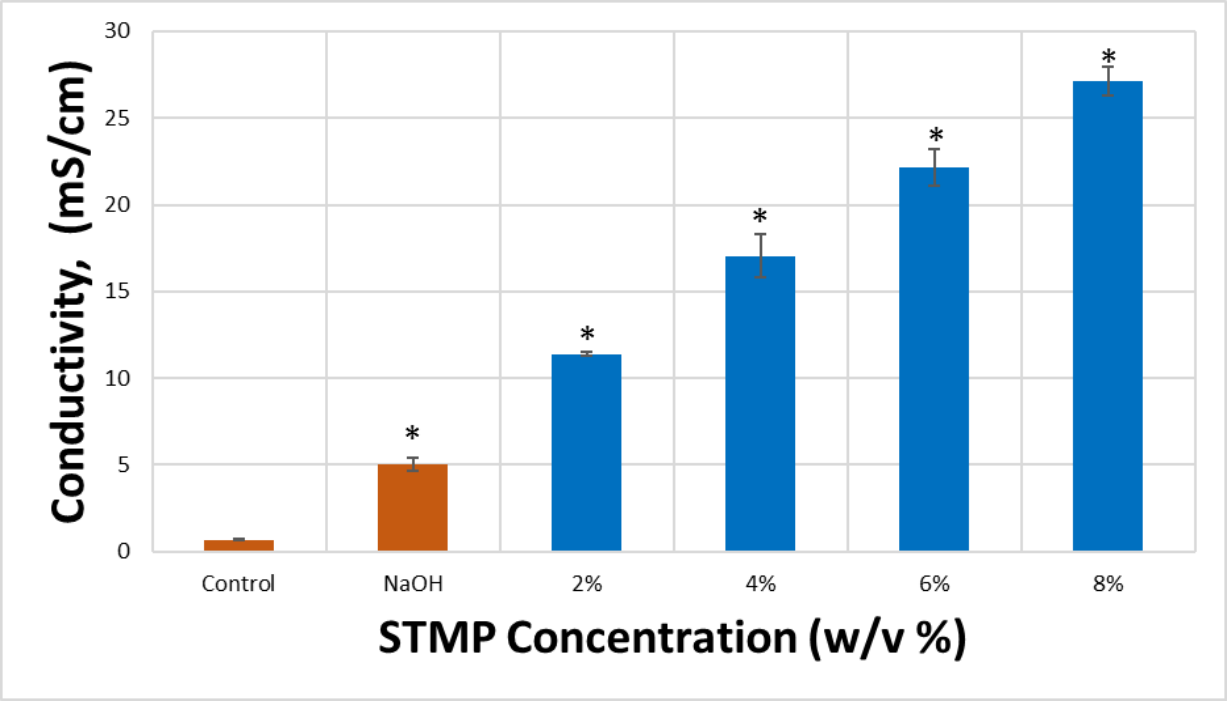

Figure 3.2: Average solution conductivity for PEO/BLG/STMP solutions (Blue) and Control (PEO/BLG) and PEO/BLG/NaOH (Orange). Differences in conductivity were statistically significant among all STMP concentrations and between control groups (*$p<0.05$, One-way ANOVA *post hoc* t-test).

The pH for PEO/BLG solutions has been previously reported to be 7.5 (Sullivan S. T., 2011). Solution pH was recorded at least 3 times for PEO/BLG plus NaOH and PEO/BLG/STMP solutions. Average pH values for each solution are reported in Table 3.4. pH measurements were recorded for all solutions, reaching as high as 12.29 ± 0.08 (2 w/v % STMP). This result was expected since NaOH is a strong base compound. pH values were lower for all other STMP concentrations, i.e. 4, 6 and 8 ($p<0.05$). The pH of the solutions decreased as STMP concentration increased (see Figure 3.3). However, an unexpected increase in pH was observed at 8 w/v % STMP. Furthermore, a *post hoc* t-test determined statistical significance between STMP concentrations of 2, 6 and 8 w/v % (Figure 3.3).

Table 3.4: pH for PEO/BLG Solutions with NaOH and STMP. (Mean ± Standard Deviation (n =3).

Solution	Mean, pH	S.D., ± pH
PEO/BLG/NaOH	12.31	0.08
2 w/v % STMP	12.29	0.08
4 w/v % STMP	11.89	0.24
6 w/v % STMP	11.68	0.12
8 w/v % STMP	12.01	0.05

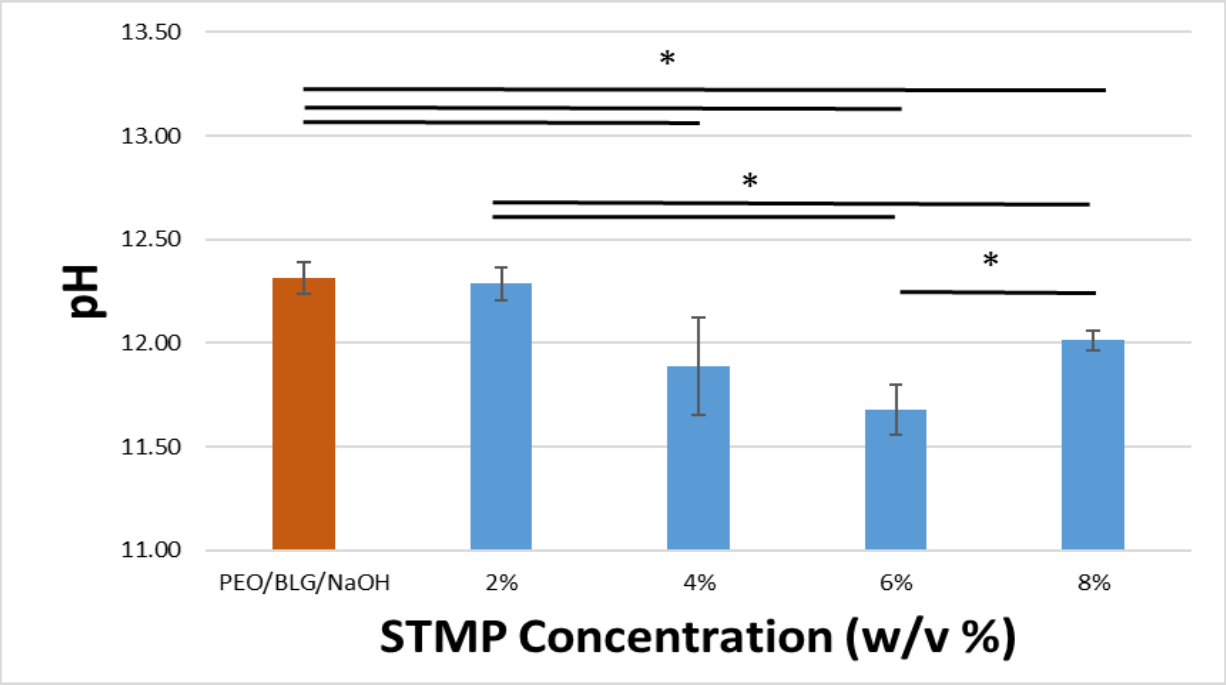

Figure 3.3: Average pH for PEO/BLG plus NaOH and PEO/BLG/STMP solutions with various STMP concentrations (*p<0.05, One-way ANOVA *post hoc* t-test).

3.2.4. PEO/BLG/STMP Scaffold Characterization

Scaffold sections were cut and prepared for fluorescent CLSM imaging. CLSM images were acquired from electrospun PEO/BLG and PEO/BLG/STMP dry scaffold sections (Figure 3.4).

