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Characterization of Fibrin Matrix Incorporated Electrospun Polycaprolactone Scaffold

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

By

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Acknowledgment

I would like to thank my entire thesis committee for their guidance and support throughout this entire process of completing my thesis project.

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Abstract

CHARACTERIZATION OF FIBRIN MATRIX INCORPORATED ELECTROSPUN POLYCAPROLACTONE SCAFFOLD

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Specific objective: Guided tissue regeneration (GTR) aims to regenerate the lost attachment apparatus caused by periodontal disease through the use of a barrier

membrane. For the GTR procedures to be successful, barrier membranes are required to be present at the surgical site for an extended period of time (weeks to months). Synthetic membranes have the advantage of prolonged presence in a wound site; however, they do not actively contribute to wound healing. Biologic membranes are recognized by the host tissue and participate in wound healing but have the disadvantage of early resorption. Therefore, the goal of this study is to create and characterize a hybrid barrier membrane that contains biologically active fibrin matrix within a synthetic polymeric electrospun scaffold.

Method: Fibrin matrices and fibrin-incorporated electrospun scaffold were created from fresh frozen plasma at three different centrifugation conditions 400g for 12 minutes, 1450g for 15 minutes and 3000g for 60 minutes. Following centrifugation, half of the membranes were crosslinked with 1% genipin. Biological stability of these scaffolds was evaluated by resistance to trypsin while their mechanical properties were characterized by MTS Bionix Uniaxial Tensile Testing System. Continuous data was analyzed by ANOVA to detect differences between groups ($p=0.05$).

Results: The addition of an electrospun scaffold to the fibrin matrix led to improvements in the mechanical properties as evidenced by an increase in the modulus ($p<0.0001$), strain at break ($p<0.0001$) and energy to break ($p<0.0001$). The effect of crosslinking was marginal but not statistically significant to the mechanical properties of fibrin matrices or the fibrin incorporated scaffold. However, crosslinking did significantly increase resistance against enzymatic degradation by trypsin ($p<0.0001$). Lastly, centrifugation speeds at 400g and 1450g showed similar mechanical properties and biologic stability; meanwhile 3000g negatively impacted the properties of the scaffold.

Conclusion: Fibrin-incorporated electronspun scaffold exhibits enhanced mechanical and biologic stability compared to fibrin matrices alone. Moreover, crosslinking improves the biologic stability of the novel biomaterial. All these characteristics of the fibrin-incorporated matrix make this membrane a potentially more ideal barrier for GTR procedures to enhance periodontal wound healing.

Introduction

Chronic periodontitis refers to the inflammation and progressive destruction of the supporting tissues of the periodontium resulting in loss of periodontal ligament and alveolar bone. [1] Restoration of the lost periodontium from periodontal disease is a paramount goal of periodontal therapy. Guided tissue regeneration (GTR) aims to regenerate the lost attachment apparatus by inducing the formation of new cementum, a new periodontal ligament and new alveolar bone through the use of a barrier membrane.[2] The principle of GTR is based on the concept of epithelial exclusion by preventing the down-growth of epithelium to allow mesenchymal stem cells from the PDL to repopulate the root surface and promote regeneration.[3]

The use of a barrier membrane in GTR also plays an important role in space maintenance and stabilization of the clot in the initial phases of wound healing.[4] Different types of barrier membranes are available on the market ranging from synthetic to biologic membranes. The benefits of biologic membranes, such as collagen-based membranes, include biocompatibility, biodegradability and capability of promoting wound healing. *In vivo* studies have demonstrated that collagen materials can positively influence chemotaxis of periodontal ligament fibroblasts[5] and gingival fibroblasts[6] to effect repair of damaged tissues. Although the biodegradable nature of biologic membranes eliminates the need for surgical membrane retrieval, this can also be a disadvantage when success of surgical treatment necessitates membrane presence at surgical site for an extended period of time. Synthetic membranes, on the other hand, due to their inert nature offer the advantage of slower resorption over biologic membranes. However, synthetic membranes do not actively contribute to wound healing. Furthermore, complications have also been reported such as infection during membrane exposures that can negatively influence clinical outcomes of regenerative procedures. [7]

