Published in final edited form as:

Biomaterials. 2007 December ; 28(35): 5298–5306.

A FIBRINOGEN BASED PRECISION MICROPOROUS SCAFFOLD FOR TISSUE ENGINEERING

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Keywords

Fibrin; Fibrinogen; Scaffold; Micropatterning; Hydrogel; Cross-linking

Introduction

In tissue engineering the scaffold plays a pivotal role in the success of the living tissue construct that is desired. Enhancement of cellular attachment, proliferation, and organized development of native structures are just a few of the attributes of a beneficial scaffold. An ideal scaffold will also match the mechanical properties of the implant location and degrade as cells create new extracellular matrix (ECM) materials to take over load bearing. The transfer of structural support from the scaffolding material to newly created ECM imposes specific demands on the scaffold degradation rate. If the burden of support is transferred to the ECM too soon, the material may fail, and if the scaffold remains around too long, the dense, avascular fibrous capsule may reduce nutrient transport to the implant[1] while the chronic inflammation associated with the foreign body reaction may inhibit healing.

Angiogenesis is another important concern for tissue engineering. Angiogenesis is the process by which preexisting vasculature gives rise to new blood vessels. This is a tightly regulated process that can be enhanced in a number of ways.[2] Numerous materials have been evaluated for their ability to support blood vessel ingrowth.[3,4] Most synthetic materials effectively inhibit angiogenesis unless impregnated with growth factors, however, some natural proteins have been shown to support vascular growth through the release of their degradation products. Fibrin is one of those materials.[5,6]

Fibrinogen is a soluble 340kDa protein found in the blood and is involved with both blood clotting and platelet aggregation. Fibrinogen is polymerized into fibrin by the removal of A and B peptides through the action of thrombin in the presence of Ca^{2+} . [7] Factor XIII then crosslinks fibrin by linking a glutamine residue on one fibrinogen residue to a lysine on another. [8] This produces the fibrin hydrogel or “clot” resulting from the coagulation cascade. Fibrin is broken down by plasmin in a process called fibrinolysis and is cleared by the kidneys and liver.

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Though fibrin has been used as a temporary scaffold for tissue regeneration, fibrin hydrogels are initially weak at concentrations that support cell growth.[9] They are often cultured for weeks to months in the presence of growth factors such as transforming growth factor beta (TGF- β) and insulin, adding time and cost to the preparation of the scaffold.[10]

The source of fibrinogen can be autologous, allogeneic, xenogeneic or recombinant.[11] Presently, the two main uses for fibrin are in the forms of a tissue adhesive/fibrin sealant, and as a non-porous hydrogel. Fibrin degrades on the order of days to weeks *in vivo*, but this time can be increased by the incorporation of protease inhibitors and increasing the degree of crosslinking in the hydrogel.[12–14] Variation of these factors increases the time frame for degradation from days or weeks up to months and is within the “window” predicted as necessary for tissue growth and ECM production.

Non-porous fibrin hydrogels have been used in many formulations and shapes to surround and support cellular growth.[10,15] Local delivery of cells to areas in need of repair using fibrin has been shown to have beneficial effects in a wide variety of applications from cartilage repair [16,17] to improving the function of the heart[18]. There are, however, limitations to using solid fibrin hydrogels as cell delivery vehicles that might be alleviated using the microporous fibrin scaffold described in this article.

Here we describe a novel way to prepare fibrinogen for use as a tissue engineered construct and methods to enhance the mechanical strength of this versatile protein in both the polymerized fibrin form and in a novel, precipitated fibrinogen form.

Materials and Methods

Scaffold preparation

Two types of fibrin scaffolds were prepared by sphere templating for this work as described in detail below. They include polymerized fibrin (PFn) and precipitated fibrinogen (PFg) scaffolds.