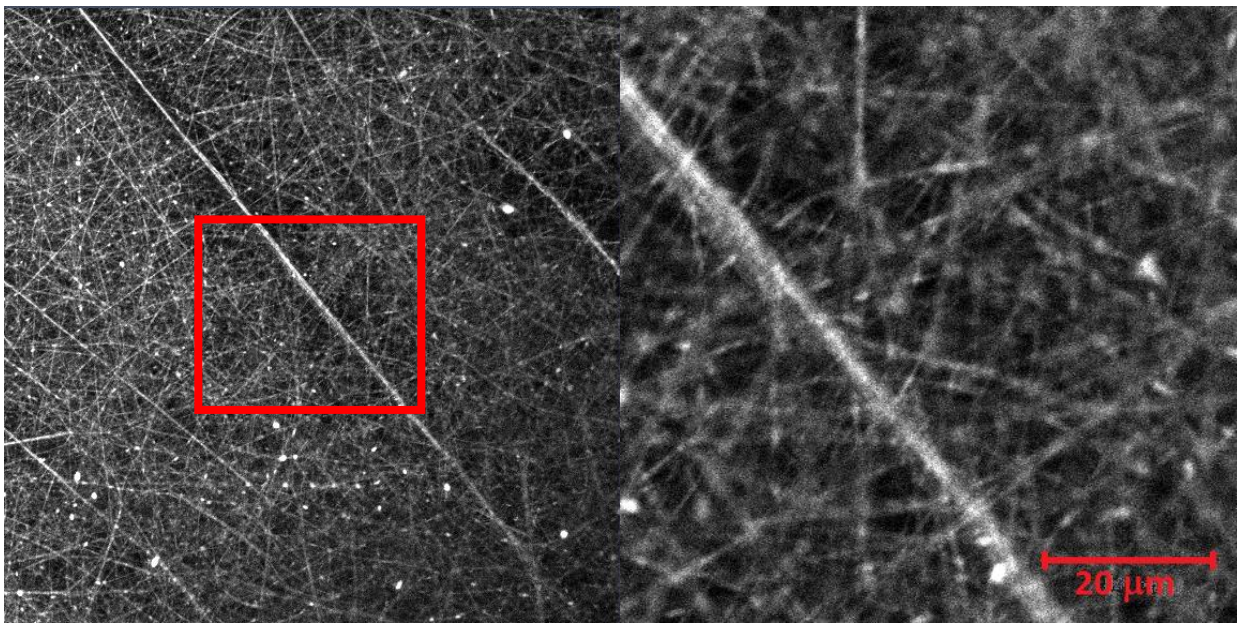

Figure 3.4: CLSM image of PEO/BLG electrospun fibers. Non-woven linear nanofiber, porous scaffold (left). Magnified image illustrating variations in nanofiber diameter (right).

Noticeable differences in scaffold topography and nanofiber morphology were observed in CSLM images acquired from scaffolds created with PEO/BLG/STMP solutions. PEO/BLG/STMP solutions resulted in beaded electrospun nanofibers with solution droplets varying in diameter throughout the nanofiber scaffolds. This pattern was observed across all STMP concentrations. Regarding nanofiber morphology and scaffold topography, solutions with 2 w/v % STMP resulted in curled and wave-like electrospun nanofibers with minimal accumulation and solution droplets with diameters up to 20 µm in diameter. At 4 w/v % STMP, linear nanofibers were created with some curved and wave-like nanofibers. The 4 w/v % STMP concentrations produced the highest nanofiber accumulation. Solution droplet diameter

decreased to approximately 12 μm, and fewer droplets were observed in the imaging field. With 6 w/v % STMP, highly beaded linear and curved nanofibers were generated. Nanofiber accumulation decreased relative to 4 w/v % STMP scaffolds and the number of observed solution droplets decreased and droplet diameter decreased to approximately 8 μm. Solutions with 8 w/v % STMP resulted in beaded linear and curved electrospun nanofibers. Observed nanofiber accumulation and number of solution droplets were the lowest at this STMP concentration. However, the diameter of the solution droplets increased up to approximately 20 μm at this STMP concentration.

Although defect-free nanofiber scaffolds from PEO/BLG/STMP solutions were not created, scaffold samples were immersed in PBS solution to ascertain if *in situ* crosslinking was accomplished. Scaffold samples were immersed in PBS for 24 hours and viewed under CLSM. The nanofibers dissolved and the scaffolds took on a membrane-like topography (figures not shown). In addition, scaffold samples examined immediately after PBS immersion yielded the same scaffold morphology and topography, indicating that the fibers were not crosslinked.

3.3. Discussion

Electrospinning defect-free nanofibers from aqueous solutions includes multiple parameters including relative humidity and temperature, flow rate, tip-to-collector distance, applied voltage, solution composition and viscosity (Doshi & Reneker, 1995). These parameters dictate the outcome of the electrospinning process pertaining to nanofiber morphology and scaffold topography. Thus the synergetic combination of these parameters govern the final outcome in electrospinning defect-free nanofibers that can serve as scaffolds for various tissue engineering application.

Sullivan *et al.* proposed the feasibility of electrospinning nanofibers from PEO/BLG solutions that produced defect-free nanofibers. Considering that PEO and BLG are both water soluble polymers, Sullivan reported that by heat treating PEO/BLG nanofibers over a 5-day period the nanofiber cross-linked, rendering their hydrophobic properties. Following Sullivan's proposed protocols, defect-free PEO/BLG nanofibers were reproduced in the Muller-Borer laboratory.

The purpose of this study was to ascertain the effects of STMP on PEO/BLG solutions and the electrospinning process with the long-term goal of developing an alternative cross-linking method to heat treatment. The primary focus of this study was to investigate various STMP concentrations and electrospinning parameters that would yield defect-free nanofibers. STMP is a sodium salt that has been reported to effectively cross-link *in situ* electrospun polysaccharide nanofibers (Shi, Le Visage, & Chew, 2011).

Defect-free nanofibers generated from PEO/BLG were electrospun using 7kV (flow rate of 0.1 mL/hr). PEO/BLG/STMP solutions required higher electrospinning voltages which differed as STMP concentrations increased. Though electrospinning voltages that lead to nanofiber initiation for PEO/BLG/STMP solutions were identified, the resulting nanofibers were not defect-free. Changes in solution composition contributed to changes in nanofiber and scaffold morphology, i.e., adding different STMP concentrations to PEO/BLG solutions, and modifications to the electrospinning parameters were expected and necessary to initiate nanofiber formation. For all combinations of STMP concentrations and applied voltages, nanofibers were beaded and non-uniform in morphology, and solution droplets were deposited throughout the electrospun scaffold. These results are consistent with previous reports concerning the effects of solution composition on the electrospinning process (Subbiah, Bhat,

Tock, Parameswaran, & Ramkumar, 2005) and for nanofibers electrospun from solutions consisting of polymers such as collagen, hyaluronic acid and PEO (Fischer, McCoy, & Grant, 2012; Sullivan S. T., 2011). Interestingly, at higher STMP concentrations, nanofibers extruded outward on the collector's z-plane making macroscale 3D nanofiber scaffold.

Given the important role of solution composition on the electrospinning process, solution viscosity, conductivity and pH were analyzed. The rheological assessments of the tested solutions revealed that adding STMP to PEO/BLG solutions presented unique effects on solution viscosity. Unlike the viscosity profile for PEO/BLG solutions which decreased, approached a limit as shear stress increased and did not exceed 10 Pa·s, the viscosity profiles for PEO/BLG/STMP solutions manifested S-shape curves (Figure 3.1). S-shape viscosity profiles are intrinsic to behavior of Non-Newtonian fluids under shear stress (Barnes, 2000). This further emphasizes that the mechanical behavior for electrospinning solutions is dependent on solution composition.

Charges in the polymer solutions are the driving mechanism for nanofiber initiation and formation during the electrospinning process. Therefore, altering solution composition changes the charge density in solutions, offering an explanation for the differences observed for solution conductivity. It was shown that adding NaOH and STMP to PEO/BLG solutions increased solution conductivity. Additional increases in STMP concentration increased solution conductivity (Figure 3.2). The increase in solution conductivity can be explain by the fact that both NaOH and STMP are ionic salts that when dissolved in water, or aqueous solutions, dissociate, freeing ions that increase charge density per volume (Subbiah, Bhat, Tock, Parameswaran, & Ramkumar, 2005; Lanigan, 2001). This further explains the directly proportional relationship between solution conductivity and STMP concentration. Changes in pH

were expected as NaOH is a strong base and increased to a pH of 12, relative to the control pH (7.5) of PEO/BLG blends previously reported by Sullivan *et al*. STMP concentration also affected pH and the results were statistically significant relative to PEO/BLG/NaOH solutions for 2 w/v % and 8 w/v % ($p<0.05$). pH values recorded for PEO/BLG/STMP solutions varied between 11 and 12. Since the pH for PEO/BLG solutions was reported as 7.5 by Sullivan *et al.*, it can be concluded that pH is mainly affected by NaOH. Increasing concentrations of STMP tended to decrease pH. However, this decrease in pH was not statistically significant, may be a factor of the small sample size and requires further investigation.