There are various techniques to create synthetic membranes, and one method is through the use of electrospinning. Electrospun scaffolds are created from polymeric solutions through application of a high electric field between a positively charge syringe tip and a negatively charged collector.[8] The benefit of using an electrospun scaffold is the ability to adjust and control the size of the produced fibers to create structures that closely simulate the architecture of the natural biologic extracellular matrix. Importantly, the electrospun nanofiber structure of the scaffolds is ideal for cell adhesion due to a greater availability of surface area for cellular interactions.[9]

The ability to add biologic activity to a synthetic membrane could have profound clinical implications in periodontal surgical treatments. In wound healing, formation of a stable fibrin clot is critical. Importantly, the fibrin clot serves as an excellent natural scaffold that provides a conductive surface for cell attachment, adhesion and migration during the initial phase of healing.[10] By incorporating a fibrin matrix into an electrospun scaffold, this novel membrane will have the stability of a synthetic membrane to function as an excellent space maintainer, as well as, the biological activity of the fibrin to positively influence wound healing.

Therefore, the objective of this study is to identify optimal conditions to incorporate the fibrin matrix into the electrospun scaffold and to characterize the biologic stability and mechanical properties of this novel biomaterial compared to fibrin matrices alone.

Methods

Electrospinning

Polycaprolactone (PCL) was dissolved in hexafluroisopropanol (HFP) at a concentration of 150mg/ml overnight. Once completely dissolved, polymer solution was subjected to previously defined electrospinning conditions (rate: 7 ml/hr, air-gap distance: 12.5 cm, voltage: 22 kV) onto a rotating mandrel revolving at 1000 rpm. [11] After electrospinning, scaffold was removed from mandrel and cut into 3"x1" uniform pieces. Scaffolds were treated with acid conditioning using 38% hydrochloric acid and washed with PBS for 5 minutes.

Preparation of fibrin-incorporated electron scaffold

Firbin matrices and fibrin-incorporated electrospun scaffolds were created at three different centrifugation conditions: 400g for 12 minutes, 1450g for 15 minutes and 3000g for 60 minutes. Twelve replicates were created for each of the centrifugation conditions. Each sample was prepared using 4mL of fresh frozen human plasma at 37^{o}C mixed with 100µL of 1M $CaCl_2$ into scaffold containing test tubes under sterile conditions. After centrifugation, the cylindrical-shaped fibrin clots and fibrin-incorporated electrospun scaffolds were gently removed and compressed to make membranes (Figure 1).

Genipin crosslinking of fibrin-incorporated electrospun scaffolds

Under sterile conditions, half of the compressed membranes created at 400g, 1450g and 3000g were placed in six well culture plates and submerged in 4mL of 1% genipin for 48 hours. After 48 hours, membranes were washed twice with PBS. All uncrosslinked (UN-XL) and crosslinked

(XL) membranes were stored at 4°C in PBS solution until mechanical proprieties of the samples were analyzed.

Biodegradation assay with trypsin

The effect of crosslinking on the fibrin clot and fibrin-incorporated electrospun scaffold was assayed by trypsin degradation. Six samples of the fibrin clot alone and six samples of the fibrin-incorporated electrospun scaffolds were created from each of the centrifugation conditions at 400g, 1450g and 3000g. Half of the samples were later cross-linked with 1% genipin for 48hours, as previously described. Following crosslinking, samples were placed in six well culture plates, mixed with 500μL of 0.01% trypsin and incubated at 37°C for 48 hours (Figure 2). All samples were individually weigh prior to degradation assay, and measured again at the completion of 48 hours (Figure 3).

Uniaxial tensile testing

The mechanical properties of the electrospun scaffold alone (S), uncrosslinked fibrin clots (UN-XL-F, crosslinked fibrin clots (XL-F), uncrosslinked fibrin-incoporated electrospun scaffold (UN-XL-S) and crosslinked fibrin-incorporated scaffolds (XL-S) were analyzed by uniaxial tensile testing. Preparation for uniaxial testing involved determining the thickness of each scaffold and cutting it into "dog bone" specimens measuring 2.75mm wide at their narrowest point with a length of 7.5mm (Figure 4). Each specimen was then mounted onto the MTS Bionix 200 testing system (MTS Systems Corp) and stretched at rate of 10.0mm/min (Figure 5). Elastic modulus, energy to break and strain at break were calculated by MTS software TestWorks 4.0 and recorded.