Template formation—An interconnected template was created using similar methods to previous studies.[19,20] Briefly, a heterogeneous mixture of poly(methyl methacrylate) (PMMA, Kupa, Inc.) beads were sieved to a homogeneous distribution using an ATM sonic sifter. PMMA beads were then placed into Teflon and glass molds to form strips of beads measuring 25mm $\times$ 5mm $\times$ 1mm. The bead constructs were next sonicated using a water sonicator (AquaSonic 75HT) to settle the beads into a closest packed arrangement. Next, the beads were heated to 145°C for 22 hours to sinter the beads together into a bead cake with a pore neck diameter 50% of the bead diameter.

Hydrogel scaffold formation—For PFn scaffolds, bovine fibrinogen (Type 1-S, Sigma) was dissolved in 0.9% NaCl at 37°C for 2 hours at a concentration of 200mg/mL. Once completely dissolved, the fibrinogen solution was poured over a stack of bead cakes placed in the bottom of a 15mL round bottom test tube, covering them with 1–2mL of solution. The test tube was placed in a vacuum chamber and a vacuum was cycled on and off to a pressure of 28in Hg for 30 minutes to ensure full infiltration of the fibrinogen solution. The infiltrated bead cakes were then removed from the test tube and scraped with a sterile razor blade to remove excess fibrinogen. Next, the infiltrated bead cakes were placed into a solution of thrombin containing 13.25 U/mL Thrombin (Sigma) and 8.3 mM CaCl₂ (Sigma) in Dulbecco's Modified Eagle Medium (DMEM, Gibco) and allowed to polymerize for 16 hours at room temperature. Then, the scaffolds were scraped to remove residual protein surrounding the bead cake. The PMMA beads were removed (solubilized) using a series of acetone rinses at room temperature on an orbital shaker. Finally, the acetone was removed from around the newly formed scaffold

with 100% ethanol before the scaffolds were rehydrated through a series of ethanol dilutions with phosphate buffered saline (PBS) and stored at 4°C until tested. For PFg scaffolds, all steps were identical except that the thrombin and CaCl₂ polymerization step was omitted. Thus, the PFn scaffolds consist of templated fibrin whereas the PFg scaffolds consist of templated, precipitated fibrinogen.

For both PFn and PFg, scaffolds were made with various pore sizes and fibrinogen concentrations. The pore sizes used in this study were 30–38µm, 50–58µm, 63–71 and 75–95µm. The fibrinogen was used in four concentrations: 50mg/mL, 100mg/mL, 150mg/mL, and 200mg/mL.

Crosslinking with genipin—PFn and PFg scaffolds were removed from acetone after 48hours and rehydrated as described above. Once rehydrated, the scaffolds were crosslinked in a 0.625% genipin solution in phosphate buffered saline (PBS) for 6, 12, 24, and 36hours. They were then stored in PBS at 4°C until tested.

Materials characterization

Electron Microscopy—Scanning electron microscopy (SEM) was used to determine scaffold morphology and the extent of fibrin polymerization. For SEM analysis, templated fibrin scaffolds were prepared as described above. They were then fixed in 2% glutaraldehyde solution, dehydrated with graded ethanol, and dried using a critical point drier (Tousimis Autosamdri-814) to retain pore structure. Specimens were cut with a razor blade to varying depths and were Au/Pd sputter coated for 30 seconds (3 angstroms/sec) (SPI Supplies, West Chester, PA). SEM images were taken using an FEI (Hillsboro, OR) Sirion field-emission microscope at the Center for Nanotechnology, University of Washington. Samples were scanned under high vacuum conditions at an accelerating voltage of 5kV and a fixed working distance of 5mm.

Digital Volumetric Imaging—Micro-porous fibrin scaffolds of varying pore size were prepared as described. They were then fixed in 10% neutral buffered formalin for 12 hours and rinsed in PBS without calcium or magnesium. Next, they were equilibrated in 0.02M citrate buffer and stained overnight in a solution of 0.1% eosin Y (Sigma) at 4°C. Samples were then rinsed in 0.02M citrate buffer for several hours and stained overnight in 0.1% acridine orange (Sigma) solution at 4°C. They were again rinsed in 0.02M citrate buffer and then dehydrated through graded ethanols into xylenes. They were next infiltrated overnight in Spurr's low viscosity embedding medium (Electron Microscopy Sciences) with sudan black B opacifier (Arcos) to prevent "out of plane" fluorescence. Samples were cured overnight at 70°C. The embedded scaffolds were placed in a digital volumetric imager (Microscience Group Inc., Los Altos, CA) and fluorescently imaged (410–490nm/510nm) with a 10X objective as previously described.[21] The imager sectioned the scaffold 0.91µm between each image. This resulted in a 1.8mm × 1.8mm × 1.8mm image with a cubic resolution of 0.91µm³/voxel.

