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Silk Fibroin-Based Scaffolds for Tissue Engineering Applications

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SILK FIBROIN-BASED SCAFFOLDS FOR TISSUE ENGINEERING

APPLICATIONS

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science in Biomedical Engineering at Virginia Commonwealth
University.

By

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Abstract

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Jennifer Morgan McCool, B.S.

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This study focused on the comparison of the electrospun silk scaffolds to the electrospun silk fibroin gel scaffolds. Moreover, this study examined the differences in cross-linking effects of genipin and methanol as well as solvents on the mechanical properties and cell compatibility of the scaffolds. Silk scaffolds were electrospun from an aqueous solution or 1,1,1,3,3-hexafluoro-2-propanol (HFIP) without genipin, immediately after 8 % (wt) genipin was added to the solution, and 18 hours after genipin blended with the solution. Uniaxial

tensile testing determined that the silk scaffolds electrospun from water exhibit a higher modulus and peak stress than that of the silk scaffolds electrospun from HFIP. *In vitro* cell culture was conducted to determine the cell compatibility of the various silk fibroin-based scaffolds. 4'-6-Diamidino-2-phenylindole (DAPI) staining and histology suggest that genipin may enhance cell compatibility, and that neither ethanol nor methanol inhibit cell interactions.

Introduction

Tissue Engineering

Tissue engineering is a cross-disciplinary field that combines applications in genetics, cell and molecular biology, biochemistry, biomedical engineering and materials science. Synthetic or naturally derived engineered biomaterials are utilized to replace damaged or defective tissues such as bone, cartilage and skin, and even organs [1]. Tissue scaffolds are designed to be a temporary structure onto which new tissue grows and eventually replaces the implanted scaffold. These acellular tissue scaffolds can be tailored in the laboratory to help target medical conditions and wounds that would be detrimental to a patient's health and recovery. Scaffolds combined with drug delivery techniques provide advanced medical therapies as well. Acellular tissue scaffolds can be fabricated using a technique known as electrospinning. This method can be used to tailor the various properties of the scaffold to better suit the application for which it is intended.

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Electrospinning

Electrospinning is a technique that fabricates nano-scale nonwoven materials for the use of tissue engineering. It incorporates a high voltage power source with a translating and rotating mandrel and a polymer of choice. Figure 1 depicts a typical electrospinning setup with a grounded collector.

High Voltage Power Supply

Electrospinning Jet
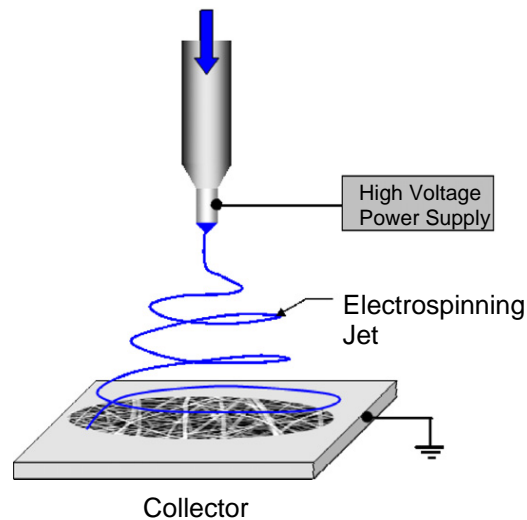
Collector

Figure 1: A typical electrospinning setup with a grounded collector [2].

Fiber formation is caused by the electrostatic repulsion between the charges at the surface of the solution droplet, as well as by the force generated by the electric field between the needle tip and the target [3]. The various process parameters, such as flow rate, voltage, rotation, air gap distance and solution concentration, can be altered to tailor the scaffold's properties. The size and shape of the mandrel used determines the size and shape of the scaffold as well. A variety of synthetic and natural polymers produce quality electrospun scaffolds for tissue engineering applications [4]. These polymers can be electrospun alone or blended together to help modify the properties of the

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scaffold. The type of polymer(s) and fabricating conditions altered the porosity, mechanical properties, biocompatibility and degradation rate. In terms of electrospun silk fibroin scaffolds, the fiber diameters are controlled by altering the solution concentration, viscosity, electric field, type of solvent, air gap distance, flow rate and the diameter and angle of the spinneret [5].

Silk as a Biomaterial

Silk fibroin is a natural polymer that has been used in textiles for centuries. More recently, it has been used for medical and tissue engineering applications due to its high tensile strength and biocompatibility. In nature, silk fibroin is coated with a gum-like protein, sericin, which must be removed for silk fibroin purification. Sericin acts like an adhesive that is meant to help maintain the structure of the cocoon [6]. However, in terms of using silk as a biomaterial, sericin can cause an adverse immune response if implanted [7]. The gummy sericin is easily removed by boiling the cocoons in water with salts. Figure 2 shows a scanning electron micrograph of a silk fibroin protein covered with sericin.
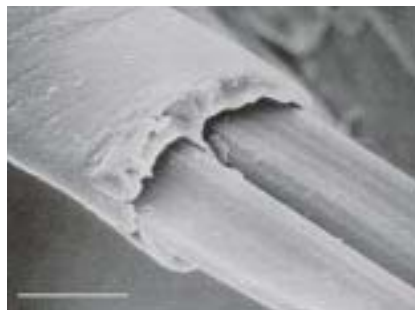
Figure 2: A scanning electron micrograph of a silk fibroin protein and sericin coating [8].

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The fiber consists of two cores of fibroin covered with a layer of sericin [6]. Silk fibroin consists of a heavy and light chain (350 kDa and 25 kDa), that are linked together by a disulfide bond [9]. Overall, silk fibroin is a negatively charged protein at a neutral pH and has an isoelectric pH of about 3.8 [10]. Hydrophobic interactions cause the protein's random coil formation to change to a β-sheet formation, which is responsible for the protein's exceptional tensile strength [11].

Electrospinning Silk Fibroin

Silk fibroin was first electrospun from HFIP by Shahrzad Zarkoob in 1998 and patented in 2000 [12, 13]. Soon after, Sukigara reported the effects of the various electrospinning parameters on the morphology and fiber diameter. He found that the silk fibroin concentration played a key role in producing uniform fibers [5, 14]. In 2002, Jin *et al.* successfully electrospun silk fibroin from an aqueous solution. This was achieved by adding poly(ethylene oxide) (PEO) to the silk solution in order to increase the viscosity [15]. Further studies, however, suggested that residual PEO in the silk fibroin scaffolds inhibited cell attachment and proliferation as well as adversely affecting the mechanical properties of the scaffold [16]. Regenerated silk fibroin electrospins as a random coil structure, but β-sheet formations is achieved by treating the scaffold with methanol or other cross-linking agent [17].

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Silk Fibroin Gel

Silk fibroin, when in solution, can transition into a gel when certain conditions are changed. If the pH decreases, the concentration increases or the temperature increases, the silk fibroin molecules will change from the random coil formation to a β-sheet formation and the solution will gel. The regenerated silk fibroin solution becomes very unstable at a low pH and at high temperatures which will trigger the transition. The mechanical properties and pore sizes of the hydrogel can be manipulated by altering the concentration of the regenerated silk fibroin solution as well [9]. The transition can be triggered from the addition of calcium chloride with or without the combination of water or ethanol, the addition of ethanol, methanol or PEO, sonication, freeze drying, salt leaching or gas foaming [18-23]. The gel is a porous white opaque gel that can withstand compression. Silk fibroin gel can be used for tissue engineering applications due to its strength, biocompatibility and porous structure.

Silk for Tissue Engineering Applications

Silk fibroin is an important natural polymer for tissue engineering because of its natural strength, biocompatibility, slow degradation rate, good water vapor and oxygen permeability, minimal inflammatory response and ability to be used in several forms [11, 24]. In addition, the resulting silk fibers

are thermally stable up to 245°C which ensures its stability at body temperature [25]. Silk fibroin can be used as an electrospun scaffold, hydrogel, or film. It can be applied to vascular, bone or ligament engineering with its many options. Table 1 displays examples of regenerated silk fibroin as a tissue engineering material.

Table 1: Examples of silk's utility as a matrix material in tissue engineering [26]

Form	Supported cell type *in vitro*	Comments
Film	L-929 mouse Fibroblast	Comparable growth rates to collagen films
Film	SE1116 (human colon adenocarcinoma); KB (human mouth epidermoid carcinoma); Colo201 (human colon adenocarcinoma); QG56 (human lung carcinoma)	Comparable growth rates to collagen films as well as rates of protein production of carcinoembryonic antigen (CEA)
Film	Saos-2 (human osteoblast-like cells)	Bone formation was evident on fibroin films, but was enhanced on RGD-coupled matrices
Film	hBMSC (human bone marrow stromal cells)	Supports bone nodule formation from adult stem cells
Fibers	hBMSC; human adult anterior cruciate ligament fibroblasts	Supports ligament specific development *in vitro*

Vascular Tissue Engineering

Silk fibroin scaffolds are a feasible option for vascular grafts with their unique mechanical properties and flexibility. Silk fibroin produces tubular grafts that are porous and exhibit a high tensile strength which would be suitable for vascular applications. A porosity of 80% and above is ideal for endothelialization of vascular grafts [27]. Furthermore, tubular silk scaffolds electrospun out of formic acid can resist up to 575 mmHg, which is more than four times the upper physiological pressure of 120 mmHg, and twice that of pathological upper pressures of 180-220 mmHg [28]. A downfall of using

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organic solvents, such as formic acid and HFIP, is that traces of the organic solvents may be present in the electrospun scaffolds which in turn may affect cytocompatibility. For vascular applications, silk may be electrospun out of an aqueous solution with the addition of PEO. Human aortic endothelial cells and coronary artery smooth muscle cells have been successfully cultured on electrospun silk scaffolds. However, the addition of a large quantity of PEO may eventually affect the structural integrity and stability of the vascular graft, making PEO another adverse residual as are organic solvents [29]. Studies show that electrospun aqueous silk scaffolds promote aortic endothelial cell and arterial smooth muscle cell growth and proliferation while withstanding vascular pulsating pressure [30, 31].

Bone Tissue Engineering

Electrospinning silk fibroin out of water provides a way to introduce growth factors and other components into the scaffold. In this way, silk fibroin scaffolds are a potential polymer for bone tissue engineering. When bone morphogenetic protein-2 (BMP-2) or nanoparticles of hydroxyapatite (nHAp) are incorporated into the electrospinning solution, the *in vitro* bone formation from mesenchymal stem cells greatly increased [32]. Such proteins and nanoparticles may not withstand the electrospinning process if the scaffolds were electrospun out of organic solvents. To increase the viscosity of the aqueous silk solution, PEO may be added to aid in the electrospinning process.

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Studies show that while electrospun silk scaffolds support bone marrow stromal cell attachment and proliferation, residual PEO may initially inhibit cell attachment. However, after a few days in growth media, PEO will automatically be extracted from the scaffolds and cell attachment and growth will commence [16]. Silk hydrogels are also suitable for bone engineering with its porous structure and low inflammatory response like nonwoven mats [11]. Moreover, the mechanical properties of silk scaffolds make it a desirable candidate for bone tissue engineering.

Ligament Tissue Engineering

Ligament tissue engineering also benefits from silk-based scaffolds. An ideal scaffold for ligament tissue engineering must be biodegradable, porous, mechanically strong and promote the formation of ligament tissue. Silk-based scaffolds meet all of these requirements. Studies show that anterior cruciate ligament (ACL) fibroblasts and mesenchymal stem cells grow well on silk scaffolds as well as combined knitted silk scaffolds and silk sponges [7, 33, 34]. The versatility of silk as a biomaterial as well as its controllable mechanical properties makes it a strong candidate for ligament and tendon tissue engineering. After sericin extraction, bundles of silk fibers can be wound into cords and arranged to make the ligament matrix. This configuration is similar to that of the collagen fibers found in ligaments and tendons. The twisted structure provides the scaffold the mechanical integrity close to that of native

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ligament. A maximum load of 2337 ± 72 N, an elastic modulus of 354 ± 26 N/mm and a strain at failure of 38.6 ± 2.4% have been observed from these silk matrices, which are similar to anterior cruciate ligament (ACL) [35]. The silk matrices demonstrate the natural ligament and tendon structure while retaining the biocompatibility of silk scaffolds. Vunjak-Navakovik *et al.* designed a six-cord silk fiber matrix that decreased the scaffold stiffness while maintaining the tensile strength. Moreover, the configuration allowed more void space (>90%) for enhanced tissue infiltration and increased surface area for cell proliferation and tissue growth [36].

Mechanical Properties

Silk is a very versatile biomaterial with its significant crystallinity, high elasticity, strength and toughness, and resistance to failure in compression (even compared to Kevlar). The combination of the β-sheet crystals, the interphase between the crystals, the semi-crystalline regions and the shear alignment of the molecular chains are the basis for silk's unique mechanical properties. While the highly organized β-sheet regions of the protein provide the tensile integrity, the semi-crystalline regions are the basis for the protein's elasticity [26]. The β-sheet structure affects the tensile properties, degradation rate and elasticity of the scaffold, so the tailoring of these properties can be done in part with the cross-linking process. The transition depends on the length of time exposed to the solvent as well as the solvent concentration.

Methanol treatment is a widely used process to induce β-sheet formation although it does not transform all molecular regions. Ethanol, 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide [3] hydrochloride (EDC), glutaraldehyde or genipin also cause the transition from random coils to β-sheet configurations [37-40]. Studies show that regenerated silk fibers can hold their initial tensile integrity for 21 days under immune deficient *in vitro* culture conditions [41]. Moreover, the solvent used for electrospinning can effect the β-sheet formation of the scaffold's secondary structure, which in turn can alter the mechanical properties. Formic acid, HFIP and water have been used to electrospin silk scaffolds, and of those, water and formic acid seem to enhance the mechanical properties of the scaffolds [42, 43].

Degradation

According to the US Pharmacopia, an absorbable biomaterial loses most of its tensile strength 60 days post-implantation. Even though silk is considered non-degradable by this definition, it does in fact degrade but over a longer period of time. Silk will lose most of its tensile strength within a year *in vivo*, and will be unrecognizable at the implantation site within 2 years. However, the rate of degradation depends on the animal model and the tissue implantation site. Silk is considered biodegradable due to its vulnerability to bacterial and enzymatic degradation. Studies show that proteases will cleave

the protein at the less-crystalline regions after which the resulting peptides can be phagocytosed by the cell [26, 37].

The solvent used to electrospin the silk scaffold may affect the degradation of the scaffold *in vitro* and *in vivo*. Yongzhong Wang and colleagues demonstrated that electrospinning from an aqueous solution instead of an organic solvent like HFIP can increase the degradation rate while promoting cell proliferation and penetration. The silk scaffold electrospun from an aqueous solution degraded between 2 and 6 months while those electrospun out of HFIP lasted over a year. Furthermore, methanol treatment can significantly decrease the degradation rate [37, 44].

There is a wide variety of biocompatible polymers used for tissue applications aside from silk fibroin. However, the degradation rates of other polymers cannot be tailored within such a high range as that of silk. Collagen, which is a widely used biomaterial, degrades between 1 to 4 weeks and sometimes longer depending on the cross-linking process [45]. Polycaprolactone (PCL) can last within the body for more than 2 years [46]. Another synthetic polymer, poly(lactic-co-glycolic acid) (PLGA) (85:15) usually degrades within 26 weeks, while PLGA (50:50) degrades between 6 and 8 weeks *in vitro* [47-49]. Silk scaffolds, however, can be modified to have similar degradation rates by changing the solvent for electrospinning [44].

Purpose of Study

This study compares the physical, mechanical and *in vitro* properties of electrospun regenerated silk fibroin and silk fibroin gel. The effects of solvents, specifically water and HFIP, as well as cross-linking agents were also investigated. The dry porosity and mean fiber diameters of the electrospun scaffolds were compared, as well as the peak stress and modulus of the samples. Human dermal fibroblasts were also cultured under static conditions to determine any cytocompatibility differences among the various types of silk-based scaffolds.

Materials and Methods

Extraction and Purification of Silk Fibroin

Bombyx mori (B. mori) silkworm cocoons were obtained from The Yarn Tree (Brooklyn, New York). Silk fibroin extraction and purification were performed following an established protocol [16]. The silkworm cocoons were cut into pieces and added to a boiling aqueous 0.02M Na_2CO_3 (Sigma Aldrich) solution to remove the sericin coating. After 30 minutes, the silk fibroin was washed thoroughly in deionized (DI) water. The raw silk fibroin air dried overnight and then added to a 9.3M LiBr (Fisher Scientific) solution and placed in a 60˚C oven for 4 hours. The silk-LiBr solution was then inserted into dialysis tubing (3500 Molecular Weight Cut Off) (Fisher Scientific) and dialyzed against DI water. The water was refreshed 6 times over the course of 3 days. The aqueous silk solution was centrifuged at 9000g at 10˚C for 20 minutes twice to remove any impurities. Half of the aqueous silk solution was stored at 4˚C while the other half was lyophilized and stored at -20˚C.