A 2005 study reported the influence of pH on electrospinning poly (vinyl alcohol) (PVA) solutions by assessing solution viscosity, surface tension and conductivity as a function of pH (2-12.9). The researchers modified pH by using NaOH and showed that conductivity was significantly affected by pH, i.e. increasing pH resulted in increased solution conductivity. However, it was also demonstrated that solution viscosity and surface tension was not affected by changes in pH (Keun Son, Ho Youk, Seung Lee, & Park, 2005). From evidence provided by Keun Son *et al.*¸ it can be concluded that, in this study, the effects of NaOH on PEO/BLG/STMP solutions only contribute to solution conductivity. The observed changes in nanofiber morphology are associated with the effects of STMP concentration on the mechanical properties of the solution and solution conductivity.

Electrospinning solutions are continuously forced through a syringe pump. The solution forms a droplet at the tip of the spinneret. Due to the applied voltage potential between the spinneret and the collector, the solution is exposed to an electric field. The charges in the solution move towards the electrode of the opposite polarity which forms a jet and results in nanofiber initiation and formation (Figure 3.5).

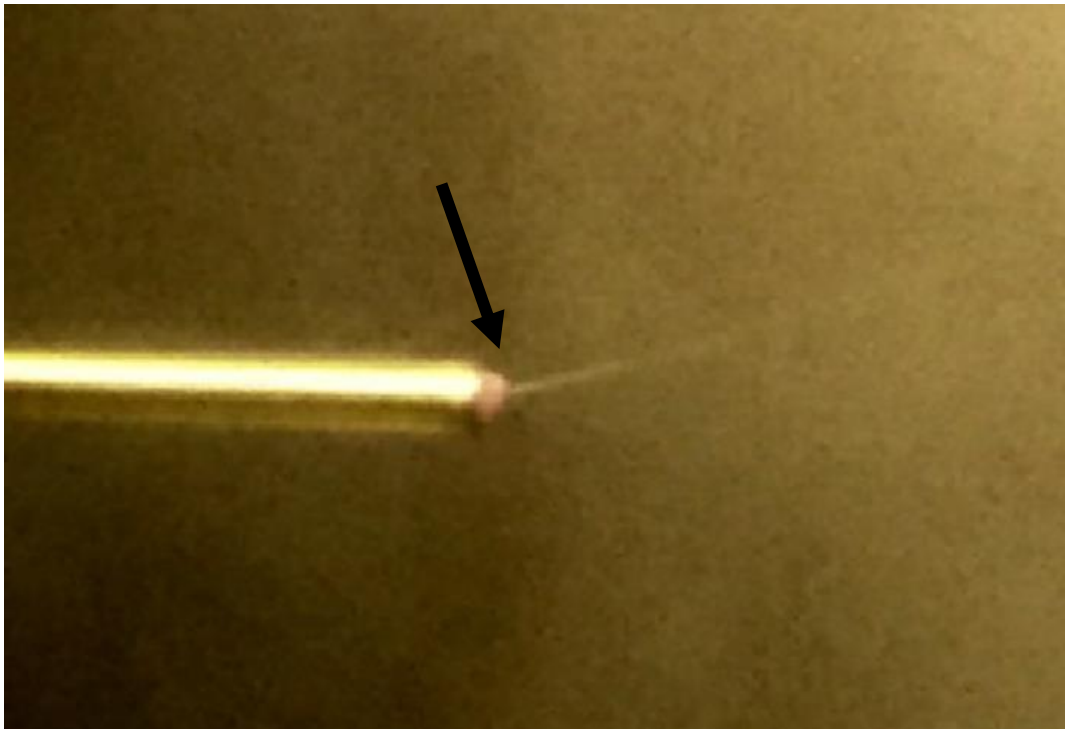

Figure 3.5: PEO/BLG nanofibers at needle tip during electrospinning process. Figure illustrates solution droplet, jet formation and initiation nanofiber.

In order to establish a nanofiber jet, the electrostatic forces must overcome tensile forces in the solution droplet at the tip of the spinneret. Solution viscosity and conductivity influence the tensile and electrostatic forces during electrospinning, respectively. During the electrospinning of PEO/BLG solutions, once nanofiber formation has initiated, the droplet at the tip of the spinneret reduces in size. However, fiber generation continues uninterrupted due to the constant flow through the spinneret. This pattern was not observed for PEO/BLG/STMP solutions. Solutions with STMP produced large droplets that were pulled as a whole from the spinneret. Nanofiber jets were initiated, forming nanofibers, but nanofiber formation ceased as a new droplet developed at the spinneret. Nanofibers were not produced continuously despite the constant flow rate.

The continuous and uninterrupted formation of nanofibers is the result of the electrostatic forces that overcome the surface tension of the droplet at the spinneret (Doshi & Reneker, 1995). It was previously mentioned that the main effect of adding salts (NaOH and STMP) results in an increase in solution conductivity. Although solution conductivity significantly increased with STMP concentration (Table 3.3), the changes in solution composition by the addition of STMP to PEO/BLG solutions is the main factor contributing to nanofiber morphology. Beaded nanofibers were created, nanofiber accumulation deceased and scaffold porosity increased with increased STMP concentration. These changes coincide with various studies that report on electrospinning polymer blends with different weight ratios and the effects on nanofiber morphology (Aluigi, et al., 2008; Colín-Orozco, Zapata-Torres, Rodríguez-Gattorno, & Pedroza-Islas, 2015; Fischer, McCoy, & Grant, 2012; Sullivan, Tang, Kennedy, & Tawlar, 2014).

PEO/BLG/STMP solution viscosity increased considerably relative to the PEO/BLG solution. The S-shape viscosity profiles recorded for PEO/BLG/STMP solutions (Figure 3.1) has been described for other Non-Newtonian fluids which experienced a rapid decrease in viscosity at a very small shear stress. This region of rapid decrease in viscosity has been identified as shear-thinning, which to the behavior of nanofiber formation for PEO/BLG/STMP solutions (Barnes, 2000). Large electrostatic forces were necessary to overcome the high viscosities imposed by low shear stresses, as the droplet at the spinneret increased in size, charge density increased. Upon reaching sufficient magnitude, the solutions experienced shear-thinning, and decreased viscosity, allowing for the electrostatic forces to overcome tensile forces. Verifying this requires further research to assess charge density and current measurements for STMP solutions for the electrospinning conditions reported in this study

Despite being unsuccessful in producing defect-free nanofibers, scaffold samples were immersed in PBS solution to determine if *in situ* crosslinking due to STMP and NaOH had occurred. BLG has two intrachain disulphide bridges and a free thiol group within its primary structure in the form of cysteine amino acids, which due to their high reactivity can served as potential crosslinking sites (Hernández-ledesma, Recio, & Amigo, 2008). Crosslinking was not accomplished since upon immersion in PBS the nanofibers dissolved (images not shown). The disulphide bridges and free thiol group are encapsulated within the internal structure of BLG. It was anticipated that an increase in pH due to NaOH would denature and unfold BLG, exposing cysteine reactive groups to influence crosslinking. Base-induced denaturation for BLG has been reported. These studies report that BLG unfolding occurs in a transitional manner from pH 9-13 and pH 5-12 where small portions of the secondary structures are preserved (Taulier & Chalikian, 2001) and complete unfolding occurs at high pH (Partanen, et al., 2011). Due to the different results reported on BLG unfolding and denaturation at basic pH it is unclear why there was no evidence of nanofiber crosslinking. According to Taulier *et al.*¸ if small secondary structures are preserved, it can be argued that these structures may contain the disulphide bridges. Therefore, a reducing agent may be necessary to reduce the disulphide bonds and making the thiol group available for crosslinking; however, this does not explain why crosslinking did not occur on the free thiol group.

3.4. Conclusion

This work represents efforts to develop an alternative crosslinking technique to prolonged heat treatment for PEO/BLG nanofibers. In this study, PEO/BLG solutions were prepared with various STMP concentrations. PEO/BLG/STMP nanofibers were electrospun to assess electrospinning parameters that may yield defect-free nanofibers. Specific parameters were

identified to generate electrospun nanofiber scaffolds. However, CLSM imaging revealed that defect-free nanofibers were unattainable under the tested electrospinning parameters. Viscosity, conductivity and pH were assessed for PEO/BLG/STMP solutions to determine the effects of adding STMP and NaOH to PEO/BLG solutions. STMP in combination to NaOH has been shown to chemically crosslink polysaccharide nanofiber during the electrospinning process. Ultimately, adding STMP did not chemically crosslink PEO/BLG and defect-free nanofibers were not obtained. This approach was determined to not be suitable or efficient for producing biological scaffolds for TE. Additional research in polymerchemistry is suggested to advance this project.