Pore morphology throughout the microporous fibrin scaffold was characterized with the digital volumetric imager. The DVI software, RESView™, was used to calculate average pore size, interconnected neck size, and porosity of the templated fibrin scaffolds from rendered 3-D images of the scaffold as shown in the supplementary video.

The average pore size was calculated by measuring a representative number of pores in the samples at their largest point from top to bottom. The interconnectedness of the pores was measured by calculating the width of the necking between each of the pores along the same direction. The porosity was calculated by measuring the inverse of the total fluorescence in a representative section of the scaffold and dividing it by the total area of a region. Background fluorescence was reduced by carefully setting fluorescence thresholds.

Mechanical Properties—Microporous fibrin scaffolds measuring 20mm × 5mm × 1mm were fabricated using the described methods. Tensile properties of each scaffold were determined using an Instron 5543 mechanical tester set up with a 10N load cell and pneumatic grips. Samples were strained to failure at a rate of 10mm/min. The Young's modulus was calculated from the initial 40% strain. The maximum strain % and ultimate tensile strength (UTS) were also measured.

Cell culture and characterization

Cell Culture—NIH-3T3 cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco-Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS) (HyClone, Logan, UT) and 100µg/mL penicillin/streptomycin (Gibco-Invitrogen, Carlsbad, CA). They were grown in T-75 vented tissue coated polystyrene plates (Corning Life Sciences, USA) and incubated at 37 °C in a humidified atmosphere of 5% CO₂. The culture media was changed every other day, and cells were passaged by detaching with 0.05% trypsin-ethylenediaminetetraacetic acid (Gibco-Invitrogen, Carlsbad, CA).

Cytotoxicity—A standard cytotoxicity test was performed on the fibrin scaffolds using the elution method. Briefly, 3 cm² of the scaffold's surface area was placed in DMEM supplemented with 10% fetal bovine serum for 24 hours while any soluble factors leached into the culture media. The media was then removed and placed atop NIH 3T3 fibroblast cells grown to 70% confluency in a 6-well plate. The cells growth was imaged at 24 and 48 hours.

AlamarBlue™ Assay—The cytocompatibility of PFn scaffolds was measured using alamarBlue™ (Biosource, Camarillo, CA) in accordance with the manufacturer's instructions with some modifications for use with cell seeded scaffolds. 8mm diameter × 1mm thick PFn discs were prepared as described, were kept sterile following the series of ethanol rinses, and stored in culture media at 4°C for 1 week. Immediately prior to cell seeding, the scaffolds were placed in 12 well plates and lightly compressed to remove the liquid within the pores. NIH-3T3 cells were trypsinized and resuspended at 4×10⁶cells/mL in culture media. 25µL of the cell suspension was added as a drop to the top of each scaffold. 1mL of culture media was carefully added around each scaffold so as to not disturb the cells. After 4 hours, the scaffolds were covered with an additional 2mL of culture media. At 24, 48, and 96 hours the scaffolds were moved to a new 12 well plate to eliminate cells on the plate surface and covered with 1.5mL of 10% alamarBlue™ in DMEM supplemented with 0.5% FBS and 100µg/mL penicillin/streptomycin. The plate was then incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 4 hours. 100µL aliquots were then taken from each well in triplicate and placed into a 96-well plate. The scaffolds were then recovered with culture media and placed in the incubator. The 96-well plate was read on a SafireII fluorescent plate reader (TECAN, Männedorf, Switzerland) with an excitation/emission of 530nm/590nm and an excitation/emission bandwidth of 10nm. To correlate fluorescence intensity with cell number, NIH-3T3 cells were grown on TCPS, assayed using alamarBlue™ as described, trypsinized, and quantified using a Coulter counter (model Z1; Beckman Coulter, Miami, FL).