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Electrospinning Aqueous Silk Fibroin Solution

50 mg/mL PEO (900,000 MW) (Sigma Adrich) aqueous solution was added to the 80 mg/mL aqueous silk solution to make a 90:10 silk:PEO weight:volume solution. The solution remained on a shaker plate at room temperature overnight to ensure thorough blending. Prior to electrospinning, 0.5 mL PEO in ethanol was electrospun onto a rectangular metal mandrel (2.5 cm wide x 10.2 cm long x 0.3 cm thick) to help the aqueous solution electrospin properly. The aqueous solution was loaded into a 10 mL Becton Dickinson syringe with a blunt-end 18 gauge needle and dispensed at a rate of 2 mL/hr by a KD Scientific syringe pump. A charging voltage of 30 kV was applied to the needle and a grounded 15.2 cm diameter aluminum target behind the mandrel. The mandrel rotated at 400 rpm covering a distance of 6 cm/s with an air gap distance of 28 cm between the needle and mandrel. All electrospinning was conducted at room temperature. The samples were immediately cut off the mandrel using a razor blade and stored in a desiccator chamber. The solutions that included genipin (Wako), 8% (wt) genipin was added to the solution and remained at room temperature for either up to an hour or for 18 hours prior to electrospinning. The operating parameters remained the same. Scanning electron micrographs (Zeiss EVO 50 XVP) were taken of the scaffolds and the fiber diameters were determined using ImageTool software 3.0 (UTHSCSA).

Electrospinning Silk Fibroin out of HFIP

For the solutions electrospun out of HFIP (TCI America, Inc), PEO was not used to make the solution more viscous. Moreover, PEO was not electrospun onto the mandrel first to assist in electrospinning. 80 mg/mL lyophilized silk fibroin was measured and dissolved in HFIP (TCI America). The solution remained at room temperature overnight on a shaker plate prior to fabrication. 3 mL silk solution was loaded into a 5 mL syringe and an 18 gauge blunt end needle was attached. The high voltage power supply was attached directly to the needle and the target was grounded. The operating parameters included a charging voltage of 30 kV applied to the needle, a grounded target, 28 cm air gap and a flow rate of 10 mL/hr. Electrospinning was conducted at room temperature and the samples were stored in a desiccator chamber. For the solutions that incorporated genipin, 8% (wt) genipin was added to the solution and left to blend at room temperature for either up to an hour or for 18 hours. The operating parameters remained the same.

Electrospinning Silk Fibroin Gel out of HFIP

50 mL aqueous silk fibroin solution was stored at room temperature and gelled within 3 days. The gel was lyophilized and dissolved in HFIP to make an 80 mg/mL solution. The solution was left overnight on a shaker plate to ensure thorough blending. The solution was loaded onto a 3 mL syringe and

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an 18 gauge needle was attached to the tip. The high voltage power supply was attached to the needle and another voltage supply was attached to the target. The fabrication parameters included: airgap, +30 kV applied to the needle, -10 kV charging voltage applied to the target, a 10 mL/hr flow rate and a distance of 20 cm between the needle and the mandrel. Electrospinning was conducted at room temperature and the samples were stored in a desiccator chamber. For the samples that incorporated genipin, 8% (wt) was added to the solution and blended at room temperature for either up to an hour or for 18 hours prior to fabrication. The operating parameters remained the same.

Uniaxial Tensile Testing

Dog bone-shaped samples (2.75 mm wide at the most narrow space and 7.5 mm long) were punched out of the electrospun samples. The average sample thickness was 185 ± 44 µm and all samples were measured using a micrometer (Mitutoyo Corp). The dog bones were treated with either methanol or ethanol for one hour and then PBS for another hour prior to testing. The samples (n>4) were tested to failure on a MTS Bionix 200 testing system (MTS Systems Corp) at an extension rate of 10.0 mm/min. The elastic modulus and peak stress were calculated by the MTS software TestWorks 4.0 and recorded.

Scaffold Porosity

The porosity of the scaffolds were measured to determine if there is a significant difference between electrospinning from HFIP versus an aqueous solution as well as a difference between electrospun silk fibroin and silk fibroin gel. Six 10 mm diameter discs were punched out of dry, uncross-linked scaffolds from each group (54 samples total). The dry thickness and mass of each disc were measured and the porosity was calculated using the following equation:

$$\text{Void Fraction} = 1- \left[\frac{\text{Calculated Scaffold Density}}{\text{Known Material Density}} \ \text{x100} \right]$$

The porosity measurements were calculated using Microsoft Excel 2000.

***In vitro* Cell Culture**

To evaluate the effects of the various cross-linking agents as well as solvents, *in vitro* cell culture was carried out for 7 and 28 days. Two 10 mm discs were punched out of each sample and were disinfected with either ethanol or methanol and then rinsed in PBS. The discs were placed in 48-well plates with cloning rings and were seeded with 50,000 human dermal fibroblasts (Cascade Biologics). The scaffolds were soaked in DMEM-F12 (Invitrogen Corp.) supplemented with 10% fetal bovine serum and 1%

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penicillin-streptomycin (10,000 units/mL each) (Gibco BRL Life Technologies) in an incubator under standard culture conditions (37˚C and 5% CO_2). The media was changed every 3 days and after the allotted time period the scaffolds were taken out of culture, cut in half, and placed in formalin. One half was sent to Harris Histology Relief Services for histological evaluation (hematoxylin and eosin stain(H&E) and Masons Trichrome) while the other half was used for confocal imaging using DAPI staining (Zeiss LSM META NLO multiphoton laser scanning microscope).

The H&E slides were evaluated using optical light microscopy (Eclipse TE300, Nikon) and all images were taken at 10x. DAPI staining determined the extent of cell growth and proliferation across the surface of the scaffolds. The scaffolds are washed with PBS and then soaked with DAPI stain (1 µg/mL solution) for 5 minutes. The samples are then washed with PBS again and placed on microscope slide for viewing. All images were taken at 10x.

Statistical Analysis

Statistical analysis was performed on the porosity and uniaxial tensile testing using the JMP IN 8.0 statistical software (SAS Institute, Inc). The analysis of the mean porosity as well as the modulus and peak stress was a one-way analysis of variation and a Tukey-Kramer comparison with apriori level of significance set at $\alpha=0.05$. The averages and standard deviations were also determined and graphically depicted using Microsoft Excel 2000.

Results

Electrospinning Aqueous Silk Solution

Electrospinning 80 mg/mL aqueous silk solution with 10% PEO resulted in randomly oriented, non-woven scaffolds. 90:10 silk fibroin:PEO yielded an average outside surface fiber diameter of 300 ± 130 nm and an inside surface fiber diameter of 540 ± 220 nm (Figure 3). The addition of 8% (wt) genipin to the solution within an hour of electrospinning made an average outside surface fiber diameter of 450 ± 140 nm and an inside surface average fiber diameter of 500 ± 240 nm (Figure 4). Electrospinning the aqueous silk solution with 8% (wt) genipin after 18 hours of blending yielded an average outside surface fiber diameter of 540 ± 250 nm and an average inside surface fiber diameter of 550 ± 180 nm (Figure 5).
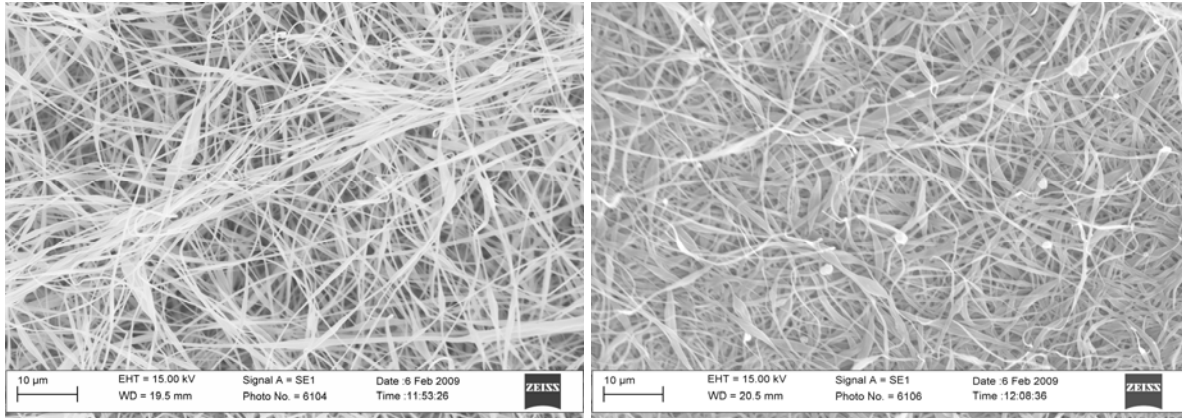
Figure 3: 90:10 Silk Fibroin:PEO outside (left) and inside (right) surfaces at 3000x.
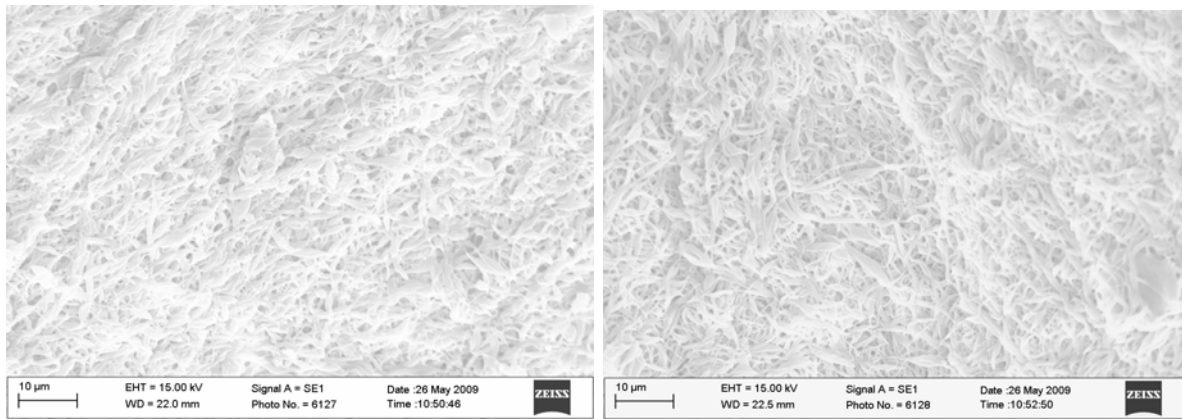

Figure 4: 90:10 Silk Fibroin:PEO with 8% (wt) genipin electrospun immediately outside (left) and inside (right) surfaces at 3000x.
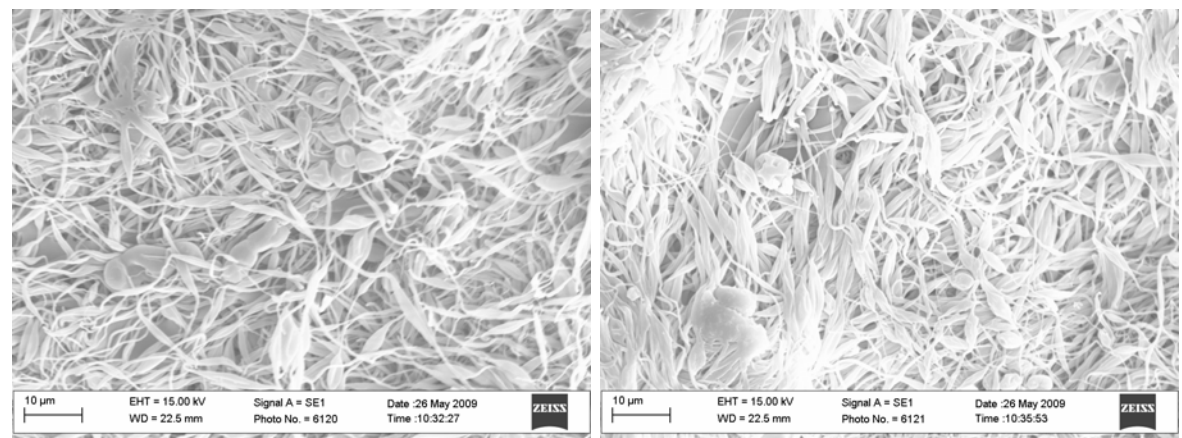

Figure 5: 90:10 Silk Fibroin:PEO with 8% (wt) genipin electrospun after 18 hours outside (left) and inside (right) surfaces at 3000x.

Electrospinning Silk Fibroin out of HFIP

Electrospinning 80 mg/mL silk fibroin out of HFIP yielded nonwoven fibrous scaffolds with an average outside surface fiber diameter of 318 ± 303 nm and an average inside surface fiber diameter of 298 ± 265 nm (Figure 6). The addition of 8% (wt) genipin within an hour of electrospinning the silk fibroin made a scaffold with an outside surface fiber diameter of 500 ± 250 nm and inside surface average fiber diameter of 510 ± 360 nm (Figure 7). Electrospinning silk fibroin with 8% (wt) genipin after 18 hours of blending at ambient temperature yielded a scaffold with an average outside surface fiber diameter of 450 ± 180 nm and an average inside surface fiber diameter of 370 ± 120 nm (Figure 8).
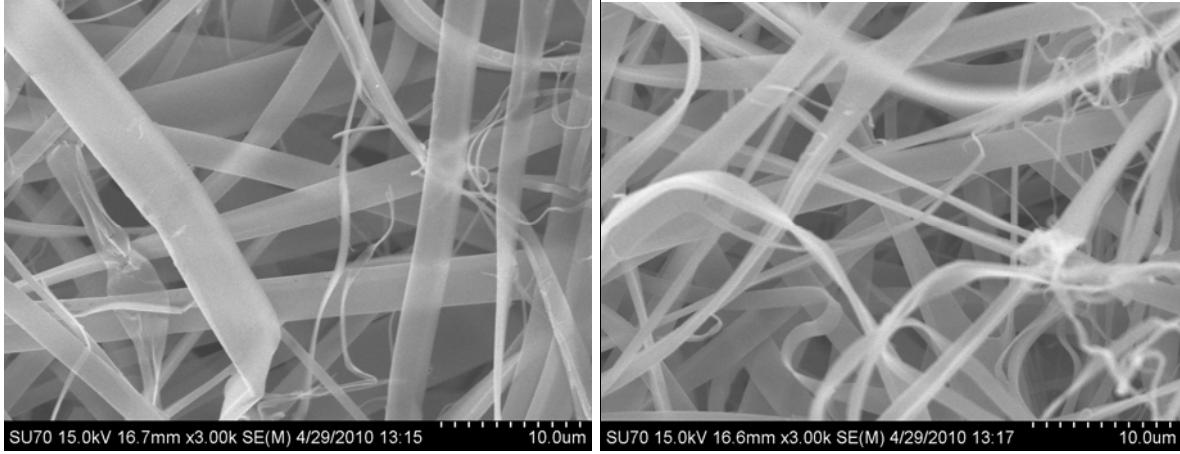
21

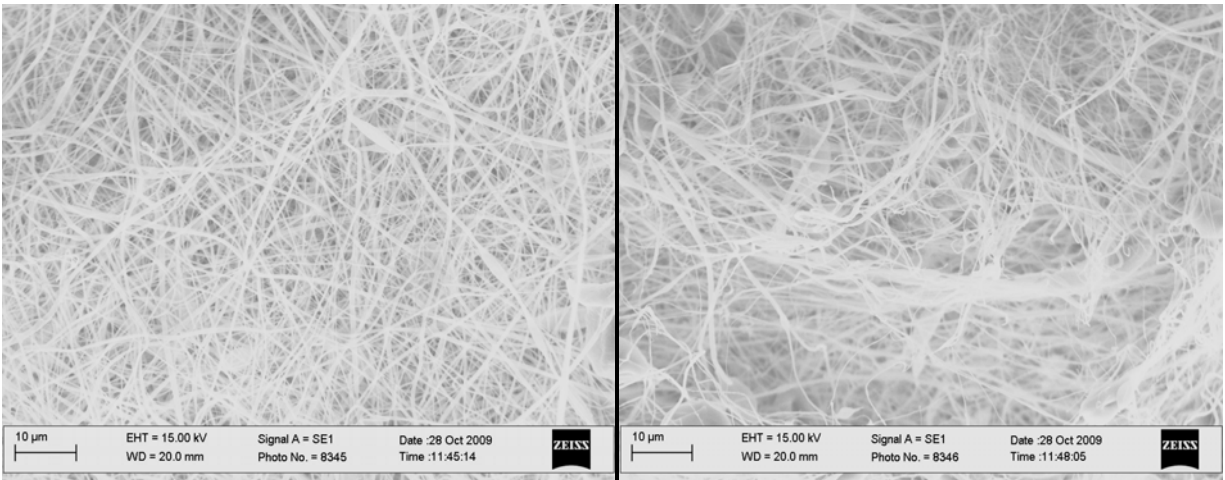Figure 6: 80mg/mL Silk Fibroin outside (left) and inside (right) surfaces at 3000x.