Statistical Analyses

Multi-way ANOVA models were used to assess the relationship between various measures (degradation, modulus, strain at break, energy to break) based on the presence or absence of the scaffold, crosslinking, and centrifuge speed. A three-way interaction was fit to allow for differences based on the combination. Post-hoc pairwise comparisons were performed to determine where there were differences in materials combinations. A conservative Tukey's HSD adjustment was used to account for multiple comparisons. A significance level of 0.05 and SAS EG v6.1 was used for all analyses.

Result

ANOVA model results are presented in Table 1 for each of the measures of interest. This table shows what factors were associated with differences in stability, modulus, strain at break, and energy to break. Specifically, presence of a scaffold is statistically significant for all outcomes, but was influenced by other factors (centrifuge rate and/or crosslinking) on trypsin degradation, modulus, and strain at break. Crosslinking was significant for all outcomes except modulus (p-value=0.0969). Centrifuge rates were significantly associated with differences in trypsin degradation and modulus.

Baseline scaffold mechanical properties

Results from the uniaxial tensile strength test of the electrospun scaffold alone reported a mean modulus value of 47MPa. The strain at break was 4.1 and the energy to break was 100.7N*mm (Table 2).

Trypsin Degradation

Results from ANOVA model of the percent of the sample remaining after 48 hour degradation with trypsin demonstrated a significant 3-way interaction for the presence of a scaffold, cross-linking, and the centrifuge rate (p-vlaue= 0.0064). The highest percent remaining was seen with crosslinked samples with scaffold when spun at 1450g, though this combination was not significantly different from that at 400g, or the samples with no scaffold when spun at 400g or 1450g and crosslinked. The general trend is that increased centrifuge rates decrease the degradation, cross-linking greatly increases the percent remaining, and the addition of the

scaffold provides marginal, but not significant increased stability. Estimated percent remaining is presented in Figure 6. All pairwise comparisons of interest are presented in Table 3.

Modulus

There was a significant interaction between the presence of the scaffold and the centrifuge rate when modeling modulus (p-value=0.0007). Specifically, there were no significant differences in modulus based on centrifuge rate for fibrin matrix alone, but with fibrin+scaffold, as the centrifuge rate increased the modulus decreased. There was a statistically significantly higher modulus for samples with scaffold when spun at 400g vs 3000g (p-value<0.0001) (Table 4). Overall, modulus was greatly increased with the presence of the scaffold (average 52.9 vs 1.1MPa). The effect of crosslinking was marginal but not statistically significant (0.0969). Figure 7 presents the estimated modulus for each sample combination. Pairwise comparisons by scaffold and centrifuge rate are presented in Table 4.

Strain at Break

The effect of crosslinking on strain at break was different based on the presence of a scaffold (p-value<0.0001) (Table 1). Centrifuge speed was not significantly associated with a change in strain at break (p-value=0.2116). The effect of crosslinking was negligible for samples with no scaffold (p-value-0.9116), but significant in presence of scaffold. For samples with scaffold, crosslinking significantly decreased the strain at break by an average of 1.44mm/mm (p-value<0.0001). The greatest strain at break was seen in un-crosslinked samples with a scaffold, spun at 1450g, but this was not significantly different from samples at either of the other two

centrifuge rates (400 or 3000g). Figure 8 presents the estimated mean strain at break and Table 5 presents the pairwise comparisons for the interaction of scaffold and crosslinking.

Energy to Break

The only statistically significant predictor of energy to break was the presence or absence of a scaffold (p-value<0.0001) (Table 1). The presence of a scaffold increased the energy to break by over 90N*mm (Table 5). After adjusting for scaffold, the effects for the crosslinking and centrifuge rate were not significantly different. Estimated mean energy to break is presented in Figure 9.