Cell Morphology—The morphology of cells cultured on PFn scaffolds was evaluated using SEM imaging. PFn scaffolds were prepared as described and placed in culture media over night prior to seeding. NIH-3T3 cells were seeded on PFn scaffolds as they were in the AlamarBlue™ assay, but at twice the concentration. The scaffolds were removed from incubation after 24hours, rinsed with sterile PBS, and fixed. They were then fixed in 2% glutaraldehyde solution, dehydrated with graded ethanol, and dried using a critical point drier (Tousimis Autosamdri-814) to retain pore structure. Specimens were Au/Pd sputter coated for 60 seconds (3 angstroms/sec) (SPI Supplies, West Chester, PA). SEM images were taken using an FEI (Hillsboro, OR) Sirion field-emission microscope at the Center for Nanotechnology,

University of Washington. Samples were scanned under high vacuum conditions at an accelerating voltage of 5kV and a fixed working distance of 7.8mm.

RESULTS

Scanning Electron Microscopy (SEM)

SEM imaging was used to examine the morphology of the polymer at varying depths in the scaffold over a range of magnifications. Figure 1 illustrates a representative section of the micro-porous PFn scaffold viewed as a depth profile. It shows that the polymer formed throughout the entire depth of the 1mm thick scaffold. It also showed the regular pore shape, interconnectedness, and spacing that is representative of this processing technique.[20] Figure 2 shows increasing magnification of a PFn scaffold pore wall. In the highest magnification image in figure 2c, polymerized fibrin fibers in the size range of 40–80nm in diameter could be seen at the edge of the pore surface. This is within the range of what would be expected for a typical fibrin network[22] and was representative of the PFn scaffolds. As expected, PFg scaffolds lacked these fibers.

Digital Volumetric Imaging

Resulting scaffolds made from each bead size fraction were analyzed using digital volumetric imaging. As shown in figure 3, sonically sifted microspheres used to form the scaffolds gave a narrow, homogeneous distribution in pore sizes. The three bead distributions, 30–38 μ m, 50–58 μ m, and 75–95 μ m, resulted in average pore diameters of 34.0 μ m, 50.8 μ m, and 83.7 μ m with standard deviations of $\pm 2.9\mu$ m, $\pm 3.9\mu$ m, and $\pm 6.4\mu$ m respectively (n=30). Calculations of the interconnected neck sizes for the 30–38 μ m, 50–58 μ m, and 75–95 μ m scaffolds were found to be 17.0 μ m, 26.8 μ m, and 39.4 μ m with deviations of $\pm 2.4\mu$ m, $\pm 3.0\mu$ m, and $\pm 4.8\mu$ m respectively (n=30). The roughly 50% neck size was achieved by slow sintering, 22hrs, at 145°C. The overall porosity of the scaffolds created by the templating procedure described was calculated to be 73.7% $\pm$ 0.6% (n=3).

Mechanical Properties

Mechanical testing of microporous fibrin strips showed the hydrogels to exhibit a nearly linear stress vs. strain profile. Table 1 shows a compilation of the bulk mechanical properties for PFn and PFg scaffolds. PFn scaffolds showed increased mechanical stiffness with increasing fibrinogen concentrations, but all had similar maximum strain values of ~190%. The mechanical properties of PFn scaffolds with varying pore sizes were found to be statistically similar in each category, showing no significant differences between the groups (student's t-test, $p < 0.05$). In contrast, different properties were observed when PFn and PFg scaffolds were placed in acetone for increasing times, as shown in Figure 4. The PFn scaffold Young's modulus increased with time in acetone up to 48hours before leveling off at ~80kPa. In contrast, PFg scaffolds continued to increase with time with a maximum at 14.3kPa after 96hours. The maximum strain % for the PFn and PFg scaffolds increased initially, but decreased with extended time in acetone as the scaffolds become brittle. Preconditioning cycles of 30% strain were performed for both PFn and PFg scaffolds, however this did not significantly alter their measured mechanical properties and was not included in the table.