Figure 7: 80mg/mL Silk Fibroin with 8% (wt) genipin electrspun within an hour of blending outside (left) and inside (right) surfaces at 3000x.
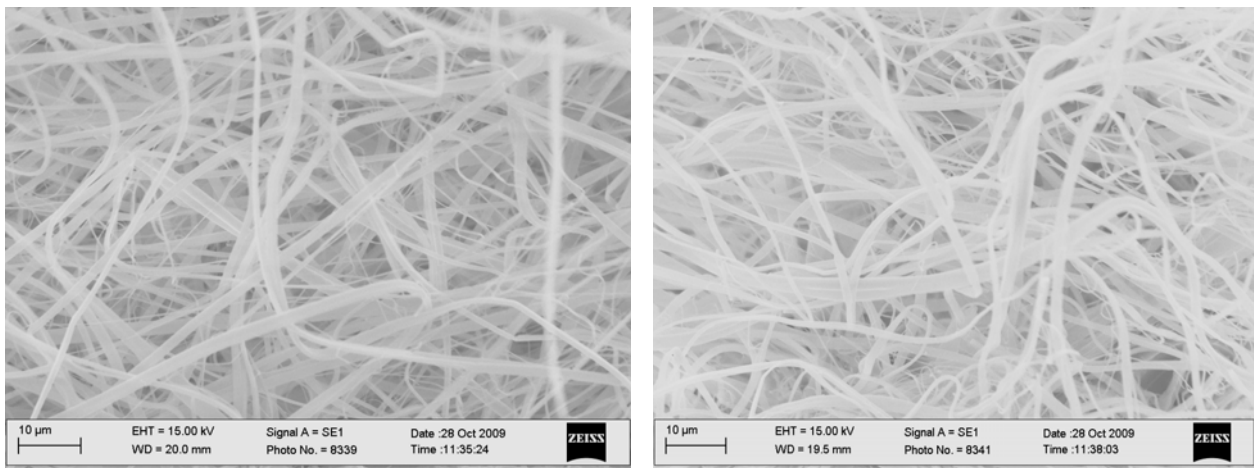

Figure 8: 80mg/mL Silk Fibroin with 8% (wt) genipin electrospun after 18 hours of blending outside (left) and inside (right) at 3000x.

Electrospinning Silk Fibroin Gel out of HFIP

Electrospinning silk fibroin gel out of HFIP yielded a nonwoven fibrous scaffold with an outside surface average fiber diameter of 720 ± 360 nm and an average inside surface fiber diameter of 510 ± 200 nm (Figure 9). The addition of 8% (wt) genipin within an hour of electrospinning made a scaffold with an average outside surface fiber diameter of 690 ± 370 nm and an average inside surface fiber diameter of 510 ± 200 nm (Figure 10). The electrospun 80 mg/mL silk fibroin gel with 8% (wt) genipin with 18 hours of blending yielded a scaffold with an average outside surface fiber diameter of 870 ± 560 nm and an average inside surface fiber diameter of 710 ± 310 nm (Figure 11).
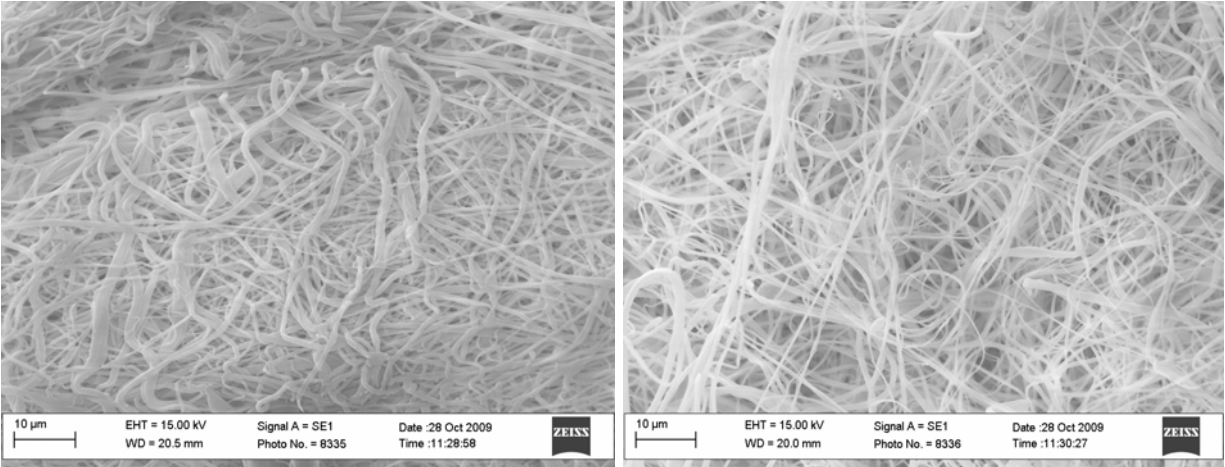
Figure 9: Electrospun 80mg/mL Silk Fibroin Gel outside (left) and inside (right) at 3000x.
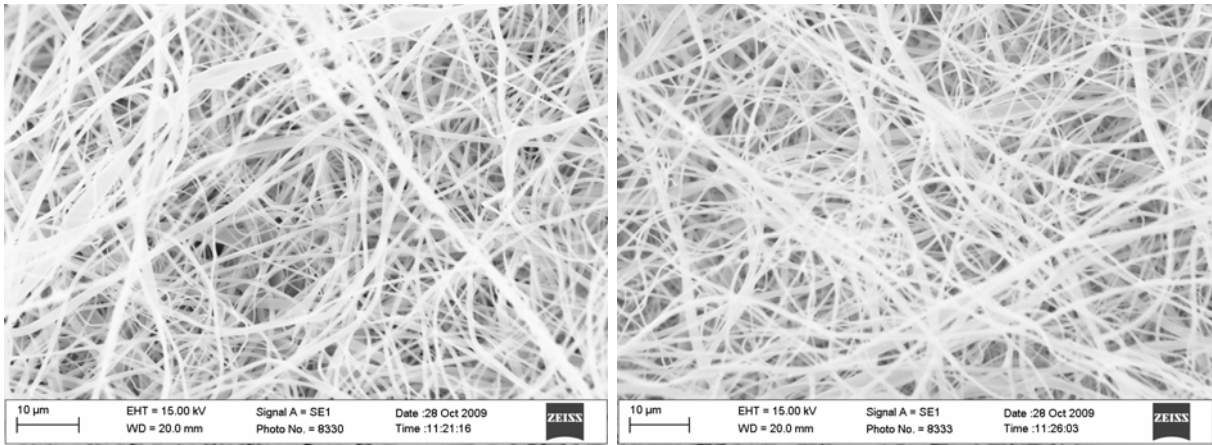

Figure 10: 80mg/mL Silk Fibroin Gel with 8% (wt) genipin electrospun immediately outside (left) and inside (inside) surfaces at 3000x.
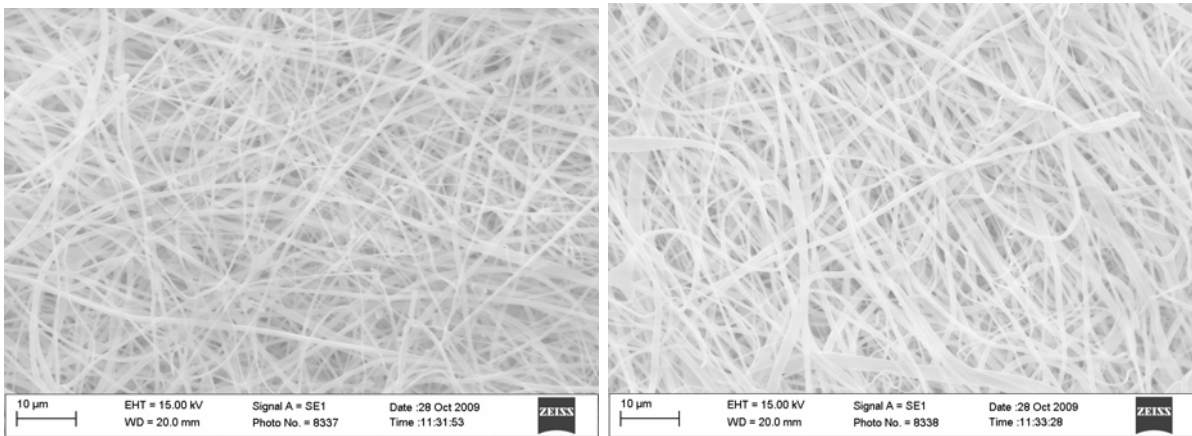

Figure 11: 80mg/mL Silk Fibroin Gel with 8% (wt) genipin electrospun after 18 hours outside (left) and inside (right) surfaces at 3000x.

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Figure 12 compares the various electrospun scaffolds' average fiber diameters and standard deviations. As seen in Figure 12, there is some variation in fiber diameters among the various scaffolds. The silk scaffolds electrospun out of water have the more uniform average fiber diameters, even after the incorporation of genipin. The smallest average fiber diameter was 300 ± 130 nm (Silk:PEO, inside surface) and the largest being 550 ± 180 nm (Silk:PEO with genipin electrospun after 18 hours, outside surface). There is a decrease in the average fiber diameter for the silk scaffolds electrospun out of HFIP after the addition of genipin. Without genipin, the average fiber diameter was 860 ± 680 nm (inside surface) and drops to 370 ± 120 nm after genipin has blended with the solution for 18 hours (inside surface).

Electrospun silk fibroin gel experienced a slight increase in the average fiber diameter after genipin was added to the solution. Without genipin, the scaffold's mean fiber diameter was 510 ± 200 nm (inside surface), while electrospinning the solution after 18 hours of blending with genipin generated an average of 870 ± 560 nm (inside surface).

Average Fiber Diameters of Silk:PEO, Silk and Silk Fibroin Gel
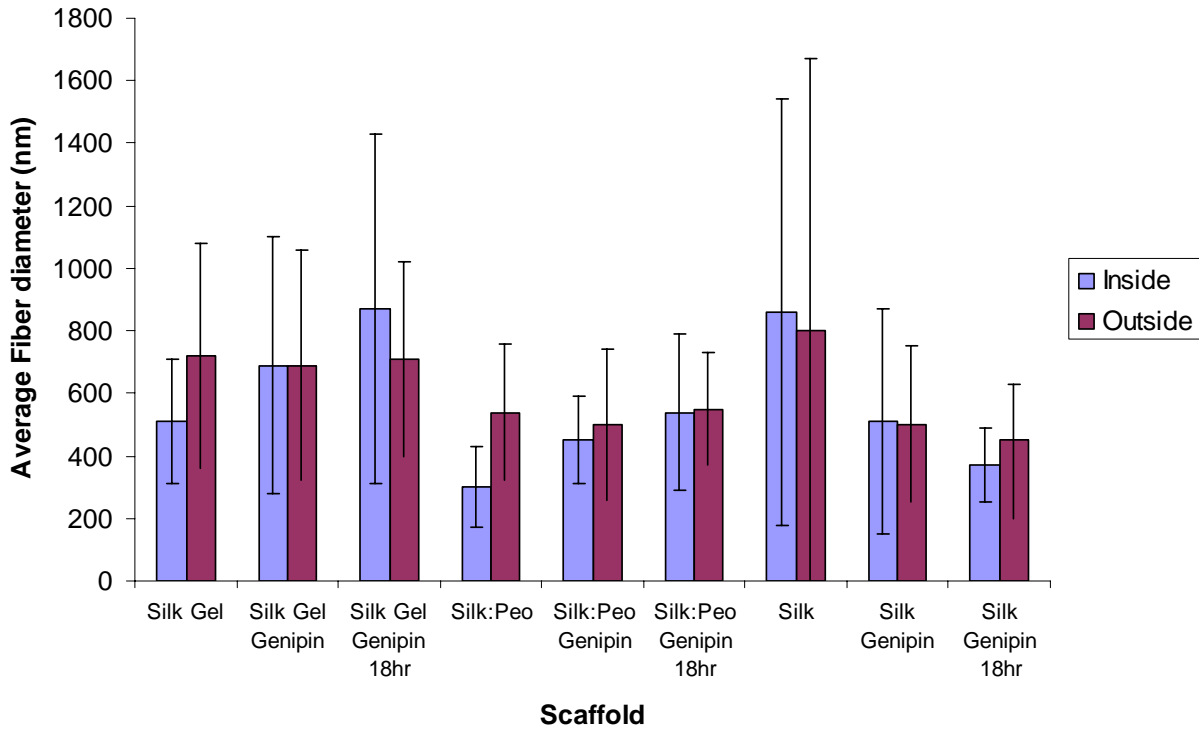
Figure 12: Compares the average inside and outside surface fiber diameters for the various scaffolds.

Uniaxial Tensile Testing

The uniaxial tensile testing compared the effects of solvents and cross-linking agents on the mechanical properties of the silk scaffolds. Figure 13 compares the peak stress of the silk scaffolds and silk fibroin gel electrospun out of HFIP. There were no statistically significant differences among the mean peak stresses among the scaffolds electrospun out of HFIP ($p < 0.05$).

Peak Stress of 80mg/mL Silk Fibroin Gel vs Silk after Methanol and Ethanol Soaks
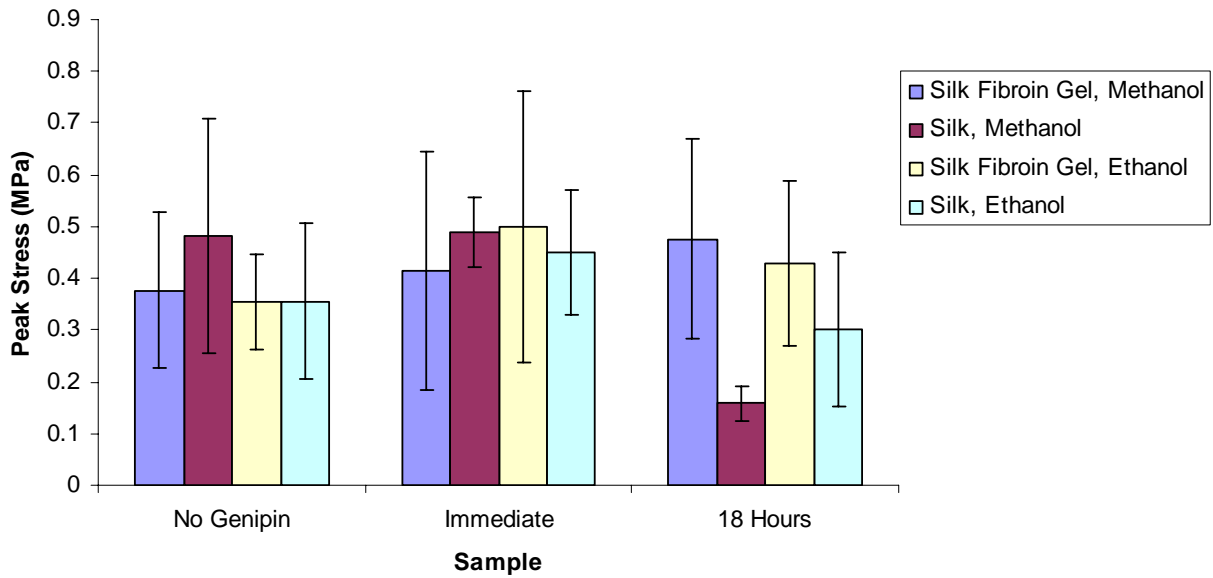
Figure 13: Compares the peak stress of silk fibroin and silk fibroin gel with and without genipin after ethanol and methanol soaks.

There were similar results with the modulus, which are displayed in Figure 14. There was not a significant difference in the modulus or peak stress when the genipin was added to the solution prior to electrospinning. Additionally, there was not a significant difference between the peak stress and modulus of the scaffolds electrospun from HFIP that were soaked in ethanol before testing and those that were soaked in methanol. The moduli among those scaffolds ranged from 2.4 ± 3.3 MPa (silk electrospun out of water with genipin after 18 hours, cross-linked with methanol) down to 0.26 ± 0.04 MPa (silk electrospun out of HFIP with genipin after 18 hours, cross-linked with methanol). The only scaffolds that were significantly different than the others

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were the silk electrospun out of water and cross-linked with methanol and those treated with ethanol.