Discussion

In this study, the initial objective was to identify the optimal centrifugation conditions to create a biologically active membrane by incorporating fibrin matrix into an electrospun scaffold. It is known, based on the principle of centrifugation, that altering the centrifugation force will influence the rate of sedimentation.[12] Specifically, that increasing the centrifugation force leads to greater compaction and formation of a denser construct. Applying this concept, three varying centrifugation speeds (400g, 1450g and 3000g) were selected to evaluate effects on the mechanical properties of the novel membrane. 400g was selected as the benchmark since this is the centrifugation protocol currently used for the creation of L-PRF through the Intra-Spin system (Intra-Lock)[13]. 3000g was selected as the upper limit of the centrifugation speed as this was the greatest speed permitted on the centrifuge. Lastly, 1450g was selected as the relative halfway mark between 400g and 3000g. It was hypothesized that the membranes created at 3000g would exhibit enhanced mechanical properties due to greater condensation of the fibrin matrix at an increased centrifugation force.

Based on the results from the uniaxial mechanical test, the mechanical properties of the fibrin-incorporated electrospun scaffold exhibited similar characteristics at 400g and 1450g with no statistical significant difference in the modulus, energy to break or strain at break. Meanwhile, the novel membrane created at 3000g exhibited a statistically significant decrease in the modulus, irrespective if the membrane was crosslinked, compared to membranes created at 400g or 1450g (Figure 7). However, centrifugation speed at 3000g did not show any statistical significant difference in the strain at break or energy to break compared to 400g or 1450g.

The modulus describes the elastic properties of a material and measures the resistance of the material to elastic deformation under load[14]. In other words, the modulus refers to the

stiffness of the material. Our study reported that an increase in the centrifugation speed altered the stiffness of the membrane. In clinical practice, a membrane with a higher modulus would be preferred since this would imply that the material can withstand greater stress and minimally changes its shape under elastic load. It is possible that a significant increase in the centrifugation force did not allow the fibrin matrix to properly engage with the nanostructure of the electrospun scaffold during the polymerization process. Rather, the fibrin matrix was simply layered on top of the scaffold and thus membranes created at 3000g lacked the inherent stiffness of the scaffold and could explain the observed decrease in modulus.

The ability to affect the stability of the novel biomaterial was also examined through the effects of crosslinking. Crosslinking is the process of chemically joining two or more molecules together with the purpose of stabilizing the protein structure[15]. Fibrin is generally present up to 1 week in the body before it is remodeled during wound healing[16]. It was hypothesized that crosslinking can increase the stability of the fibrin matrix and increase resistance against enzymatic degradation to trypsin. Trypsin is a serine protease found in the human body and functions to hydrolyze protein[17]. The trypsin degradation assay is an indirect method to evaluate the stability of the fibrin matrix after crosslinking and to assess if the addition of an electrospun scaffold would alter the stability of the fibrin clot.

Our study showed a general trend that crosslinking significantly increased the stability of both the fibrin clots and fibrin-incorporated electrospun scaffolds (Figure 6). This was evidenced by a statistically greater percentage of remaining constructs after the 48-hour incubation period with trypsin. Moreover, in the absence of crosslinking, the addition of scaffold was beneficial and decreased the degradation of the fibrin matrix. However, in the presence of crosslinking, the addition of the scaffold provided marginal but not significant increase in

stability. The one exception was seen in the 3000g group where the addition of scaffold to crosslinking significantly decreased the rate of degradation. It is likely that the increase in centrifugation speed altered the fibrin polymerization process and negatively impacted the structural architecture of the fibrin matrix, especially in the absence of a scaffold. However, with the addition of the scaffold to the fibrin matrix at 3000g, it was able to compensate for changes in the structural integrity of the fibrin clot and improve the overall stability.

The effect of crosslinking was also observed in the mechanical properties of the novel biomaterial, specifically decreasing the strain at break. In fact, in the presence of crosslinking and scaffold, the strain at break is reduced to the same levels of the fibrin matrix alone irrespective of crosslinking. The strain at break of a material refers to the maximum stress a material can stand before it breaks as stress is applied to the material[18]. A decrease in the strain at break translates into a material that exhibits a decrease in the ability to be stretched. One possible explanation is that crosslinking can result in significant dehydration making the material more stiff and reduces ability to be stretched. However, when the membrane is uncrosslinked, the presence of scaffold significantly increased the strain at break allowing the membrane to be stretched.