Crosslinking the scaffolds with genipin dramatically increased the stiffness of the scaffolds but decreased the maximum strain values. The trends for Young's modulus and maximum strain with an increase in genipin cross-linking time are detailed in figure 5. The correlation between time in genipin and Young's modulus is shown in Figure 5(a). This shows the ability of genipin to more than double the mechanical strength of the PFn material in 24 hours and increase the strength of the PFg material more than 10 fold in 12 hours. However, as shown in figure 5(b), time in genipin also significantly reduces the flexibility of these materials.

Cytotoxicity testing

NIH3T3 cells plated in the presence of micro-porous fibrin scaffold lysates showed increased cell proliferation when compared with controls at both 24 and 48 hours. No toxicity was seen (data not shown).

Cytocompatibility

PfN scaffolds supported a significant increase in the metabolic activity of NIH-3T3 cells over 6 days in culture. This was represented by an increase in fluorescence intensity which was found to linearly correlate with cell number ($R^2=0.9715$) as shown in figure 6(a). The PfN scaffolding provided a suitable substrate for cell growth. As shown in figure 6(b), cell number significantly increased at each time-point over the 6 day culture period. Figure 7 depicts cell spreading on the PfN scaffolding. Cells are able to adhere to the pore walls and spread.

Discussion

In this report we describe the development and characterization of novel sphere templated, fibrinogen-based hydrogels. Our results indicate that sphere templating does not interfere with the enzymatic conversion of fibrinogen to fibrin, and maintains excellent fibrin protein nanostructure within tightly controlled pore sizes. Furthermore, we found that acetone and genipin treatment could be used to enhance and tightly control mechanical properties of both PfN and PfG scaffolds. Scaffolds created in this manner were stable and nontoxic. These studies suggest that sphere-templated fibrinogen-based materials may be of value for tissue engineering scaffolds.

Fibrin hydrogels have been a popular choice for tissue engineering for more than a decade. They have been investigated for the development of many tissues including cartilage,[15] heart valves,[9] and bioartificial arteries.[10,23] However, the mechanical strength of fibrin hydrogel constructs is initially weak. Promising *in-vitro* studies performed by Jockenhoevel *et al.* using typical fibrin hydrogels in cardiovascular tissue engineering have been conducted, however, disadvantages were noted.[9] Low mechanical stiffness and shrinking of the fibrin hydrogel did not allow for direct implantation of newly formed structures. The Young's modulus of native heart tissue has been reported to be upwards of 67kPa[24] and esophageal tissue has been reported to be 60kPa[25]. In comparison, typical fibrin hydrogels are inherently weak constructs with a Young's modulus of 0.94kPa and 6.49kPa when starting fibrinogen concentrations are 0.5mg/mL to 3.0mg/mL respectively[26]. At higher fibrinogen concentrations, such as those of the commercially available fibrin based adhesives, hydrogels made with 30mg/mL and 70mg/mL fibrinogen have Young's modulus of 15.8kPa and 31.1kPa respectively[27]. However, at these high fibrinogen concentrations cells were unable to spread, proliferate, or ultimately survive.

We have focused on preparing porous fibrin constructs to make them suitable for immediate implantation. Cell seeded constructs are often cultured for weeks to months in the presence of growth factors while they produce extracellular matrix proteins to enhance the mechanical properties the engineered tissue.[28] Alternatively, fibrin has been mixed with polymers and knitted fabrics[24] to increase construct initial strength. In order to increase the strength of fibrin without extended culture periods or creating a composite material, we turned to physical and chemical treatments of fibrin. While scaffold templating has been recognized as a method to improve porosity it does not increase mechanical properties, though in creating voids for cells to adhere and grow we were able to increase the fibrinogen concentration to levels that made the scaffold mechanically sound. Proteins such as fibrinogen have solubility restrictions that present formidable challenges for most common methods of scaffold formation such as gas foaming[29] and salt leaching[30]. However, a sphere templating process using materials

that are not water soluble, described by Xia et al.[31] and later improved upon by Marshall *et al.*[20], provided a good starting point for templating fibrinogen. Polymeric microspheres did not dissolve in the fibrinogen solution during infiltration and held the fibrinogen solution in place during precipitation of the PFg scaffolds and polymerization of the PFn scaffolds.