Modulus of 80 mg/mL Silk Fibroin Gel vs Silk after Methanol and Ethanol Soaks
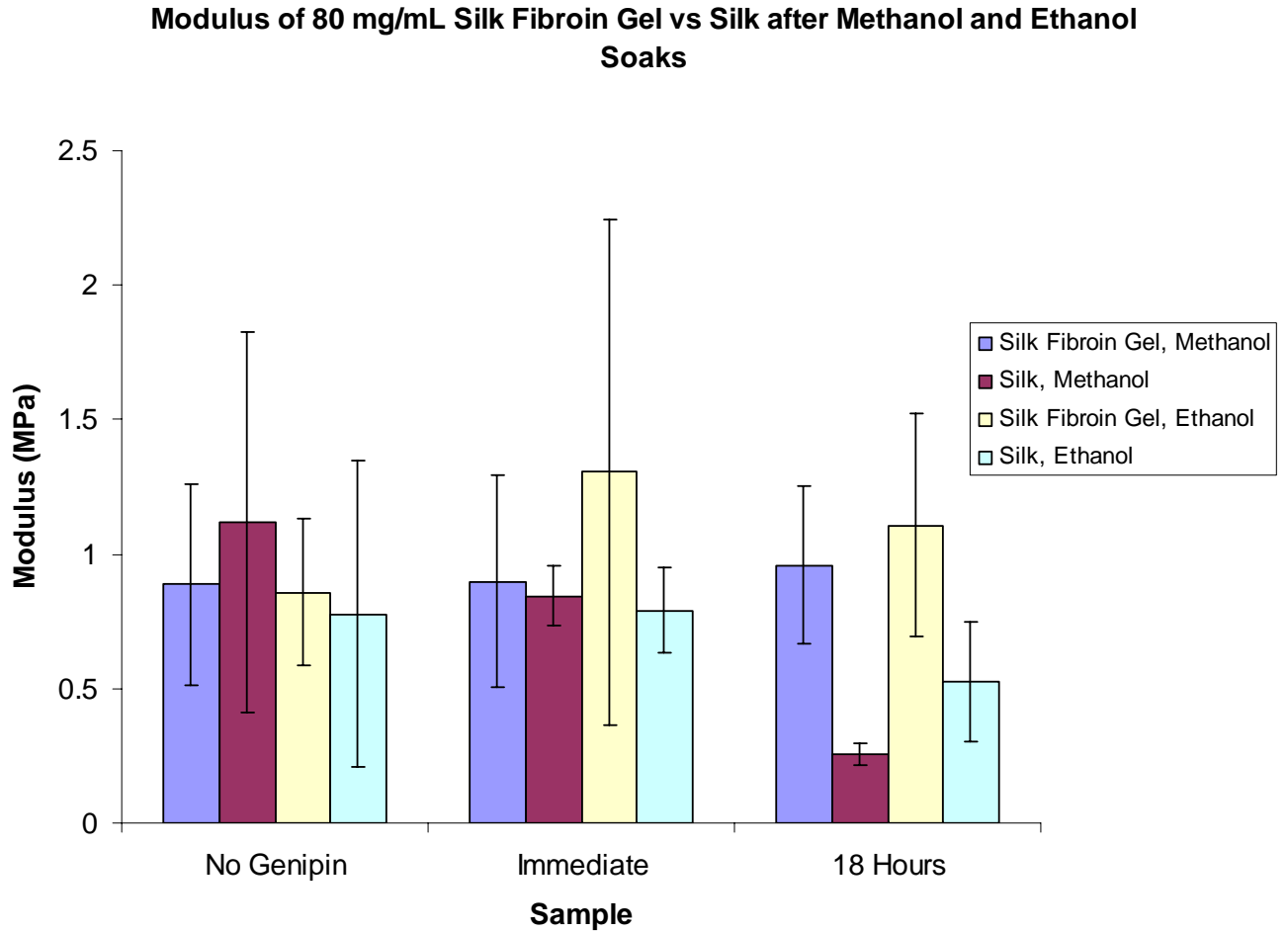
Figure 14: The modulus of silk fibroin and silk fibroin gel with and without genipin after methanol and ethanol soaks.

When comparing the peak stress of silk electrospun out of HFIP to that of silk electrospun out of water, there was a greater difference as seen in Figure 15. There is a significant difference ($p < 0.05$) of the peak stress of the silk scaffold electrospun out of water as opposed to silk electrospun out of HFIP (denoted by *). The silk scaffolds electrospun out of water and cross-linked with

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methanol had a mean peak stress of 2.0 ± 1.4 MPa. The rest of the scaffolds had mean peak stress values ranging from 0.7 ± 0.6 MPa (silk electrospun out of water and cross-linked with ethanol) to 0.13 ± 0.09 MPa (silk electrospun out of water with genipin, cross-linked with ethanol).

Peak Stress of 80mg/mL Silk Fibroin vs Silk in Water after Methanol and Ethanol Soaks
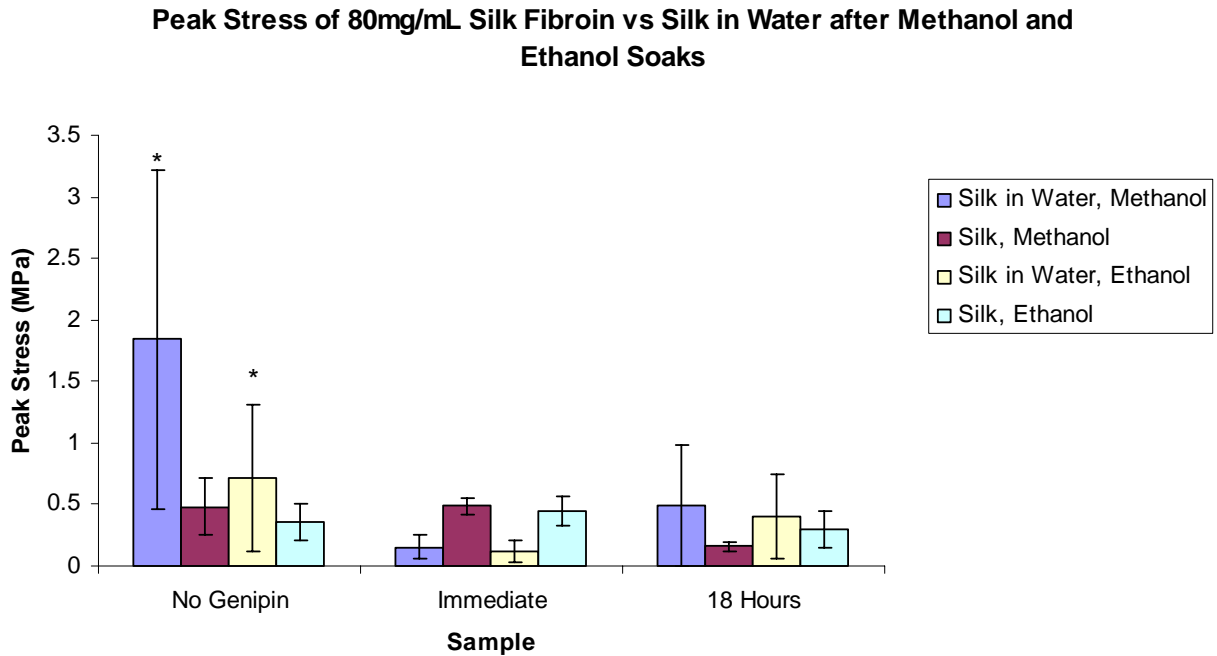
Figure 15: Comparison of peak stress between silk electrospun out of HFIP and an aqueous silk solution.

The same trend was apparent for the modulus as well, as shown in Figure 16. There was a significant difference (p<0.05) in the modulus of the samples electrospun out of water and cross-linked with methanol as well as ethanol (denoted by *). The mean modulus of the scaffolds treated with methanol was 11.8 ± 6.6 MPa. The average modulus for the scaffolds soaked in ethanol was 5.5 ± 4.8 MPa. The results shown in Figure 16 suggest that soaking the scaffold in ethanol is almost as effective at cross-linking (or fixing)

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the samples prior to testing. It also suggests that electrospinning the silk fibroin scaffolds out of water may enhance the mechanical properties more than the addition of genipin.

Modulus of 80 mg/mL Silk Fibroin vs Silk in Water after Methanol and Ethanol Soaks

Figure 16: Compares the modulus of silk fibroin samples electrspun out of water and out of HFIP.
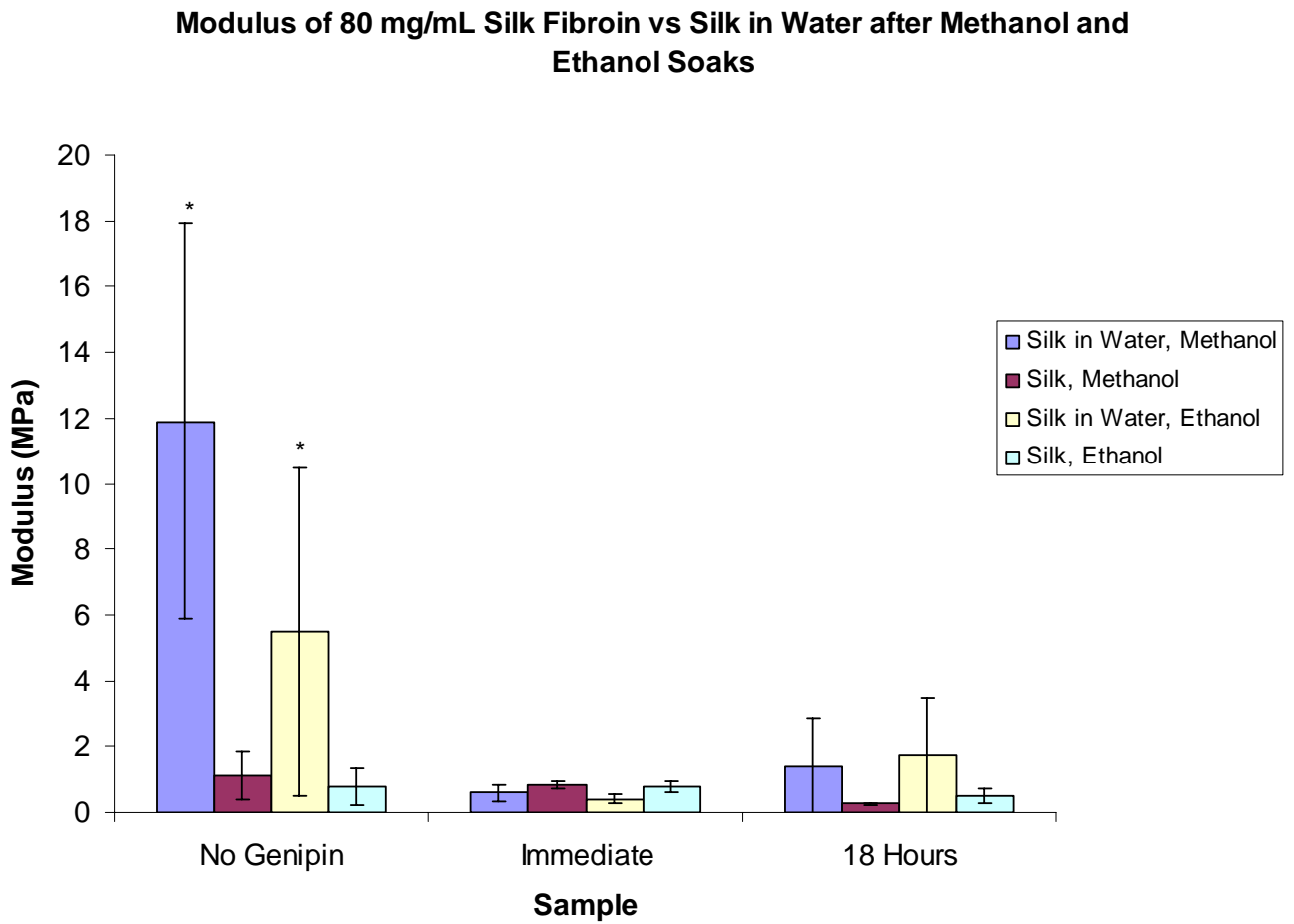
Porosity

While all of the scaffolds exhibited around a 90% porosity, the silk fibroin gel electrospun immediately after genipin was added to the solution was significantly more porous ($p < 0.05$, denoted by *) than the scaffolds electrospun from water without genipin, with genipin added immediately before fabrication, electrospun from HFIP with genipin added prior to electrospinning, as well as the electrospun silk gel with and without genipin. Additionally, the silk scaffolds electrospun from HFIP was significantly more porous than the samples electrospun from water with genipin added just before fabrication. Figure 17 shows the mean porosities of the scaffolds. The porosity was calculated only on the dry scaffolds and was not repeated after the scaffolds had been hydrated. In addition, the PEO was still present in the silk scaffold electrospun out of water. Blending the silk solutions briefly prior to fabrication lowered the porosity of the silk:PEO scaffolds more than those electrospun out of HFIP.

There is a slight increase in porosity for the electrospun silk fibroin gel when the solution was briefly blended with genipin before fabrication. For the silk fibroin scaffolds electrospun out of HFIP and water, there was a slight decrease in porosity when the genipin was introduced, but increased again with the prolonged blending. This suggests that blending the genipin with the electrospinning solution longer prior to fabrication may enhance the porosity of the silk fibroin scaffolds.

Porosity of Silk Fibroin vs Silk Fibroin Gel
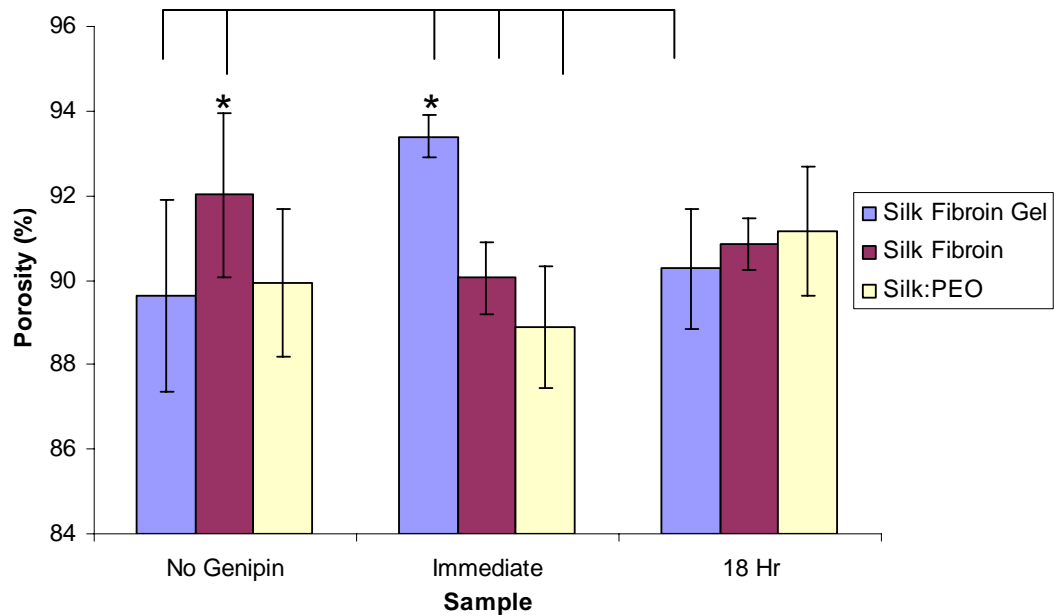
Figure 17: Shows the average dry porosity of the scaffolds.

In vitro Cell Culture

Histology

The histology provided some insight on the amount of cell penetration into the scaffold over the course of the 7 and 28 days. Figures 18 and 19 show the histology results of the silk scaffolds electrospun out of water with and without genipin. There is some cell penetration after only 7 days of static culture, especially for the silk scaffold soaked in ethanol before cell seeding (Figure 18, top left). There are a few cells seen in the scaffold after 28 days (Figure 18, top right), but not as many as the samples evaluated after 7 days.