CHAPTER 4: Assessment Stem Cell Proliferation Using Poly(ethylene oxide) and β-Lactoglobulin Electrospun Nanofibers

4.1. Introduction

Numerous methods have been reported for the fabrications of scaffolds for tissue engineering (TE) (Vasita & Katti, 2006). There are multiple reasons supporting the use of electrospun nanofibers for TE. The versatility of modification for numerous applications, cost-effective set-up and process has made the electrospinning process a common tool for research. This has allowed the fabrication of tissue specific nanofiber scaffolds with abilities to control scaffold thickness, porosity and nanofiber diameter. In addition, the technique has allowed for the fabrication of nanofiber scaffolds composed of biodegradable and natural polymers, improving the biocompatibility properties of nanofibers scaffolds. In addition, the nanoscale nature of electrospun nanofibers provides characteristics intrinsic to the extracellular matrix, promoting cellular function and interaction (Kriegel, Arrechi, Kit, McClements, & Weiss, 2008; Jiang, et al., 2012; Jiang, et al., 2015). Consequently, the use of electrospun nanofibers for TE and regenerative medicine applications has resulted in increased research interest.

Poly(ethylene oxide) (PEO) is a water soluble thermoplastic polymer that, due to its biocompatibility and low toxicity, has been extensively studied for biomedical and food applications (Colín-Orozco, Zapata-Torres, Rodríguez-Gattorno, & Pedroza-Islas, 2015). For PEO and biopolymer solutions, PEO is used as a visco-modifier to enhance electrospinnability of biopolymers and proteins for generating electrospun nanofibers. The combination of PEO and β-lactoglobulin (BLG), a globular protein found in whey, for electrospinning solutions has previously been reported (Sullivan, Tang, Kennedy, & Tawlar, 2014). Due to their composition

consisting of biocompatible and natural polymers, Sullivan *et al*. suggested that PEO/BLG electrospun nanofibers may have potential applications as TE constructs.

The purpose of this study was to evaluate, for the first time, PEO/BLG electrospun nanofibers as TE constructs for human mesenchymal stem cells (hMSCs). HMSCs were seeded onto PEO/BLG scaffolds. HMSC proliferation was evaluated on naïve PEO/BLG scaffolds and on PEO/BLG scaffolds functionalized with the wound healing protein were thymosin-β4 (Tβ4). Among the previously mentioned qualities that have promoted research interest for electrospun nanofibers in TE, recent research has demonstrated the capabilities of functionalizing nanofibers with a variety of biological factors that when used for TE can promote various cellular functions. Among these biological factors, Tβ4 has been shown to promote wound healing, angiogenesis, migration, proliferation, growth, and suppression of tissue inflammatory response (Ti, et al., 2015). Furthermore, a recent study showed that Tβ4 functionalized poly(ε-caprolactone) electrospun nanofibers promoted the growth and differentiation of murine derived cardiomyocytes (Kumar, Patel , Duvalsaint, Desai, & Marks, 2014). Additionally, the desire to investigate Tβ4 as a biological functionalizing agent advances previous research conducted in the Muller-Borer laboratory (Byrum, 2008; Crifasi, 2011). In this study a total of four experimental groups were analyzed to ascertain the effects of PEO/BLG electrospun nanofibers and Tβ4 functionalization on hMSC proliferation (Table 4.1).

Table 4.1: Experimental Groups for hMSC Proliferation Assay.

Group 1	Group 2	Group 3	Group 4
hMSCs seeded on PEO/BLG electrospun scaffolds (Control)	hMSCs seeded on PEO/BLG electrospun scaffolds treated with Tβ4 (Tβ4scaff/DMEM)	hMSCs seeded on PEO/BLG electrospun scaffolds with Tβ4 added to cell culture medium (Scaff/Tβ4DMEM)	hMSCs seeded on electrospun scaffolds coated with Tβ4 and Tβ4 added to cell culture medium (Tβ4 Both)

4.2. Results

4.2.1. HMSC proliferation

A colorimetric assay to evaluate hMSC proliferation was performed 2, 4 and 8 days after hMSC plating. HMSCs remained viable throughout the cell culturing period, and hMSC proliferation increased with time for the Tβ4 experimental groups (see Figure 4.1).

A two-way ANOVA showed statistical significance for hMSC proliferation due to Tβ4 treatment and assessment days as well as interactions between both independent variables. To identify the sources of statistical significance for each independent variable, *post hoc* analyses were conducted to compare proliferation of the experimental groups with respect to each assessment day (Table 4.2) and proliferation at all assessment days with respect to individual experimental group (Table 4.3) using one-way ANOVA followed by t-test for independent samples.

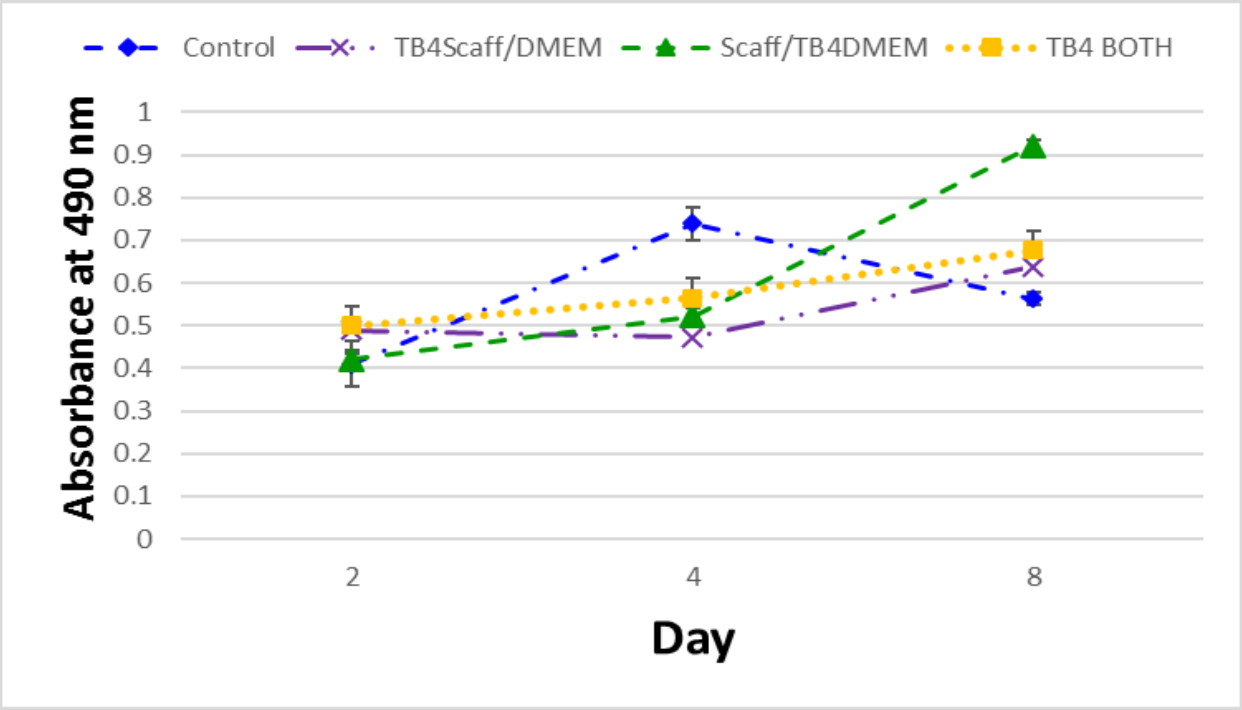

Figure 4.1: HMSC proliferation increased with time in culture. HMSC proliferation increased for all groups from day 2 to day 8.

One-way ANOVA comparing proliferation of the experimental groups for each assessment day showed statistical significant differences only for Day 4 and 8 (Figure 4.2). Furthermore, t-test pairwise comparison demonstrated statistically significant differences in hMSC proliferation among all the experimental groups at Day 4 and 8 (Figure 4.3). Interestingly, the Control group exhibited the highest hMSC proliferation at Day 4.

Table 4.2: One-way ANOVA of hMSC Proliferation with Increased Time in Culture.

Independent Variable	Comparisons	Analysis	Significance (p<0.05)	*Post Hoc*
Day 2	$H_{o:}$ Mean hMSC proliferation at assessment day are equal for Groups and Control	one-way ANOVA	No	No, Failed to reject H_o
Day 4			Yes	t-test for independent samples to identify statistical signifincance in hMSC proliferation for experimental groups vs control
Day 8			Yes	

Table 4.3: One-way ANOVA of hMSC Proliferation per Experimental Group.