Using SEM and DVI analysis, we showed that the PFn scaffolds contained a narrow size distribution of pore and neck sizes with fibrin fibers evident on the walls of the pores. In addition, the DVI software, Resview™ indicated that the scaffolds had porosity values of 74.7%, consistent with the expected value of a hexagonal closest packed arrangement of beads, 74.05%. The templating procedure described in this study could be easily made to produce more highly porous materials simply by inclusion of even smaller beads set between the larger beads after sintering. This could lead to scaffolds of greater than 90% porosity.

In an effort to improve their usefulness, we analyzed many conditions to control the strength and flexibility of the templated fibrinogen based scaffolds. The greatest difference between groups was seen between the PFn and PFg scaffolds. PFg scaffolds infiltrated with 200mg/mL fibrinogen were found to be, on average, less than 1/5th the Young's modulus of PFn scaffolds of the same concentration. This suggests that the polymerization of fibrinogen by thrombin dramatically adds to the mechanical strength of the scaffold. In addition, preconditioning of the samples did not significantly alter the young's modulus of these materials demonstrating the nearly elastic nature of this material. Pore size did not seem to affect mechanical properties, nor would it be expected to, since the planar densities of scaffolds with the same porosities are equal.

The ability to closely control pore size is also integral to the success of tissue engineered scaffolds. Pore size has been found to be a key player in various tissue engineered scaffolds including those for bone, as well as, scaffolds meant to induce angiogenesis. The effects of pore size on cell behavior has been most actively studied in bone tissue engineering where pores in the range of 300–400µm were found to be optimal for attachment, growth, and differentiation of osteoblasts[32] though this may be a secondary effect since it is hard to understand how a cell could sense a 300 µm pore. In soft tissue applications, pores 35µm in diameter have been shown to significantly increase angiogenesis when compared to 20µm and 70µm pores.[20,33] The ability to create a fibrin hydrogel with a size specific microenvironment may lead to improvements in many areas of tissue engineering.

Conclusions

Advances in tissue engineering provide the impetus for scaffolds with functional morphologies and appropriate chemical cues. The novel fabrication technique described here provides a new way to use an already popular protein, fibrinogen, in the tissue engineering field. Template fabrication of PFn scaffolds provides a material with tissue like mechanical strength and flexibility as well as a large surface area for cellular attachment, growth, and nutrient diffusion. This promising technique allows for precise tailoring of mechanical and chemical properties to fit the needs for many replacement tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Janet Cuy and Kip Hauch for their assistance with the digital volumetric imaging. Financial support for this research was provided by NIH/NIBIB (Bioengineering Cardiovascular Training Grant, T32 EB001650) and the A*Star, Singapore-University of Washington Alliance (Engineered Biomaterials and Tissue Engineering,

02-1-95-45-025). The sphere templating process was developed with funding from UWEB, an NSF Engineering Research Center.

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Figure 1. SEM image of PFn scaffold at 126X magnification showing homogenous pore distribution throughout the sample. The specimen was cut diagonally from the top of the sample (upper left) to the middle of the sample (lower right). Scale bar = 200 μ m

Figure 2.
SEM images of PFn scaffold at increasing magnification (A) 632X (B) 2527X (C) 10109X showing pore morphology (A and B) and fiber detail on pore wall of polymerized fibrin scaffold (C).

Figure 3.
Digital Volumetric image of sphere templated PFn scaffold showing 3-D architecture of scaffold.

Figure 4.