With no obvious signs of significant cell death during the *in vitro* testing, this may be due to the handling and cutting of the samples prior to the histological evaluation. There are more cells seen in these samples (Figure 19) than those that were electrospun without genipin (Figure 18). There is also more cell penetration after 7 days in Figure 19 (top and bottom left), which may be due to the addition of genipin prior to electrospinning. However, there seems to be a decrease in cell numbers after the 28 day time point (Figure 19, top and bottom right).
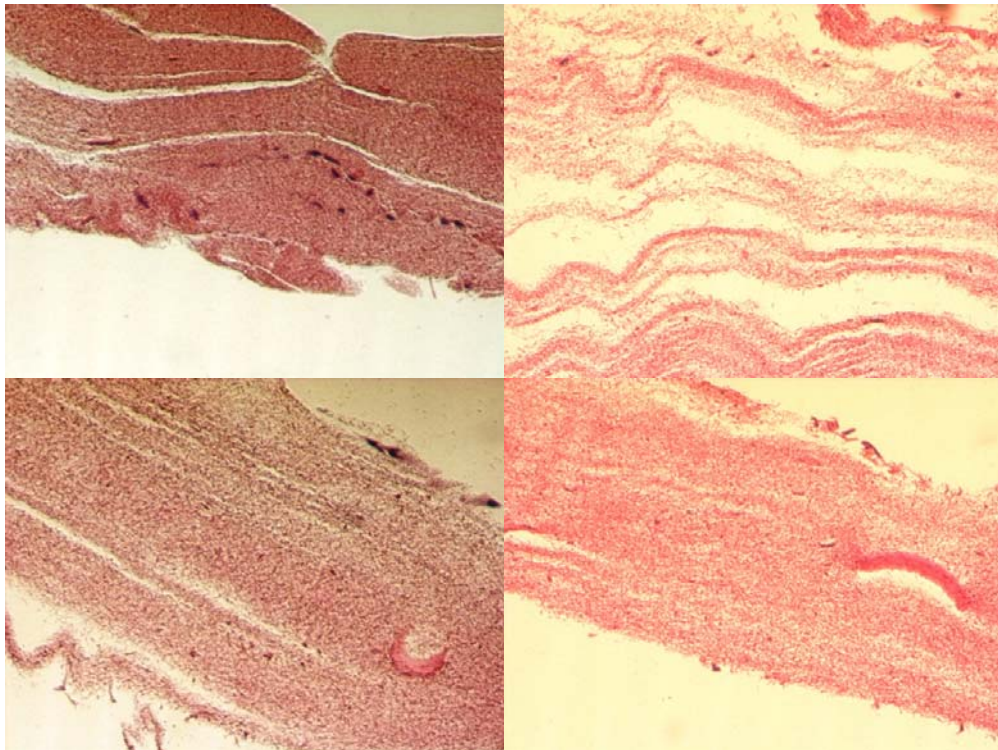

Figure 18: Silk scaffold electrospun out of water at 7 days (left) and 28 days (right) soaked in ethanol (top) and methanol (bottom) prior to cell seeding.
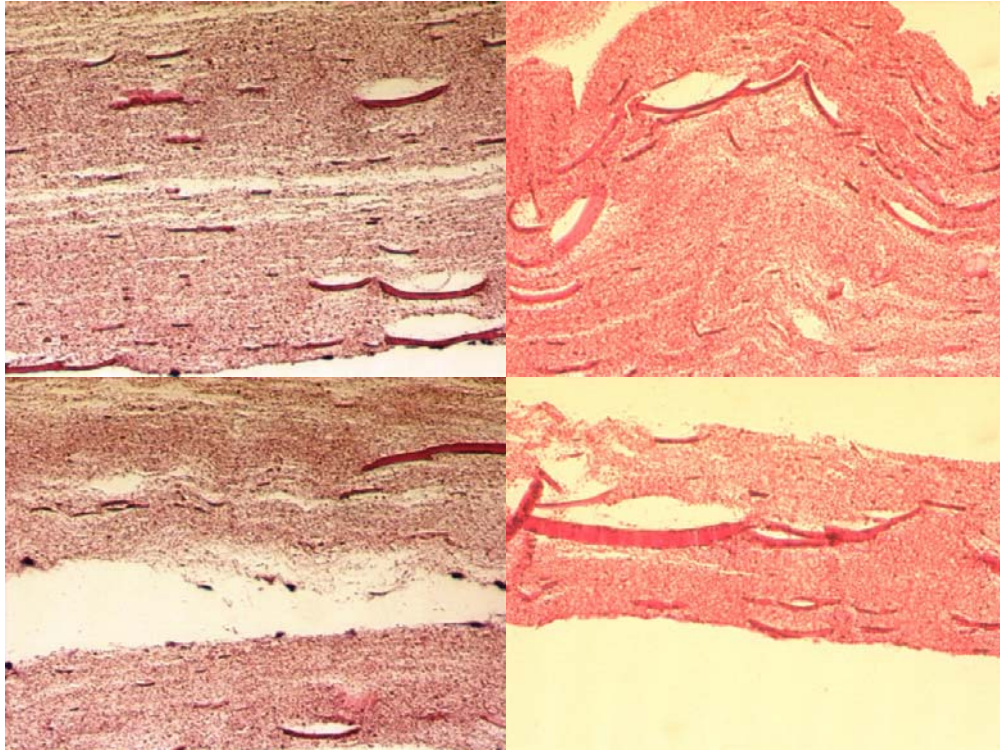
Figure 19: Silk scaffold electrospun out of water 18 hours after genipin was added at 7 days (left) and 28 days (right) soaked in ethanol (top) and methanol (bottom) prior to cell seeding.

Figures 20 and 21 display the histology results of the silk scaffolds electrospun out of HFIP with and without genipin. There was some cell migration into the scaffold after 7 days (Figures 20 and 21, top and bottom left), but not as much after the 28 days (Figures 20 and 21, top and bottom right). Again, this may be due to a difference in the handling and cutting of the samples. There were not many cells seen in these samples, but there were a few that began to migrate into the scaffolds. There does not seem to be a significant difference in the effect of the methanol and ethanol treatments prior to cell seeding in these samples. Moreover, there is not a great difference in cell proliferation or penetration in the samples electrospun out of HFIP (Figures 20 and 21) and those electrospun out of water (Figures 18 and 19).
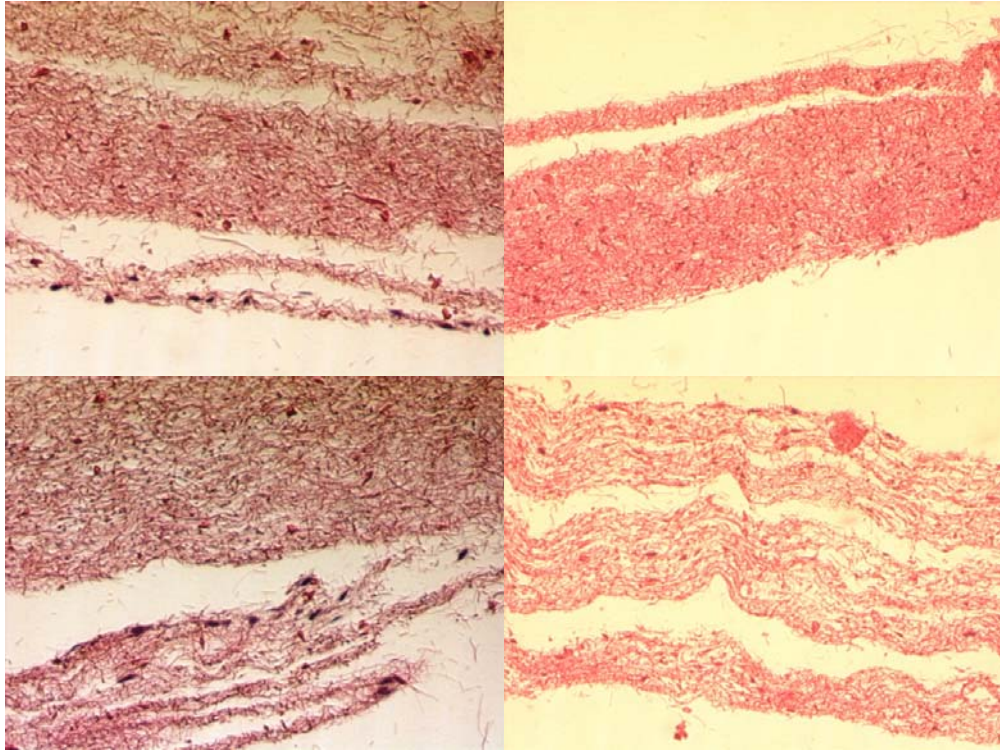
Figure 20: Silk scaffold electrospun out of HFIP at 7 days (left) and 28 days (right) soaked in ethanol (top) and methanol (bottom) prior to cell seeding.
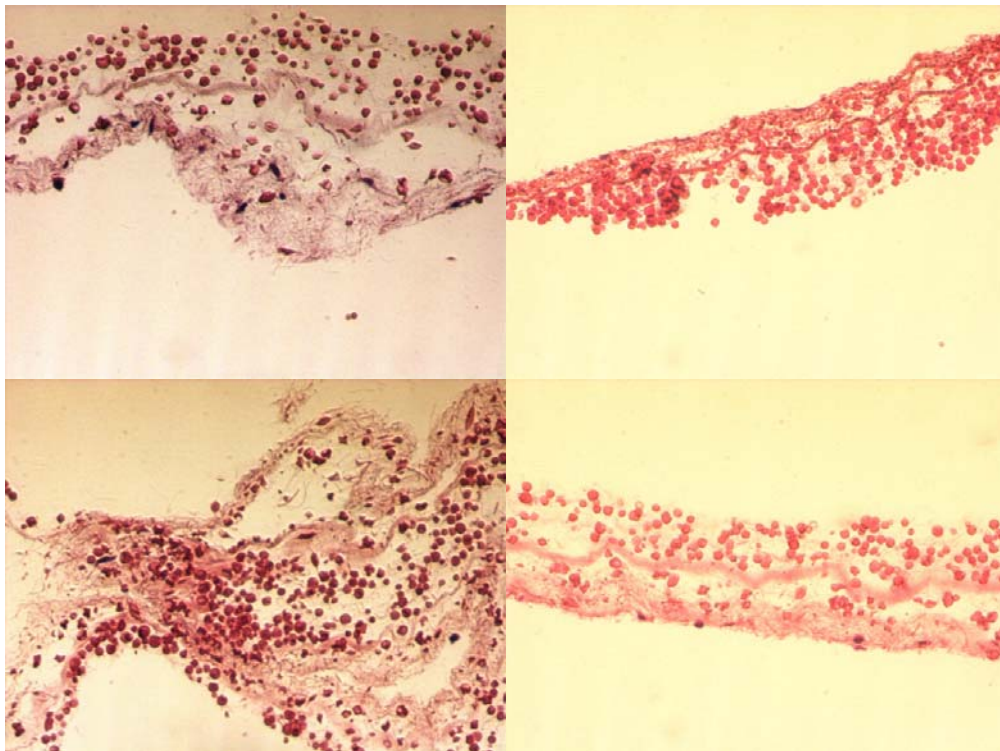

Figure 21: Silk scaffold electrospun out of water 18 hours after genipin was added at 7 days (left) and 28 days (right) soaked in ethanol (top) and methanol (bottom) prior to cell seeding.

Figures 22 and 23 show the histology results of the silk fibroin gel electrospun scaffolds. Cells migrated into the electrospun silk fibroin gel scaffolds without genipin at both time points (Figure 22). There was not a noticeable difference between the methanol (bottom) and ethanol (top) treatments either. The addition of genipin did not seem to enhance cell penetration for the electrospun silk fibroin gel scaffolds after 7 days or 28 days. There did not seem to be a significant difference between the ethanol and methanol treatments at either time point. There was noticeable cellular infiltration after 7 days in static culture after both types of alcohol soaks (Figures 22 and 23, top and bottom left). There was a slight decrease in cell counts after 28 days (Figures 22 and 23, top and bottom right), but it could be from the handling and slicing of the samples instead of cell death. The histological evaluation showed that while there was some cell penetration in all electrospun silk scaffolds, there was not an increase in cell migration after 28 days.
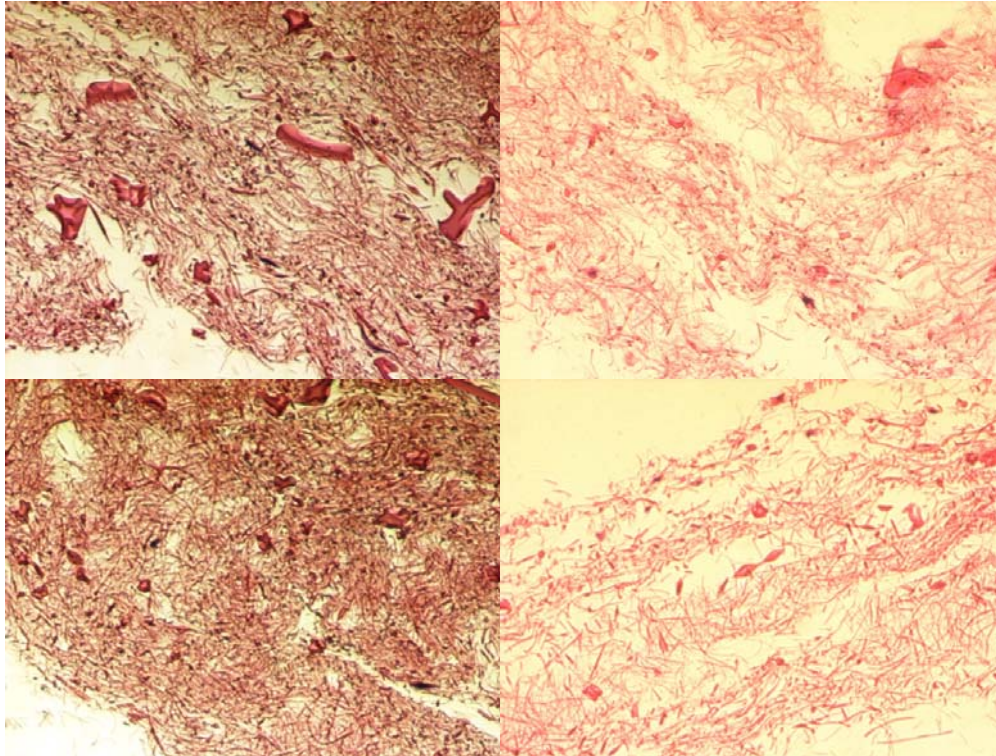
Figure 22: Silk fibroin gel electrospun out of HFIP at 7 days (left) and 28 days (right) soaked in ethanol (top) and methanol (bottom) prior to cell seeding.
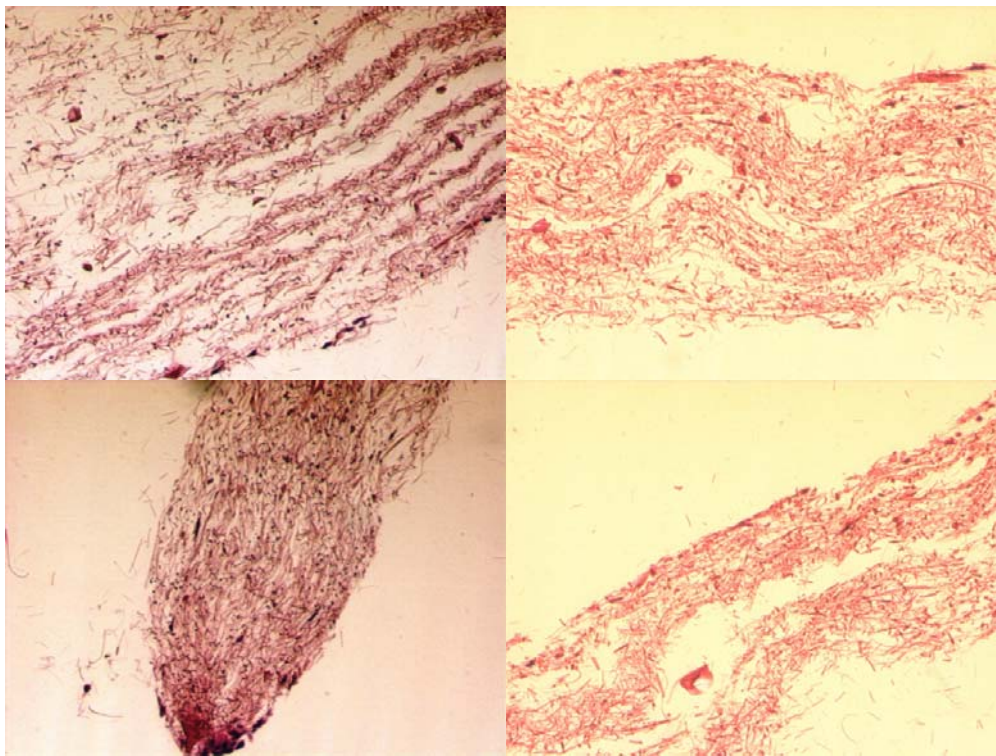

Figure 23: Silk fibroin gel electrospun out of HFIP 18 hours after genipin was added at 7 days (left) and 28 days (right) soaked in ethanol (top) and methanol (bottom) prior to cell seeding.