In conclusion, our results showed that the combined presence of both the fibrin matrix and electrospun led to statistically significant improvements in mechanical properties. Meanwhile, crosslinking enhanced the biologic stability of the fibrin matrix as evidenced by a greater resistance against enzymatic degradation following the trypsin degradation assay. Lastly, centrifugation speeds at 400g and 1450g produced membranes exhibiting similar mechanical properties. However, at 3000g the mechanical properties were negatively influenced by an increase in the centrifugation speed. Based on the results of this study, our recommendation for a

novel membrane that exhibits enhanced biologic stability and superior mechanical properties are crosslinked fibrin-incorporated scaffold generated at 400g. In summary, creation of a hybrid barrier membrane that contains biologically active fibrin matrix into a synthetic polymeric electrospun scaffold has great potential as a novel biomaterial in periodontal surgery.

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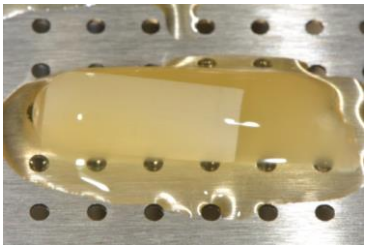
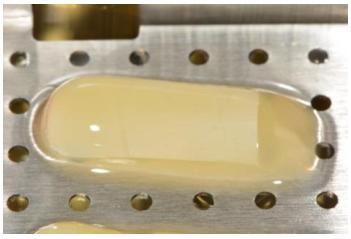
Figure 1: Preparation of fibrin-incorporated electrospun scaffold -A) Cylindrical-shaped fibrin-incorporated electrospun scaffolds were created at 400g, 1450g, and 3000g, B) gently compressed to form membranes

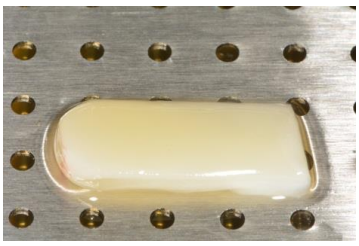
(A)



400g



1450g



3000g

(B)

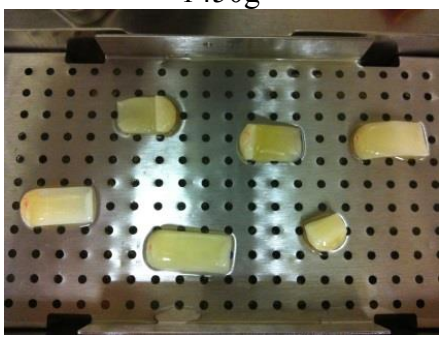
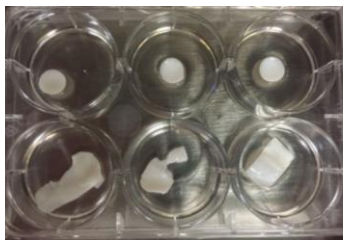
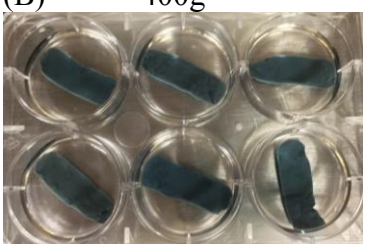


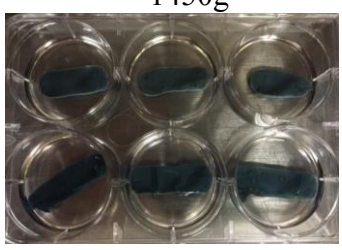
Figure 2: Biodegradation assay with trypsin - biological stability of the fibrin matrices and fibrin-incorporated scaffolds was evaluated with resistance against enzymatic degradation to trypsin (A- uncrosslinked, B- crosslinked samples)

(A) 400g 1450g 3000g



(B) 400g 1450g 3000g





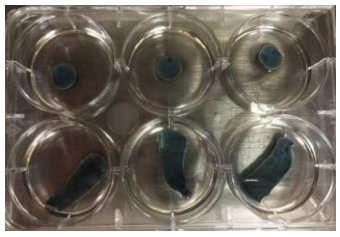


Figure 3: Biodegradation assay with trypsin - completion of biodegradation assay following 48-hour incubation period with trypsin at 37 C degrees (A- uncrosslinked, B- crosslinked samples)