Comparison and trends of Young's modulus for PFn and PFg scaffolds with increasing time in acetone. *denotes statistical significance using a two sided unpaired student's t-test ($p < 0.05$)

Figure 5. The changing (a) stiffness and (b) maximum strain of PFn and PFg scaffolds is shown with time in genipin (hours).

Figure 6.

AlamarBlue™ assay showing increase in NIH-3T3 cell number on PFn scaffolds over 6 days in culture. (a) Correlation of fluorescence intensity with cell number on TCPS as assessed by Coulter counter. (b) Cell growth on PFn scaffolds initially seeded at 100,000 cells/scaffold (n=4). Incubation time with alamarBlue™= 4 hours. Error bars indicate standard deviations. (*) denotes significance (p>0.05)

Figure 7. SEM image of NIH-3T3 cells cultured on PFn scaffold at 1000X magnification. Cells are attached and spread on the PFn matrix. Scale bar = 20 μ m

Table 1

Mechanical Characteristics of Pfn and PFg scaffolds

Fibrinogen/Fibrin Scaffold Characteristics				Mechanical Properties		
Fibrinogen Concentration (mg/mL)	Pore Size (µm)	Polymerized with Thrombin?	Time in Acetone (hours)	Young's Modulus (kPa)	Max Strain %	Ultimate Tensile Strength (kPa)
50	63–71	yes	48	28.56 $\pm$ 2.09	198.6 $\pm$ 9.2	50.85 $\pm$ 7.72
100	63–71	yes	48	36.16 $\pm$ 6.15	192.5 $\pm$ 36.9	69.81 $\pm$ 26.04
150	63–71	yes	48	53.45 $\pm$ 7.74 *	197 $\pm$ 26.8	98.17 $\pm$ 14.17 *
200	63–71	yes	48	78.84 $\pm$ 5.10 *	183.2 $\pm$ 36.7	133.56 $\pm$ 36.36
200	30–38	yes	9	38.38 $\pm$ 4.12	182.3 $\pm$ 33.1	84.19 $\pm$ 24.99
200	50–58	yes	9	37.16 $\pm$ 6.28	173.0 $\pm$ 18.5	75.16 $\pm$ 12.94
200	75–95	yes	9	39.01 $\pm$ 1.14	152.6 $\pm$ 16.4	66.81 $\pm$ 9.59
200	63–71	yes	12	45.82 $\pm$ 4.31	155.8 $\pm$ 31.5	56.44 $\pm$ 16.44
200	63–71	yes	24	70.52 $\pm$ 6.51 *	195.3 $\pm$ 34.1	114.02 $\pm$ 24.28 *
200	63–71	yes	48	78.84 $\pm$ 5.10	183.3 $\pm$ 36.7	133.56 $\pm$ 36.36
200	63–71	yes	72	80.33 $\pm$ 5.96	114.5 $\pm$ 26.2	76.27 $\pm$ 19.98 *
200	63–71	yes	96	79.55 $\pm$ 4.80	117.0 $\pm$ 7.2	75.28 $\pm$ 4.15
200	63–71	no	12	18.84 $\pm$ 14.27	187.6 $\pm$ 108.7	16.04 $\pm$ 9.90
200	63–71	no	24	8.48 $\pm$ 0.61	222.6 $\pm$ 18.5	13.04 $\pm$ 0.67
200	63–71	no	48	10.91 $\pm$ 0.83 *	186.9 $\pm$ 18.0	14.53 $\pm$ 0.71
200	63–71	no	72	14.3 $\pm$ 1.23 *	176.4 $\pm$ 11.1	18.90 $\pm$ 1.67
200	63–71	no	96	17.68 $\pm$ 2.66 *	131.55 $\pm$ 20.6	14.29 $\pm$ 2.65 *

Table 1: Shows a comparison of mechanical properties for PFn and PFg scaffolds through varying conditions. Bold type indicates varying condition in the section.

* denotes statistical significance for Young's Modulus and Ultimate Tensile Strength using a two sided unpaired t-test ($p > 0.05$) between value and preceding condition value.