Independent Variable	Comparisons	Analysis	Significance (p<0.05)	*Post Hoc*
Control	Ho: mean hMSC proliferation for group are equal for all assessment days	one-way ANOVA	Yes	t-test for independent samples to identify the assessment day that hMSC proliferation is statistical significant
Group 2			Yes	
Group 3			Yes	
Group 4			Yes	

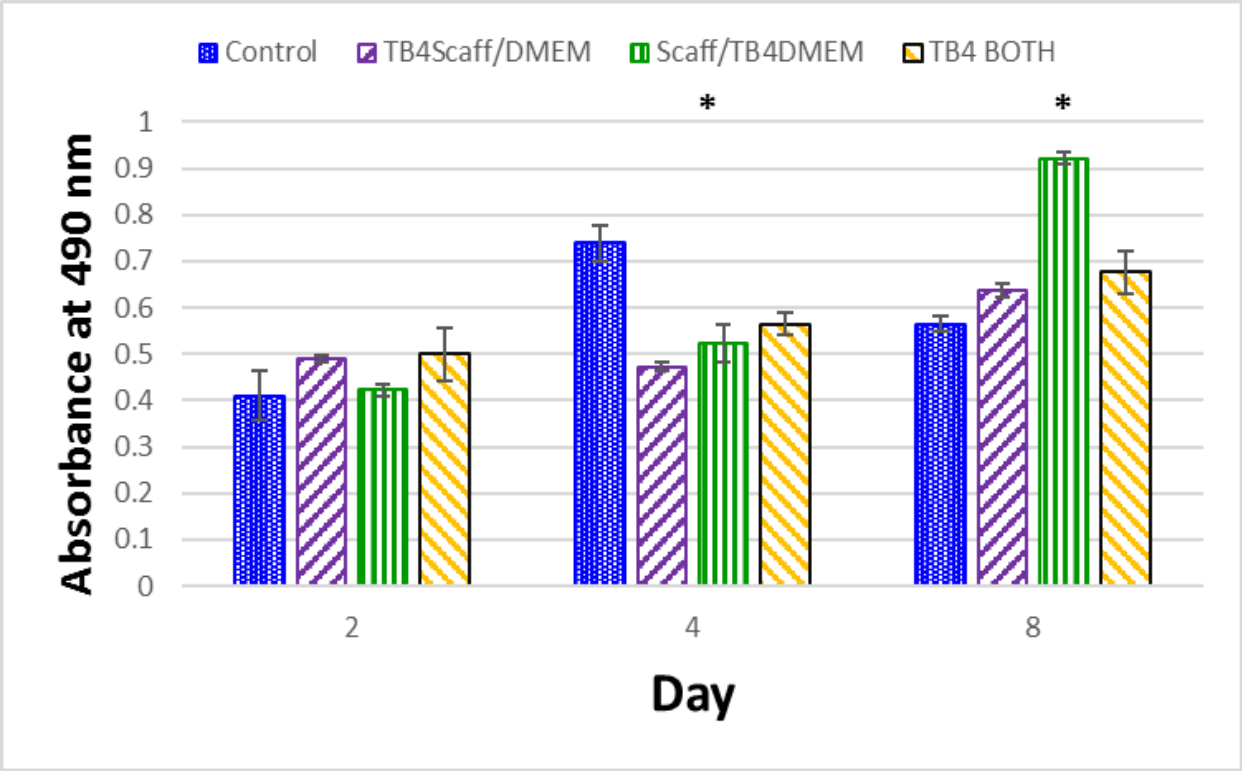

Figure 4.2: hMSC proliferation at days 2, 4 and 8 (*p<0.05, One-way ANOVA, n=3 per group). Proliferation among groups was statistically significant only between day 4 and day 8

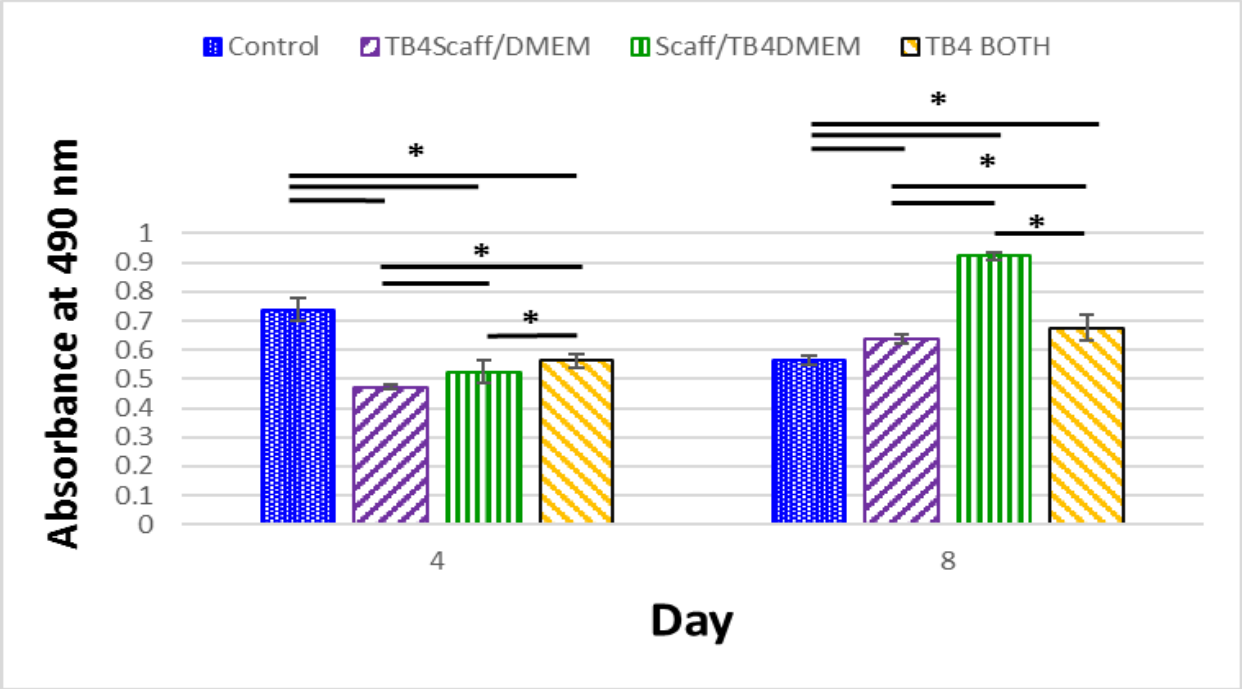

Figure 4.3: hMSC proliferation comparisons at Day 4 and Day 8 (*p<0.05, t-test for independent samples, n=3 per group). HMSC proliferation was statistically different between all groups at Day 4 and Day 8. Day 8 showed increased proliferation for Tβ4 treated groups

One-way ANOVA comparing hMSC proliferation at day 2, 4 and 8 for each experimental group revealed statistically significant differences for all experimental groups. T-test for independent samples demonstrated statistically significant differences in cell proliferation for individual experimental groups at each time point except for Group 2. HMSC proliferation increased from Day 2 to Day 4 and decreased from Day 4 to Day 8 for Group 1 (Control). HMSC proliferation increased from Day 2 to Day 8 for Group 2, and hMSC proliferation increased at each time point for Group 3 and Group 4 (Figure 4.4).