DAPI Staining

The confocal images confirmed that there was cell growth at both time points, and that there may not have been as much migration into the scaffolds as opposed to spreading along the surface. Figures 24 and 25 show the DAPI staining results of the silk scaffolds electrospun out of water with and without genipin. There was a slight visible increase in cells from the 7 to 28 day samples in both cases (Figures 24 and 25), but neither was significant. The addition of genipin did not greatly affect the cell response to the silk fibroin electrospun out of water. However, these results show a slight increase in the ethanol samples (Figures 24 and 25, top right) and a maintained cell growth for the samples soaked in methanol prior to cell seeding (Figures 24 and 25, bottom right). The corresponding histological evaluations (Figures 18 and 19) show some cell migration into the scaffolds which confirms cell proliferation along the surface as well as penetration into the scaffolds.

Figure 24: Silk electrospun out of water at 7 days (left) and 28 days (right) soaked in ethanol (top) and methanol (bottom) prior to cell seeding.

Figure 25: Silk electrospun out of water 18 hours after genipin was added at 7 days (left) and 28 days (right) soaked in ethanol (top) and methanol (bottom) prior to cell seeding.

Figures 26 and 27 are the results of the DAPI staining of the silk fibroin scaffolds electrospun out of HFIP with and without genipin. There is a significant amount of cells after 7 days in culture for the sample soaked in methanol prior to cell seeding (Figures 26 and 27, bottom left). There seems to be a decrease in cell count after 28 days (Figures 26 and 27, bottom right), but it could be possible that more cells migrated into the scaffold instead of remaining on the surface. Histology does not show an increase in cells after 28 days, however (Figures 20 and 21). As seen in Figures 26 and 27, there is a significant amount of cells after both time points. While there is a slight

decrease in cells after 28 days for the methanol soaked sample (Figure 26 and 27, bottom right), it could be because of more cell migration than proliferation along the surface. Moreover, there seems to be a greater amount of cells on the samples shown in Figure 27 which could be due to the addition of genipin prior to fabricating the scaffolds.

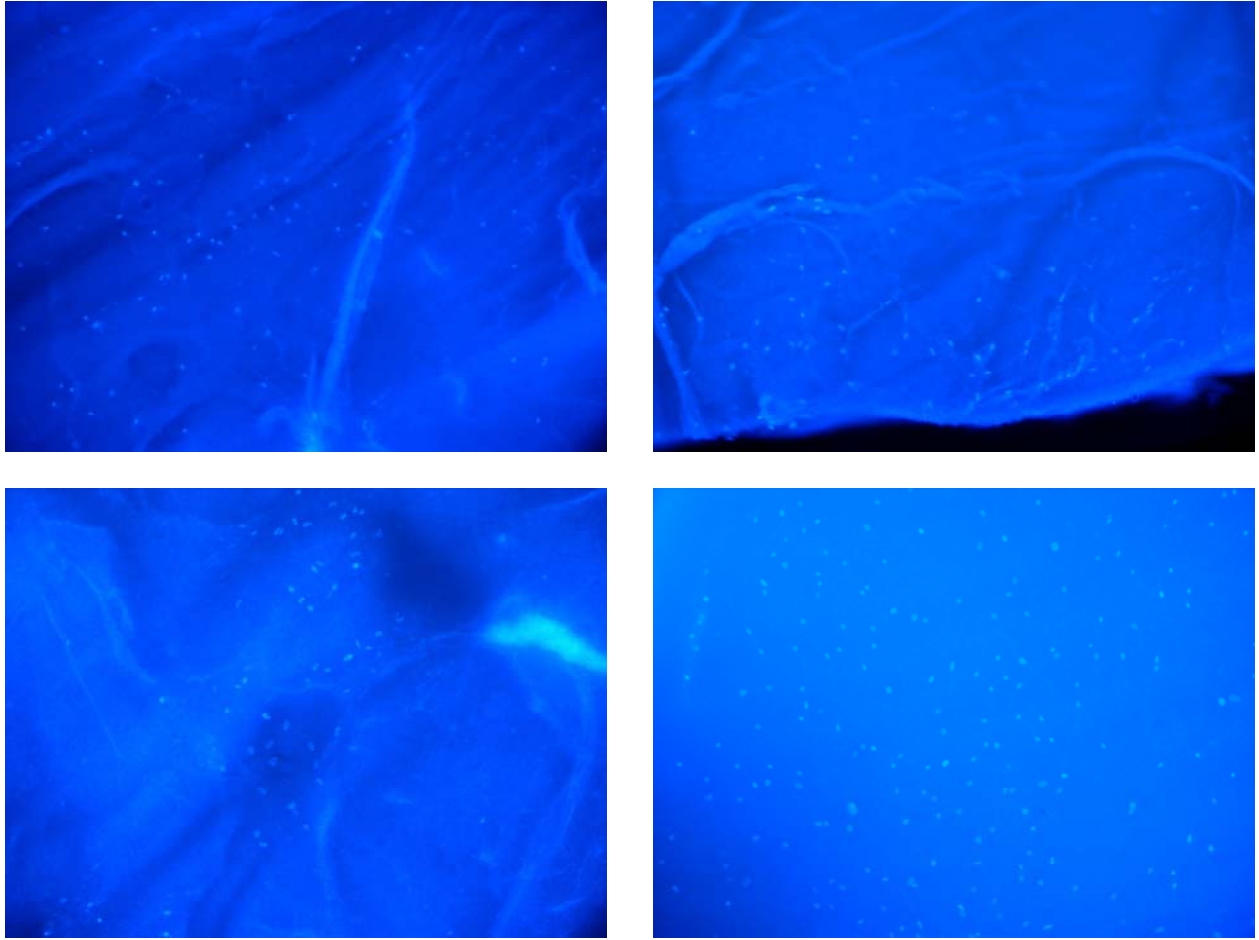Figure 26: Silk electrospun out of HFIP at 7 days (left) and 28 days (right) soaked in ethanol (top) and methanol (bottom) prior to cell seeding.
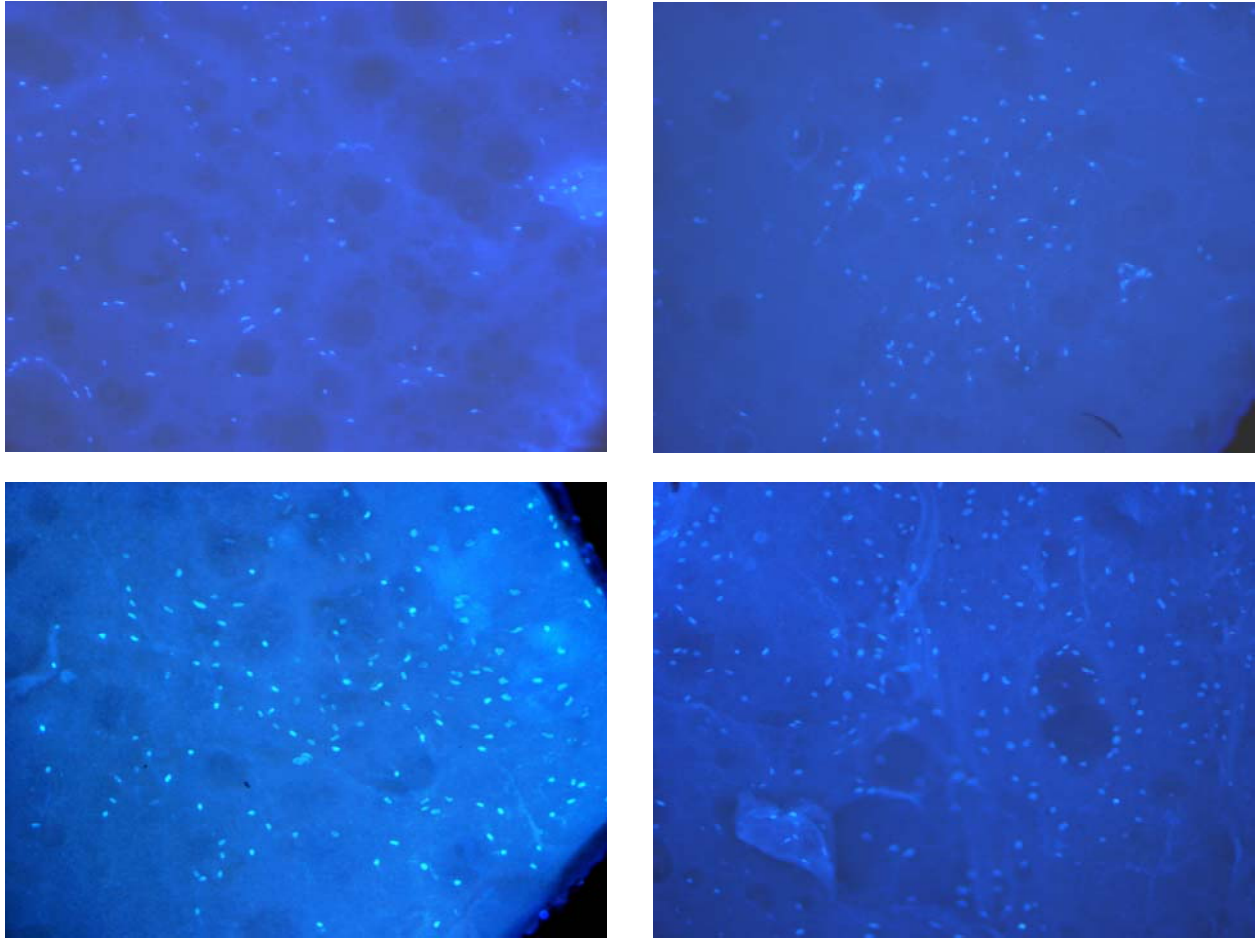
Figure 27: Silk electrospun out of HFIP 18 hours after genipin was added at 7 days (left) and 28 days (right) soaked in ethanol (top) and methanol (bottom) prior to cell seeding.

Figures 28 and 29 are the DAPI staining results of the electrospun silk fibroin gel with and without genipin. There is a slight decrease in cells after 28 days for the sample soaked in ethanol prior to cell seeding (Figure 28, top right). Since there was no apparent sign of significant cell death during the *in vitro* testing, this seemingly small visible decrease may be due to cell penetration into the scaffold. The samples soaked in ethanol prior to cell seeding (Figures 28 and 29, top right) seem to have the most cells after both time points than the other samples. This may be due to the addition of

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genipin, or the combination of genipin and the ethanol treatment with the silk fibroin gel.
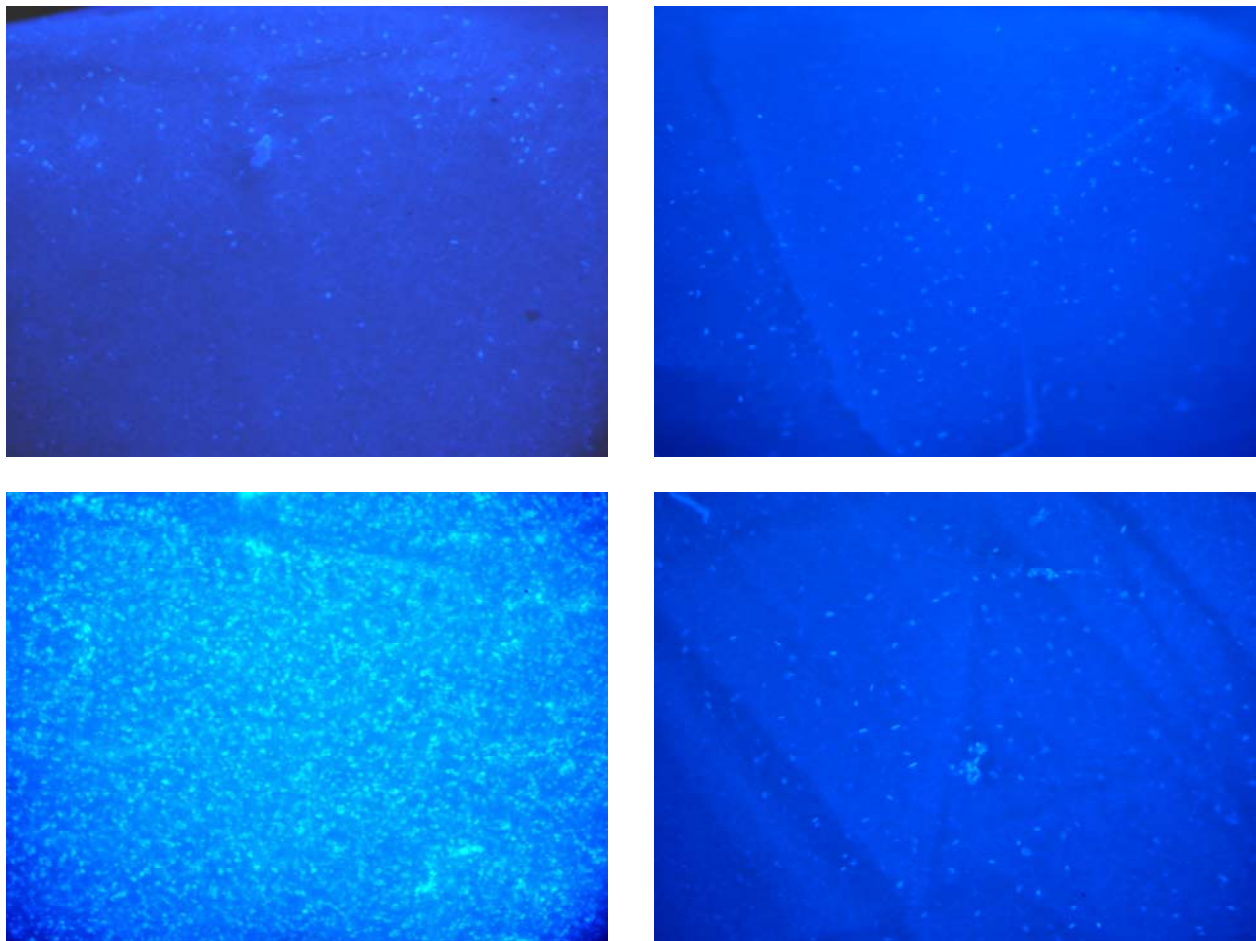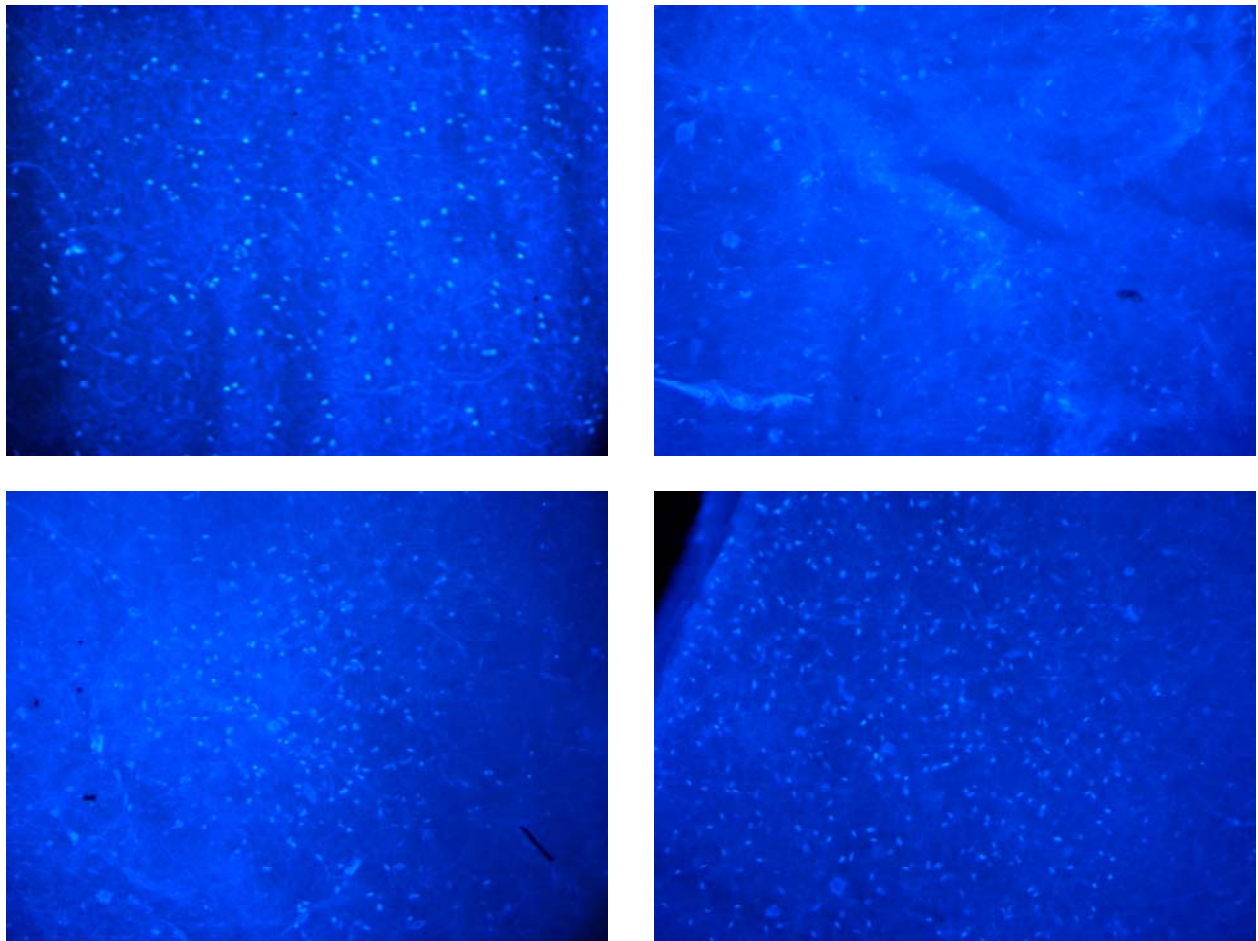