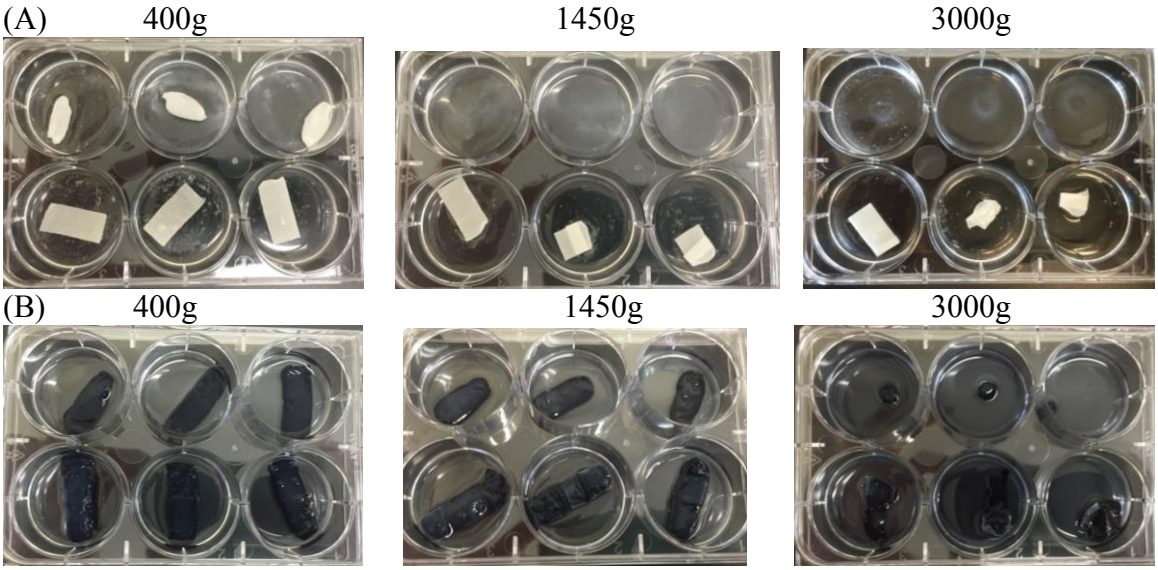


Figure 4: Preparation of specimens for uniaxial tensile testing - Preparation of samples into "dog bone" specimens measuring 2.75mm wide at their narrowest point with a length of 7.5mm

Figure 5: Uniaxial tensile testing - Illustration of mounted specimen on the MTS Bionix Uniaxial Tensile Testing System to characterize the mechanical properties of the fibrin-incorporated electrospun scaffold