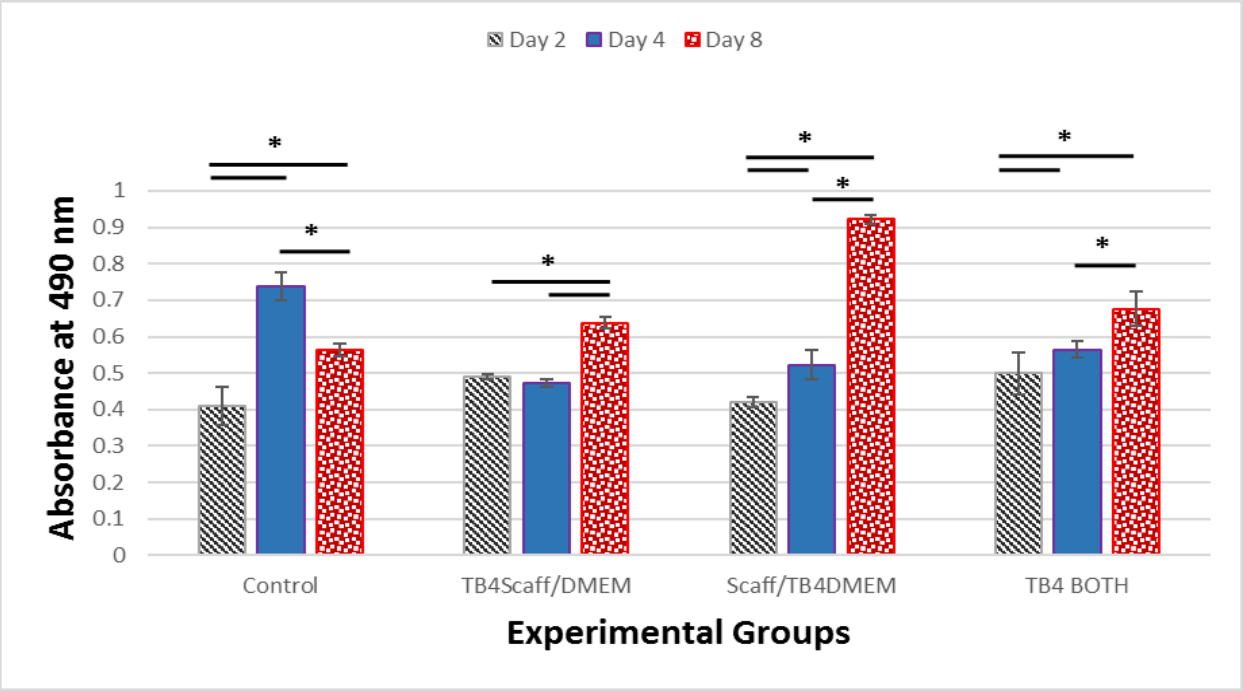

Figure 4.4: hMSC proliferation comparisons per experimental groups (*p<0.05, one-way ANOVA and t-test for independent samples, n=3 per group).

4.3. Discussion

When culturing hMSCs *in vitro*, cell proliferation is dependent on cell seeding density, the available surface area in the culturing vehicle, and time. When using standard cell culture dishes, seeded cells adhere to the bottom of the dishes and migrate to nearby cells to form small aggregates. Cell-to-cell signaling promotes proliferation allowing cells to divide and multiply. Proliferation continues until hMSCs become confluent, forming a 2D layer in the cell culture dish. Furthermore, hMSC cultures can be treated with factors to induce differentiation. By inducing differentiation, the hMSCs become tissue specific and can be used for tissue engineering (TE) applications.

When culturing hMSCs on constructs, such as electrospun nanofiber scaffolds, scaffolds increase the cell culture surface area by providing a 3D architecture for seeded hMSCs to adhere,

migrate, integrate, proliferate and differentiate. In addition, the nanofiber nature of electrospun scaffolds mimic the native extracellular matrix (ECM), which further promotes cellular function (Lodono & Badylak, 2015). These aspects have driven research efforts in using 3D constructs for developing complex, differentiated tissue *in vitro* from hMSCs for TE applications that may result in new innovative clinical applications.

PEO is a biodegradable and biocompatible polymer. BLG is a natural small globular protein found in bovine milk consisting of 162 amino acid residues (Chatterton, Smithers, Roupas, & Brodkorb, 2006). Electrospinning PEO and BLG create nanofibers with biochemical properties from both of materials. Heat treatment of PEO/BLG nanofibers partially denatures BLG by exceeding its gelation temperature, exposing the hydrophilic portions of the molecule when immersed in aqueous solutions (Oakenfull, 1996). Thus, the final outcome of these two processes are crosslinked, aqueously stable PEO/BLG nanofiber scaffolds that can be used for multiple engineering applications (Sullivan, Tang, Kennedy, & Tawlar, 2014). These characteristics provide a basis for evaluating PEO/BLG electrospun nanofibers for hMSC constructs to determine their feasibility for TE applications.

The focus of this study was to determine the effects on hMSC proliferation in the presence of PEO/BLG nanofiber scaffolds (Group 1, Control), and when scaffolds were treated with Tβ4 (Group2, Tβ4scaff/DMEM), when Tβ4 was added to the cell culture medium (Group3, Scaff/Tβ4DMEM), and the combination of Tβ4 treated scaffolds and cell culture media (Group4, Tβ4 Both).

Proliferation was expected to increase significantly for the Tβ4 experimental groups, relative to the control, since Tβ4 promotes cell migration and proliferation (Ti, et al., 2015). HMSC proliferation was statistically significant for each experimental condition at Day 4 and 8

($p<0.05$). The Tβ4 experimental groups did not provide conditions to significantly enhance hMSC proliferation in early culture (Day 2), but instead provided similar culturing conditions as the control group.

Proliferation increased at day 4 after cell plating. HMSC proliferation for individual groups significantly increased from day 2 to 4 except for Group 2. Control demonstrated the greatest increase in hMSC proliferation at day 4. HMSC proliferation results for the control group were unexpected. Given that the control group consisted of PEO/BLG scaffolds and hMSCs only, at least one of the Tβ4 groups should have exceeded the proliferation result for the control. A two-way ANOVA revealed that there were significant interactions between the independent variables. These interactions can be seen in the hMSC proliferation profiles for the Groups and control (Figure 4.1). Proliferation increased with time for Groups 2, 3 and 4, but the control did not exhibit this pattern.

At day 8, hMSC proliferation was statistically significant among the experimental groups. HMSC proliferation was greatest for Group 3 and lowest for the control. This suggests that Tβ4 treatment has a positive effect on hMSC proliferation, relative to the control group. Comparing proliferation for day 4 to day 8, hMSC proliferation increased for all experimental groups except the control.

Tβ4 treatment affected proliferation within experimental groups only. HMSC proliferation increased for Group 3 and Group 4 groups at each time point. HMSC proliferation only increased for Group 2 between day 4 and 6. The variations between Tβ4 groups at a different times did not show a consistent pattern. Therefore, a Tβ4 treatment that yielded the most significant increase in proliferation with respect to time was not identified. This may be due to the interaction between the independent variables and the difference in Tβ4 concentrations used

for Group 2, 3 and 4, which were 1 μg/mL or 10 μg/mL. However, hMSC proliferation for Group 4 was expected to be highest at all assessment days and among experimental groups since it combined the treatments for Group 2 and 3. Therefore, further research is necessary to determine an efficient Tβ4 concentration and/or treatment that will promote hMSC proliferation in PEO/BLG nanofiber scaffolds. In addition, it is also necessary to develop methods to evaluate Tβ4 interaction with PEO/BLG nanofiber scaffolds.

Despite seeding hMSCs on top of the PEO/BLG scaffolds, hMSCs migrated and adhered to the bottom of the cell culture dish for all experimental groups suggesting that the hMSCs did not integrate with the scaffolds. Thus, this investigation only showed that PEO/BLG nanofiber scaffolds do not affect hMSC viability and Tβ4 can increase hMSC proliferation given these culturing conditions.

There are multiple limitations that may have influenced the outcome of this study. Cell culture replicates are independent samples and their proliferation can be affected by many factors such as variation in cell seeding density, scaffold thickness and culture medium volume. In addition, variations on replicates also arise from the nature of samples being independent and using a small number sample size may not represent the true behavior of hMSC. This contributes to the standard deviation for the mean value for proliferation for each experimental group.

The scaffolds were observed to take on a concave structure in the culture dish when medium was added. The scaffold's edge adhered to the culture well's wall at the top of the culture medium volume with scaffold centers reaching the bottom of the well. Variations in scaffold size can limit the available area in the culture well for cells to adhere and proliferate. Also, tissue-grade culture dishes were used for this study which indicates the resulting low of

integrations of hMSC to PEO/BLG nanofiber scaffolds. Tissue-grade culture dishes are functionalized with negative charges on culturing surface. Compared to the distributions of amount of negatively charged amino acids within the primary structure of BLG, negative charges on the culturing surface are free to electrostatically interact with positively charged cell-wall binding proteins in hMSC. In addition to hydrogen-bond interactions among the primary structure of proteins, polar and charged amino acids play a major role in defining the secondary structure and tertiary structure of proteins. Therefore, this further decreases the availability of free amino acid charges in BLG/PEO electrospun nanofibers to interact with hMSC binding proteins. To improve this study, non-tissue grade culture dishes, smaller scaffolds and less culture medium should be considered to improve hMSC interactions with PEO/BLG scaffolds.