Figure 28: Silk fibroin gel electrospun out of HFIP at 7 days (left) and 28 days (right) soaked in ethanol (top) and methanol (bottom) prior to cell seeding.
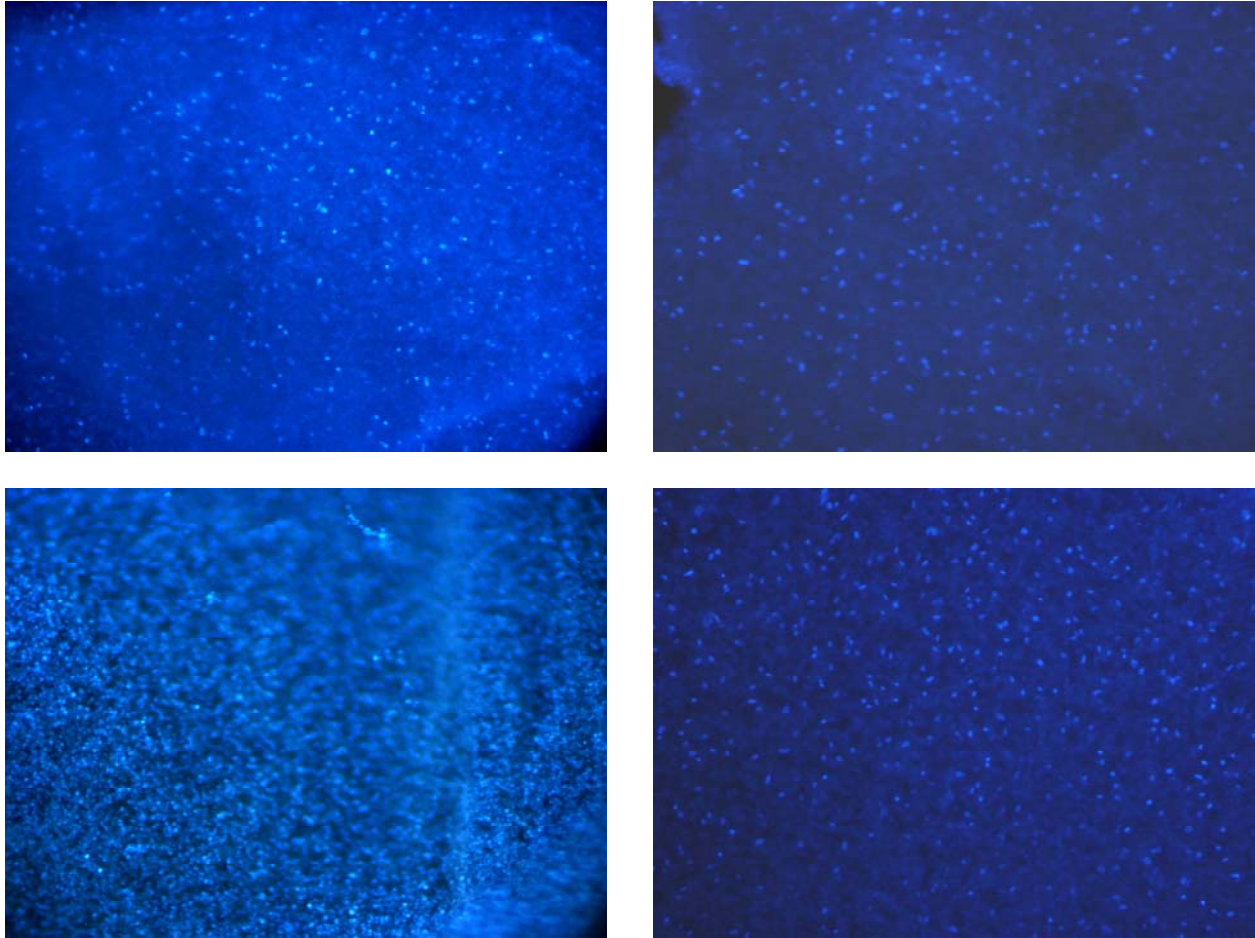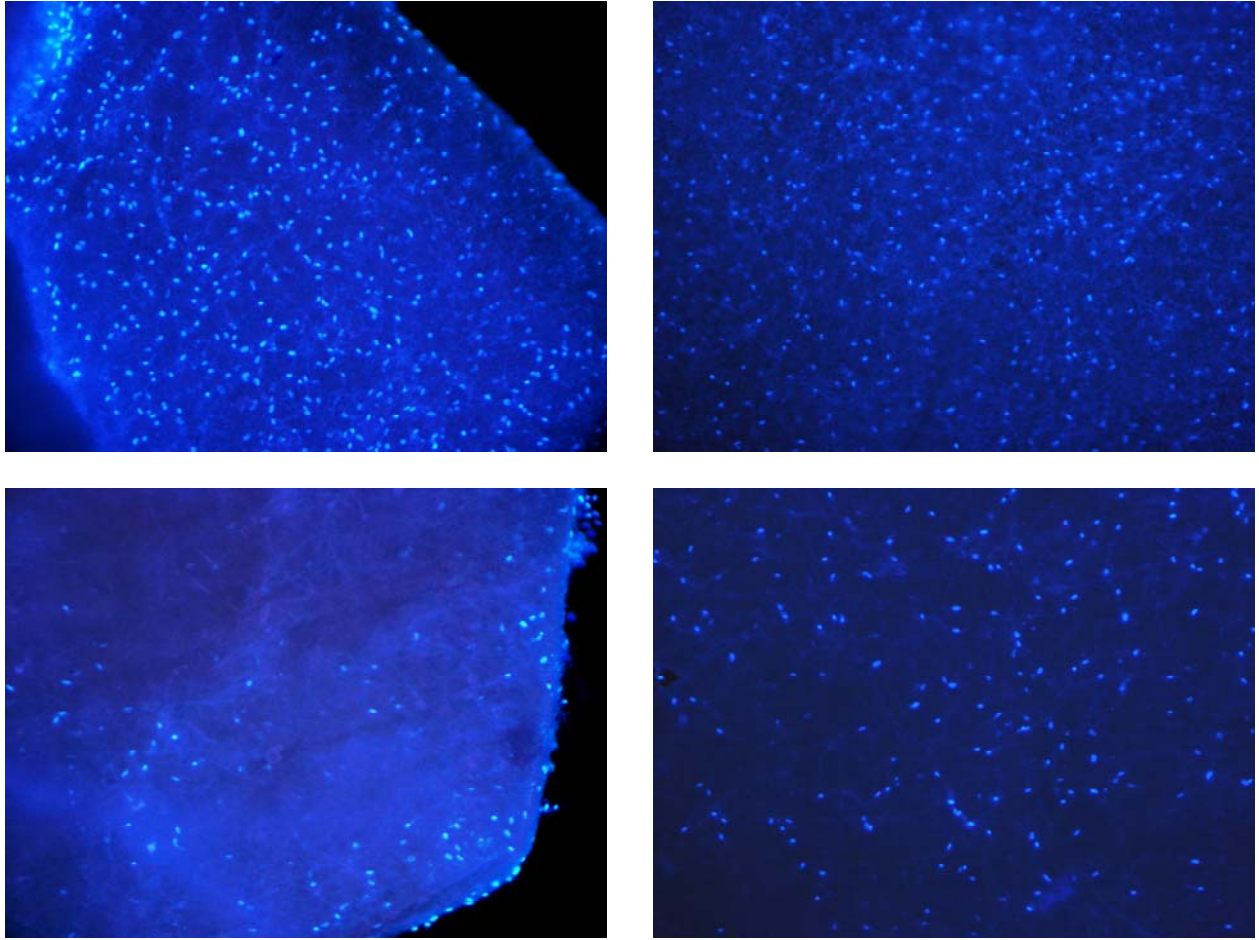
Figure 29: Silk fibroin gel electrospun out of HFIP 18 hours after genipin was added at 7 days (left) and 28 days (right) soaked in ethanol (top) and methanol (bottom) prior to cell seeding.

Discussion

Uniaxial Tensile Testing

The only significant difference in the mean peak stress was the samples electrospun out of water and cross-linked in methanol. The scaffolds electrospun out of water and cross-linked with ethanol and methanol generated the significantly different mean moduli as well. These results agree with those found by Mao Wang *et al.* in that an aqueous silk fibroin solution may produce a scaffold with a higher modulus [43]. The addition of genipin did not appear to have a great effect on the mechanical properties of the scaffolds. Moreover, there was not a significant difference between the silk fibroin scaffolds and electrospun silk fibroin gel scaffolds. These results suggest that there may not be a significant difference in the mechanical properties between electrospun silk fibroin and electrospun silk fibroin gel.

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Porosity

Calculating the dry porosity of the scaffolds showed a significantly higher porosity among the electrospun silk fibroin gel samples with genipin added to the electrospinning solution just before fabrication. The silk samples electrospun from HFIP without genipin were also significantly more porous than the scaffolds electrospun from water with genipin added to the solution prior to electrospinning. Furthermore, all scaffold variations tested were over 80% porous. This indicates that any combination of silk, solvent and cross-linking agent would produce an acceptable vascular graft based on porosity. Additionally, the silk electrospun from HFIP, silk gel electrospun immediately with genipin and silk electrospun from water with genipin after 18 hours of blending exhibited porosities greater than 90% which would be suitable for ligament grafts. The silk matrices electrospun from an aqueous solution included PEO in the calculated porosity, so the hydrated porosity should be higher after the removal of the PEO fibers.

In vitro Cell Culture

The results from the DAPI staining and histological evaluation suggest that ethanol and methanol are effective in cross-linking scaffolds without jeopardizing cell compatibility. The addition of genipin seemed to contribute to the increase in cell proliferation in the DAPI staining, especially when paired

with the ethanol treatment prior to cell seeding. The results from the DAPI staining showed more cell growth than that of the histological evaluation. This may be due to the time allotted for the cells to migrate and penetrate into the scaffold. In most cases, it appears that the 7 day results have more cell proliferation and penetration than the 28 day results. Since there were no prominent signs of cell death during the *in vitro* testing, it may be the result of handling during the histological evaluation that caused poorer results after 28 days. Each scaffold was cut in half and one half was used for histology while the other half was used for DAPI staining, which supports the possibility of human error during handling and cutting. The silk fibroin gel electrospun scaffolds appeared to have the most cell growth after the 28 day period which suggests that there may be distinct differences between electrospun silk fibroin and silk fibroin gel.

Electrospinning the scaffolds from an aqueous solution as opposed to HFIP did not have a significant effect on the *in vitro* cell testing. There were cells found in the middle of the scaffolds after the histological evaluation that show using water instead of HFIP may increase cell penetration but not cell growth. The confocal imaging further confirmed this with a greater amount of cells on the scaffolds' surfaces that were electrospun out of HFIP.

Future Work

More testing must be done to help determine the effects of the cross-linking agents on the silk-based scaffolds. Silk fibroin should be electrospun from an aqueous solution as well as HFIP and blended with genipin for 48 hours prior to electrospinning. The silk-based scaffolds should also be soaked in genipin post-fabrication for the same time points and the experiments repeated. Tests should be conducted which involve soaking the scaffolds in genipin instead of incorporating it into the solution to have a better comparison. The experiments should be repeated with scaffolds that have either soaked in genipin post-fabrication or blended with genipin for 48 hours prior to electrospinning.

DAPI staining of 80 mg/mL electrospun silk fibroin gel that blended with genipin for 48 hours prior to fabrication suggests that more time may enhance the cell compatibility of the silk:genipin scaffolds. Figure 30 compares the day 7 DAPI staining results of electrospun silk fibroin gel with genipin that blended for 18 hours (left) and for 48 hours (right). Figure 31 compares the day 28 DAPI staining results of electrospun silk fibroin gel with genipin that blended for 18 hours (left) and for 48 hours (right) prior to electrospinning. The confocal images suggest that the prolonged blending of genipin with the silk

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solution prior to electrospinning may enhance cell growth, especially when the scaffold is treated with methanol before cell seeding (Figure 30, bottom right). Moreover, there is no visible decrease in the amount of cells along the surface when the solution blends with genipin for 48 hours as opposed to 18 hours (Figures 30 and 31).
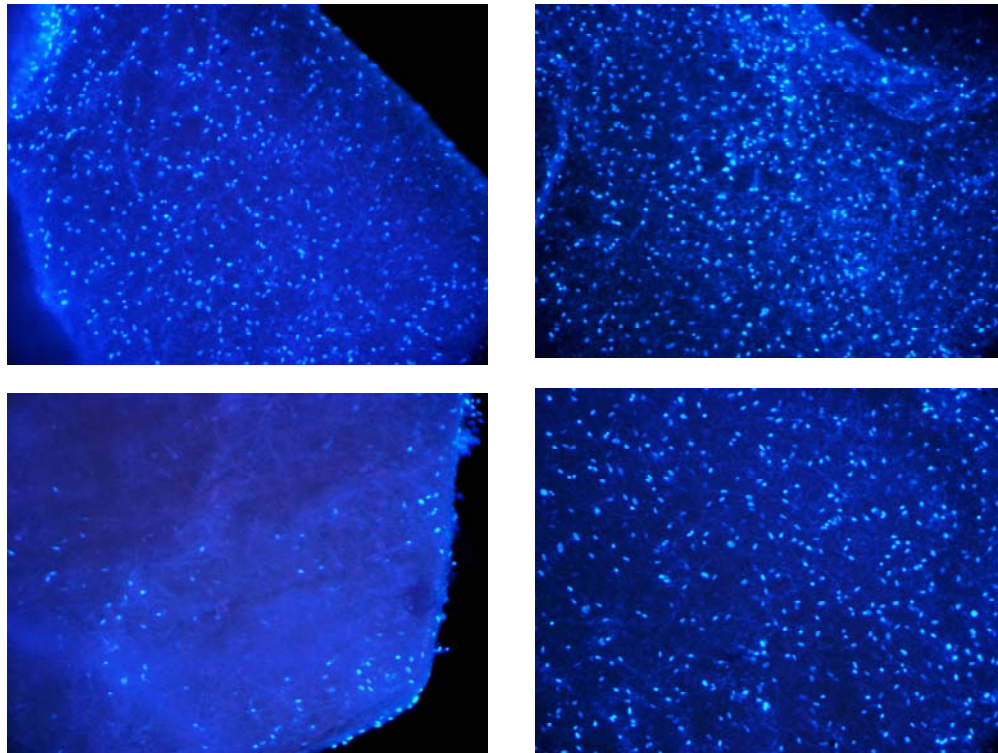

Figure 30: Day 7 DAPI staining results of electrospun silk fibroin gel with genipin after 18 hours (left) and 48 hours (right) in solution and soaked in ethanol (top) and methanol (bottom).