4.4. Conclusion

The effects on cell viability and proliferation due to seeding and culturing hMSCs onto PEO/BLG nanofiber scaffolds and PEO/BLG nanofiber scaffolds treated with Tβ4 were demonstrated. HMSC cultures were prepared and kept for 8 days. HMSCs did not integrate into the nanofiber scaffolds; however, PEO/BLG scaffolds did not have a negative effect on hMSC viability. HMSC proliferation increased for the Tβ4 treated groups at different levels. This study reports on using PEO/BLG nanofibers as TE constructs for the first time, and demonstrates that hMSC cell proliferation increased throughout the culture period demonstrating their potential for future research in TE. Challenges ahead and research directions for using PEO/BLG scaffolds for TE may include: determining how to integrate cells into the scaffold, determining an effective Tβ4 concertation that will promote cell proliferation, determining an alternative crosslinking mechanism to expedite scaffold readiness for TE purposes and modifying the

electrospinning device to produce and investigate PEO/BLG scaffolds with different nanofiber architecture.

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APPENDIX A – Electrospinning Solution Preparation Protocol

1. Introduction

Electrospinning is a process in which liquid solutions are used to produce solid structures by exposing the solutions to an electric field. Depending on the electrospinning device set-up and type of electrospinning solution, thin fibers can be produced within the micro- and nano-scale. Set-up simplicity and relative ease-of-use has made electrospinning a favorite among the modalities for producing thin fibers. This has allowed the development of numerous applications throughout different engineering disciplines including scaffolds for tissue engineering (TE) applications.

A previous study by Sullivan *et al.* reported the on the formulation and parameters to produce electrospun nanofibers from solutions containing poly(ethylene Oxide) (PEO) and β-lactoglobulin (BLG). Sullivan's formulation was modified by adding sodium trimetaphosphate (STMP) and sodium hydroxide (NaOH) to investigate their potential as an *in situ* chemical crosslinker. The formulation for making PEO/BLG/STMP solutions including STMP at 2, 4, 6 and 8% (w/v) into Sullivian's formulation for PEO/BLG electrospinning solution are described in the following sections

2. PEO/BLG/STMP Electrospinning Solution preparation

Materials

- Ultra-pure deionized water
- Poly(ethylene oxide) powder
- β-lactoglobulin powder
- Sodium trimetaphosphate powder
- 0.02 % Rhodamine-B

- 10 w/v % Sodium hydroxide
- Sterile 50 mL glass containers with lid (2x)
- Stir plates (2x)
- Sterile stir bars (2x)

Protocol

Aliquot 20 mL of ultra-pure deionized water into two 50 mL containers and place stir bar into the container. Place containers on stir plates and turn on the stir plates to approximately 120 rpm. Measure 8 w/v % PEO and 12 w/v % BLG powder using an electronic scale, approximately 1.6 g and 2.4 g, respectively. Slowly add PEO powder into one container and BLG into the second container. Secure tops and allow the powders to dissolve for approximately one hour or until the BLG powder has completely dissolved. Combine both solutions by pouring the BLG solution into the PEO solution, note that the PEO powder may not be fully dissolved or may be have formed droplets. Add 10 w/v % NaOH at a 1:10 volume-to-volume ratio (NaOH:PEO/BLG solution), stir solution overnight (approximately 12 hours). On the following morning, add the desired amount of STMP (2-8 w/v %). Rhodamine-B (RHB) is a dye which was used to stain the nanofibers and facilitate imaging with confocal laser scanning microscopy. Calculate the desired RHB concentration and add the corresponding RHB volume to the electrospinning solutions. For these experiments, RHB was added to obtain a concentration in the range of 0.0001-0.0005 v/v % RHB. RHB is photo-sensitive, cover the container with aluminum foil and stir the solution until the STMP has completely dissolve (approx. 2 hours). The solution is ready for electrospinning.

APPENDIX B – PEO/BLG Scaffold Preparation for hMSC Cultures and Thymosin-β4 Coating Protocol

1. Introduction

Nanofibers were electrospun from aqueous poly(ethylene oxide) (PEO) and β-Lactoglobulin (BLG) solutions. PEO/BLG solutions consisting of 8 w/v % PEO, 12 w/v % BLG (1:1 mixture) and 0.0001 v/v % Rhodamine-B. PEO/BLG electrospun nanofibers were evaluated as a stem cell construct for potential tissue engineering applications. Step-by-step instructions are provided for preparing electrospun nanofibers into cell-culture ready scaffolds. This section describes the following methods:

- Nanofiber preparation for crosslinking
- Crosslinking process
- Scaffold sterilization
- Scaffold hydration and thymosin-β4 (Tβ4) coating

2. Nanofiber Preparation for Crosslinking

Materials

- Scissors
- Electrospun nanofibers on collector
- 14 mm diameter circular punch (corresponding area of a 24-well plate)
- Scalpel (No. 10 blade)
- High precision needle tip and flat tip tweezers
- Heat-resistant containers with lids

Nanofibers accumulate onto an aluminum collector, which covers the collector base, during electrospinning. Once the electrospinning is completed, carefully removed the aluminum collector from the collector base. Trim the aluminum collector and dispose of the areas where there was no nanofiber accumulation. Gently place the collector into its corresponding sample bag, be sure to do this with extreme caution so the nanofibers are not damaged. You may either store the aluminum collector in a dark place for future experiments or proceed to prepare the nanofibers for crosslinking.

Nanofiber Scaffold Preparation

- Remove the aluminum collector from the sample bag and place on the bench top
- Starting on an edge, outline 14 mm circle by gently pressing the punch onto the

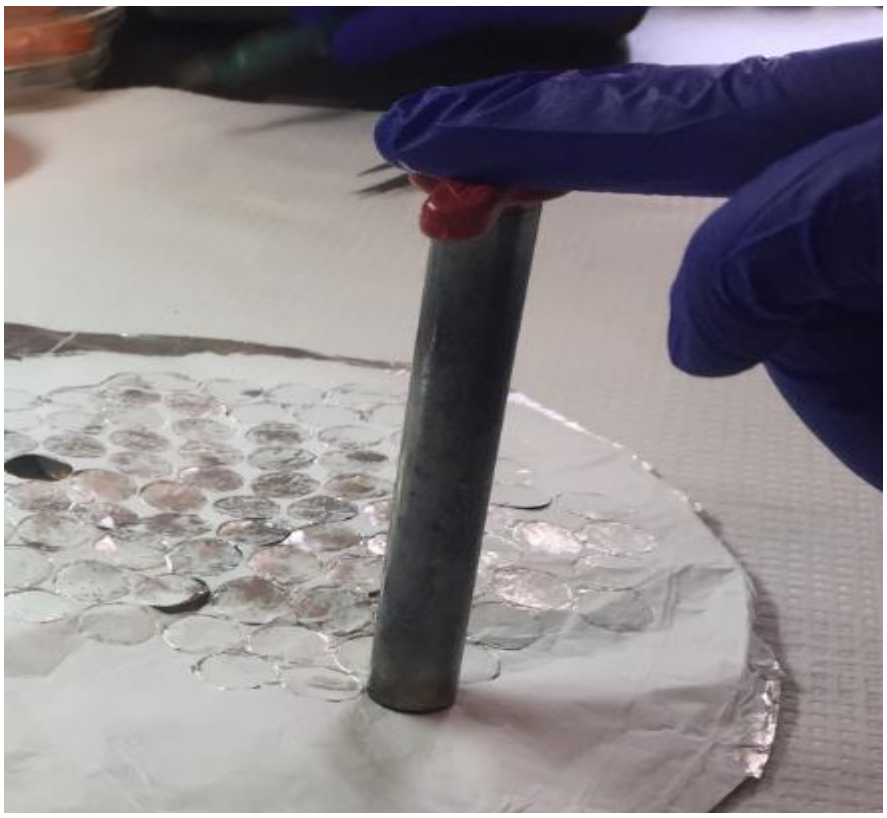

Figure B.1: Outlining PEO/BLG nanofiber scaffolds with punch

aluminum collector (Figure B.1).

- Cut along the punch's circumference using a scalpel to produce a circular scaffold sample
- Lift the punch up and collect the scaffold sample using tweezers. If the scaffold sample gets trapped inside the punch, use needle tip tweezers to tilt the scaffold sample onto its edge, then retrieve the scaffold sample from within the punch.
- Gently load scaffold sample into the heat-resistant containers. Do not stack scaffold samples. Scaffold samples are extremely light weight and tend to drift with air currents produced by body movement. Stainless steel surgical containers with lids (Figure B.2) are recommended to prevent the scaffolds samples from drifting in the air. Surgical containers can be used for crosslinking.