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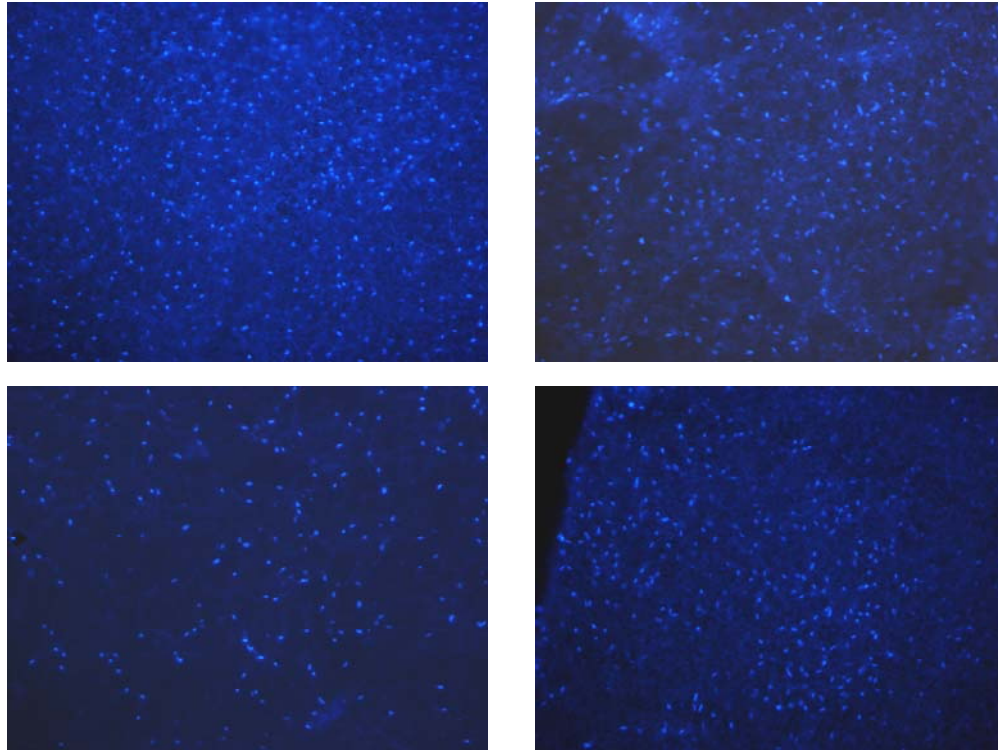
Figure 31: Day 28 DAPI staining results of electrospun silk fibroin gel with genipin after 18 hours (left) and 48 hours (right) in solution and soaked in ethanol (top) and methanol (bottom).

The histological evaluation also suggests that leaving the genipin in solution for 48 hours may enhance cell penetration. Figures 32 and 33 show the histology results for electrospun silk fibroin gel. The histological evaluation suggests that a longer blending period before fabrication may encourage more cell migration and penetration into the scaffolds. At both time points, there is a maintained, if not increased, amount of cells that have migrated into the scaffolds instead of remaining on the surface. Moreover, there is not an adverse effect of the prolonged blending with genipin when combined with the alcohol treatments.

Figure 32: Day 7 histology results of electrospun silk fibroin gel with genipin after 18 hours (left) and 48 hours (right) in solution and treated with ethanol (top) and methanol (bottom).
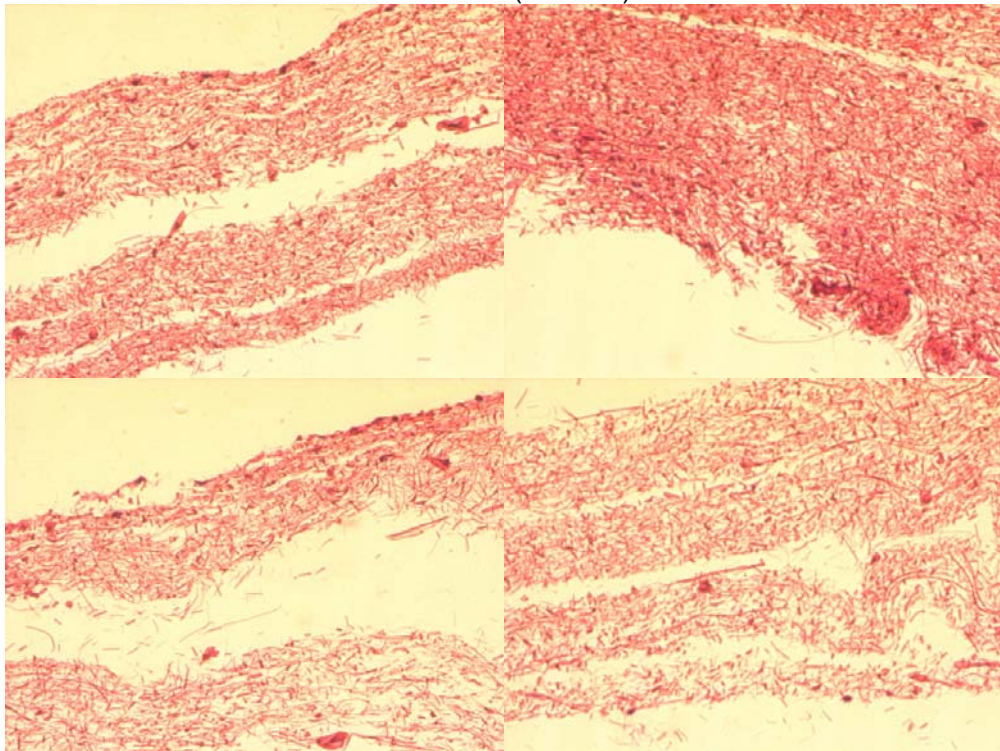
Figure 33: Day 28 histology results of electrospun silk fibroin gel with genipin after 18 hours (left) and 48 hours (right) in solution and soaked in ethanol (top) and methanol (bottom).
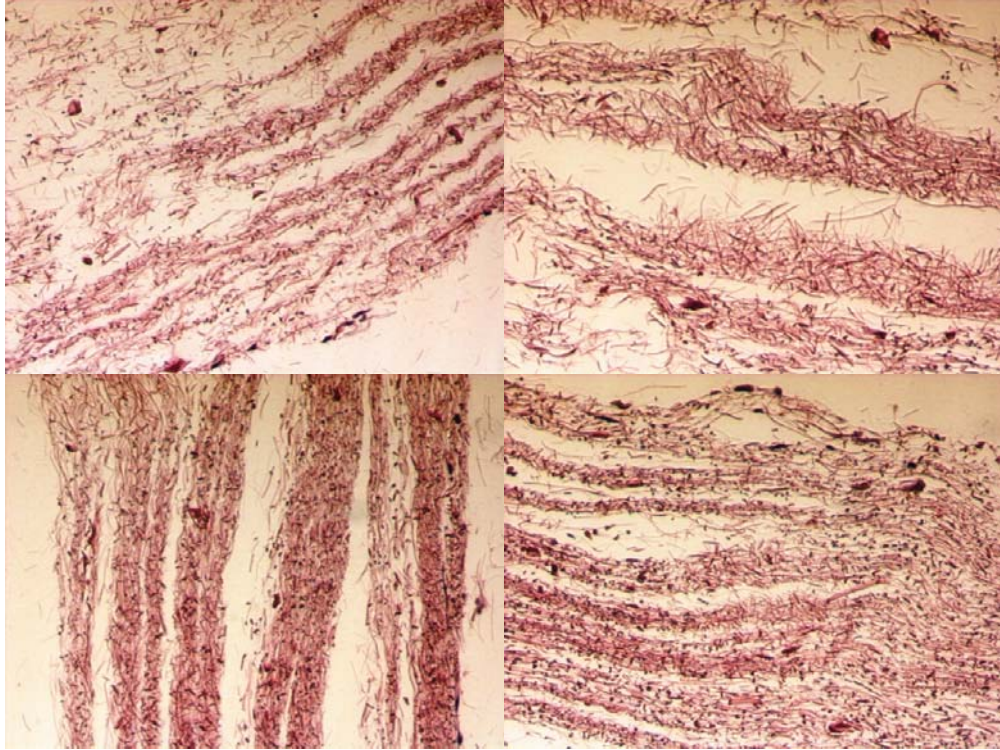
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Silk fibroin gel has potential for being another option for a vascular graft when the gelation process is controlled. Studies show that adding PEO to an aqueous silk solution can cause gelation over time [23]. A preliminary study shows that adding PEO to an aqueous silk solution of about 80 mg/mL silk at ratios of 1:1 (Figure 35), 1:2 (Figure 36) and 1:3 silk:PEO (Figure 37) will gel the silk at room temperature and retains its structure after lyophilization. Figure 34 shows the scanning electron micrographs of the air dried silk:PEO gel. Figures 35-37 display the scanning electron micrographs of the lyophilized silk:PEO gel.
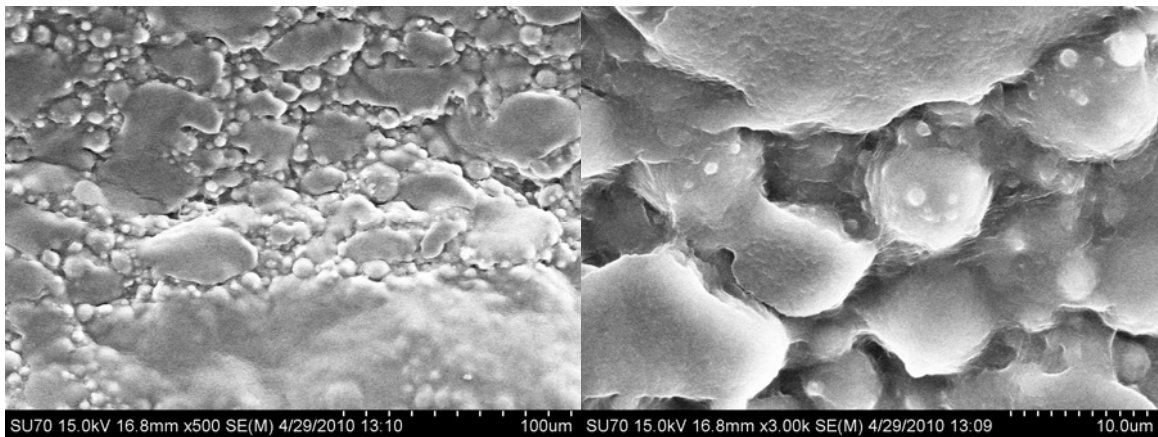
Figure 34: Air dried silk:PEO gel (1:1 ratio) at 500x (left) and 3000x (right).
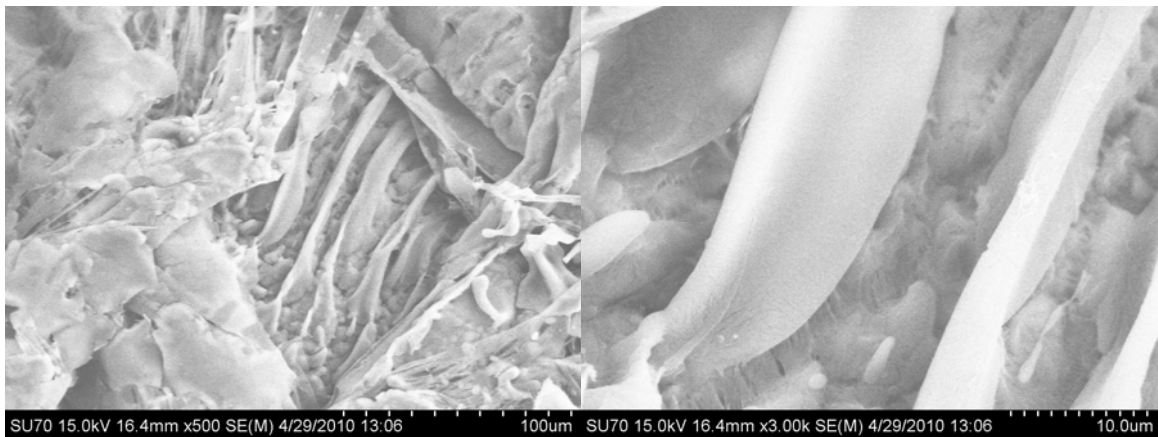
Figure 35: Lyophilized silk:PEO gel (1:1 ratio) at 500x (left) and 3000x (right).
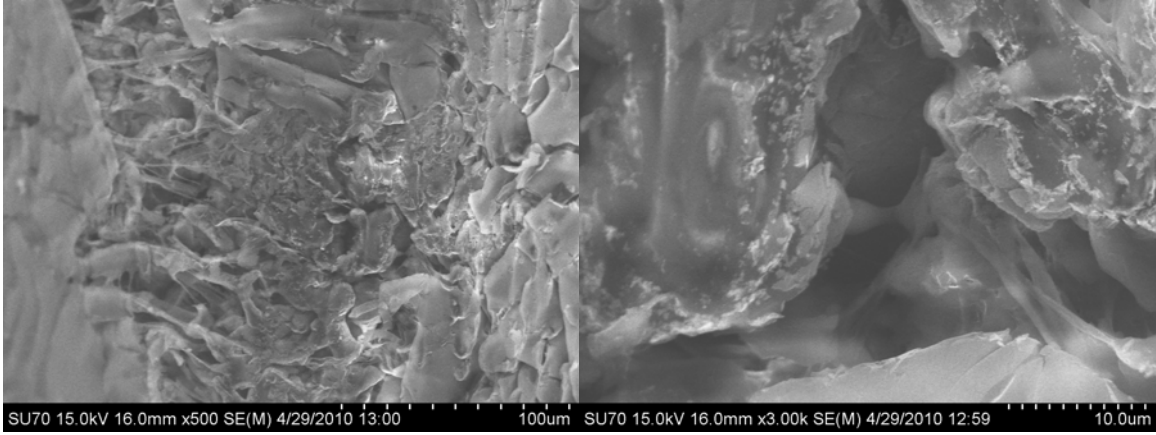
Figure 36: Lyophilized silk:PEO gel (1:2 ratio) at 500x (left) and 3000x (right).
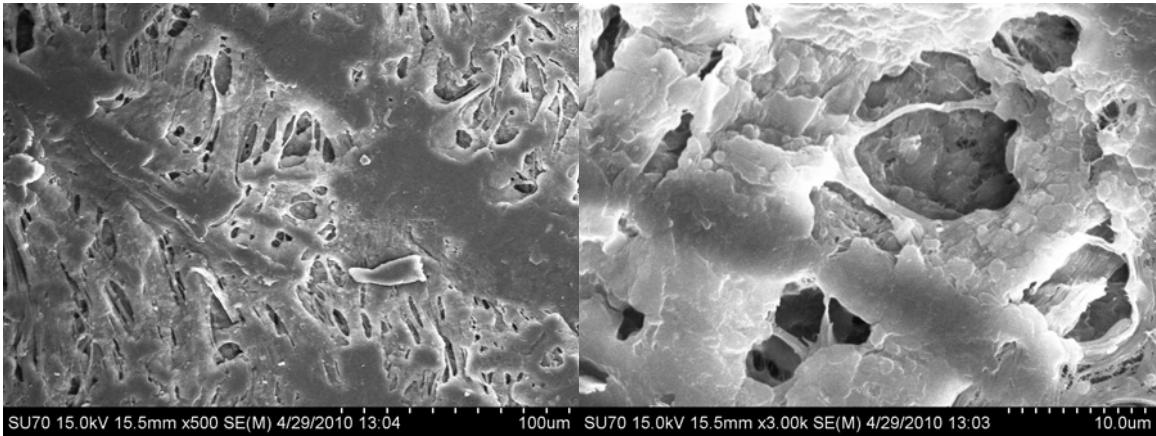
Figure 37: Lyophilized silk:PEO gel (1:3 ratio) at 500x (left) and 3000x (right).

The silk:PEO gel shows potential in forming a structure, such as a tube or sheet, that could be used as a vascular graft. Lyophilized silk gel shows promise as an electrospun nonwoven scaffold, so another study can be conducted to better understand the potential of silk gel as a biomaterial.

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Conclusion

This investigation of the effects of solvents and cross linking agents on silk fibroin-based scaffolds suggests that genipin may improve cell compatibility while not inhibiting mechanical and physical properties. Electrospinning the scaffolds out of an aqueous solution appears to enhance mechanical properties and possibly cell penetration. Ethanol and methanol had very similar effects on the scaffolds' mechanical properties. While neither have a significant affect on the cell compatibility of the scaffolds, the results from this study suggest that the combination of the presence of genipin with ethanol enhances the cell growth and penetration into the scaffold. Moreover, the results suggest that there is a difference in the physical and mechanical properties, as well as the cell compatibility, between silk fibroin scaffolds and electrospun silk fibroin gel scaffolds. Further testing is needed to investigate any future use of electrospun silk fibroin gel as a potential biomaterial.

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Literature Cited

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