Figure B.2: Scaffold arrangement for crosslinking

- Work towards the center of the aluminum collector as you continue cutting scaffold samples. Repeat process until the desired number of scaffold samples have been cut.
- If any uncut nanofibers left, place the aluminum collector back into the sample bag and store in a dark place for future experiments.

3. Scaffold Crosslinking and Sterilization

Materials

- Scaffold samples (14 mm diameter) in heat-resistant trays
- Gravity convection oven
- Multi-well plates (24-well)
- Sterile high precision needle tip tweezers
- Biological hood equipped with UV-light

Turn the oven on and set the temperature to 100°C on LOW. Allow for the temperature to stabilize. Place the cut scaffold samples in heat-resistant trays. Crosslink the scaffold samples for 96 hours. After crosslinking, turn the oven off and let it cool down to room temperature. Remove trays containing the crosslinked scaffold samples from the oven.

Post-Crosslinking Sterilization

From this point on all work should be done in a biological hood using standard sterile techniques

- Turn the biological hood ON. Allow the blower to run for 5-10 minutes
- Place multi-well plates and tweezers in the biological hood
- Remove scaffold tray(s) from the oven and place it in the biological hood
- Remove the multi-well plate from its packing

- Remove the lid from the multi-well plate
- Load scaffolds into wells, one scaffold per well, make sure that the scaffold lays flat on the well's bottom
- Once the multi-well plates are loaded, remove any tools and plastic containers from the biological hood
- Turn the UV-Light ON and expose for at least 12 hours
- After the first 12 hours, carefully turn the multi-well plate(s) upside down, turn the UV-light ON and expose for at least 12 hours.

4. Scaffold Hydrations and Thymosin-β4 coating

Materials

- 1x Phosphate buffer saline (PBS)
- Thymosin-β4 (Tβ4)
- 1000 µL pipet and pipet tips
- Multi-well plates with UV-sterilized scaffolds

Protocol

Hydrate scaffolds by pipetting 300 µL of PBS onto the scaffold, repeat for each well, then add an additional 200 µL of PBS to each well; each well should contain 500 µL PBS. Use a new pipet tip for each well. When hydrating the scaffolds, pipet PBS into the well by touching the pipet's tip onto the well's wall. Note that the scaffold may rotate as the PBS is pipetted into the well due to momentum. Place scaffolds into a 5% CO_2 atmosphere incubator and allow scaffolds to hydrate for 24 hours. After hydration, remove 350 µL PBS from the wells (3- 100 µL and 1-50 µL aspirations). Avoid aspirating the scaffold into the pipet tip by pushing the scaffold edge towards the well's center or by placing the pipet tip under the scaffold. Once the PBS has been

removed use the pipet tip to adjust the scaffold so it covers the entire well. At this point the scaffolds are hydrated and cell-culture ready.

For Tβ4 coated scaffolds, Tβ4 is added to PBS and used for hydration and coating. Prepare the Tβ4 coating solution by dissolving (or diluting) Tβ4 stock solution in PBS to a concentration of 10 μg/mL Tβ4-PBS. Pipet 200 μL of 10 μg/mL Tβ4-PBS into each well as described above. Place scaffolds into a 5% CO_2 atmosphere incubator and allow scaffolds to hydrate for 24 hours. After Tβ4-coating hydration, add 300 μL PBS to wash any unbound Tβ4 and incubate for 1 hour. Remove 350 μL Tβ4-PBS/PBS as described above

Justification for 200 μL Hydration volume & removing 350 μL PBS after Hydration

From previous preliminary experiments, we determined that 200 μL of hydration solution is the minimum volume that will not cause the scaffold to curl into itself when hydrating or coating with Tβ4. Furthermore, after hydrating, we sought to determine the maximum amount of hydration solution that when aspirated from the well no air (bubbles) was aspirated, subsequently determining the amount of hydration solutions absorbed by the scaffold. Using 500 μL of hydration solution, we determined that, on the average, scaffolds absorb 150 μL of hydration solution; no air was aspirated when removing 350 μL (100 μL three times and 50 μL once) of hydration solution.

APPENDIX C – hMSC Seeding, Cultures and Proliferation

1. Introduction

Human mesenchymal stem cells (hMSCs) viability on electrospun nanofiber scaffolds was assessed. Step-by-step instructions for seeding and culturing hMSCs on electrospun scaffold and conducting proliferation assays is provided. In this experiment, hMSCs were cultured for 8 days. Proliferation was assessed at Day 2, 4 and 8.

2. Cell Seeding and Cultures

Materials

- Frozen Adult hMSCs with green fluorescent protein
- Complete media – Dulbecco's Modified Eagle Medium (DMEM) with 16.5 % Fetal bovine serum (FBS) and 1% Penicillin-streptomycin (Pen-Strep)
- Thymosin-β4 (Tβ4)
- Cell counting Trypan-Blue
- Ice caddy

Protocol

- Calculate the volume of complete media needed. Account for volumes for diluting freezing media after thawing (hMSCs are frozen and preserved in a freezing media solution containing 10% DMSO, which must be diluted to < 1% DMSO after thawing), seeding and filling after seeding.
- Calculate the amounts of FBS and Pen-step needed
- Prepare complete media by mixing FBS and Pen-Strep into DMEM
- Aliquot complete media for filling and for mixing with Tβ4

- Carefully remove hMSCs from the liquid nitrogen Dewar and thaw cells using standard techniques. Information regarding cells, their location and contents of the freezing media documented in the liquid nitrogen log book.
- Add thawed cells into a 15 mL conical tube with enough complete media that will dilute the DMSO to < 1%. Note that DMSO is highly toxic to the cells, so this step should be promptly performed once cells are fully thawed.
- Place the conical tube in the centrifuge and spin cells for 5 minutes at 1500 rpm. This will pelletize the cells from the supernatant.
- Dispose of the supernatant by inverting the conical tube allowing the supernatant to drain into a collection container. The cell pellet should remain at the bottom of the tube.
- Add enough media so it approximates the desire cell concentration (cells/mL). Mix media with pipet (or vortex) so the cells become suspended
- Remove 25 μL of cell suspension and mix with Trypan-Blue at 1:1 volume ratio.
- Count cells to determine mortality ratio and live cell concentration

The cells are ready for seeding. Depending on the number of wells and cells needed, you may need to further dilute the cell suspension or if cell concentration is too low, you may need to pelletize the cells again to adjust for the correct cell concentration.

- Pipet 50 μL of cell suspension into each well
- Place culture dishes into incubator for 30 minutes to allow cells to adhere to scaffolds

There are four experimental groups: (1) scaffolds in complete media (control), (2) Tβ4-scaffolds in complete media, (3) scaffolds in Tβ4-complete media and (4) Tβ4-scaffolds in Tβ4-complete media. Scaffolds absorb 150 μL of hydration solution, cells are seeded using

50 μL of cell suspension and the final well volume is 500 μL. Therefore, groups 1 and 2 require an additional 300 μL. For groups 3 and 4, prepare Tβ4-complete media by diluting Tβ4 stock solution to a concentration of 1 μg/mL in complete media, then pipet 300 μL of Tβ4-complete media into each of well for groups 3 and 4. Place culture plates into 5% CO_2 atmosphere incubator.

3. Viability and Proliferation – PROMEGA KIT

HMSCs were cultured on Tβ4 treated- and non-treated electrospun scaffold for 8 days. HMSC proliferation was assessed on days 2, 5 and 8. The following protocol was used for each time point.

Materials

- Complete media – Dulbecco's Modified Eagle Medium (DMEM) with 16.5 % Fetal bovine serum (FBS) and 1% Penicillin-streptomycin (Pen-Strep)
- Thymosin-β4 (Tβ4)
- PROMEGA proliferation reagent

Protocol

The day before conducting the proliferation assay, exchange the media for the wells that will be analyzed. Therefore, calculate and prepare complete media and Tβ4-complete media. On the following day, remove 200 μL of media leaving the cell cultures in 300 μL media. The PROMEGA kit calls for 20 μL of proliferation reagent per 100 μL of cell culture media; therefore, each well will require 60 μL of proliferation reagent. PROMEGA's proliferation reagent is photo-sensitive, working with minimal light add 60 μL of proliferation reagent into each well. Place culture dishes into the incubator and incubate for 2 hours. The cell cultures will turn black or purple, this is indicative of the colorimetric reaction that the proliferation reagent

induces on the cell cultures for assessing proliferation. Remove culture dishes from incubator. Remove 200 μL from each well and pipet into a 96-well plate. Assess proliferation using a 96-well plate reader.