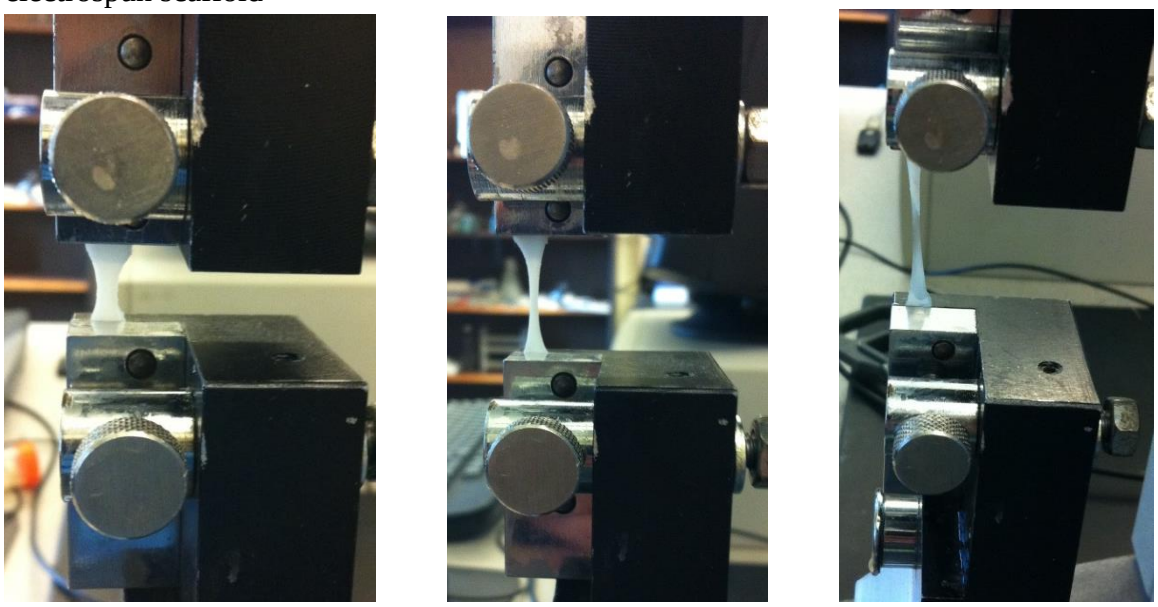


Figure 6: Mean Percent Remaining after Trypsin Degradation

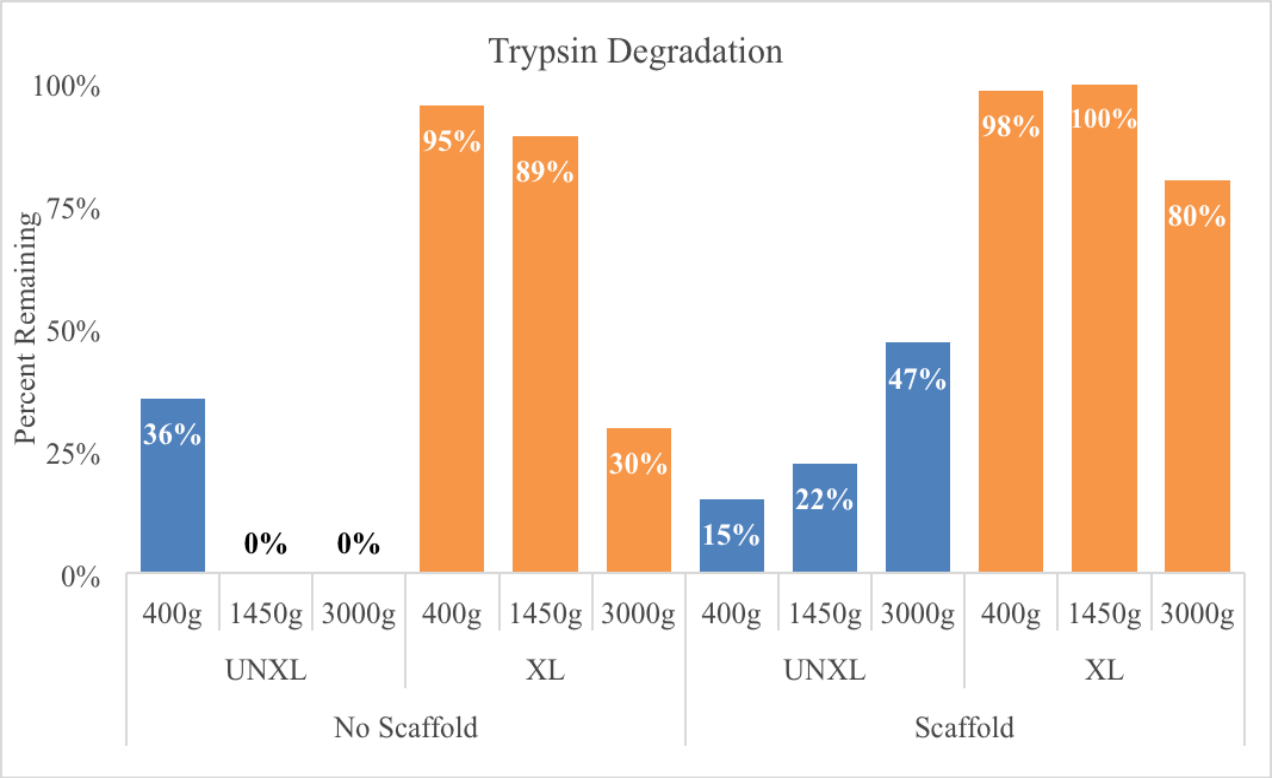


Figure 7: Estimated Mean Modulus (MPa)

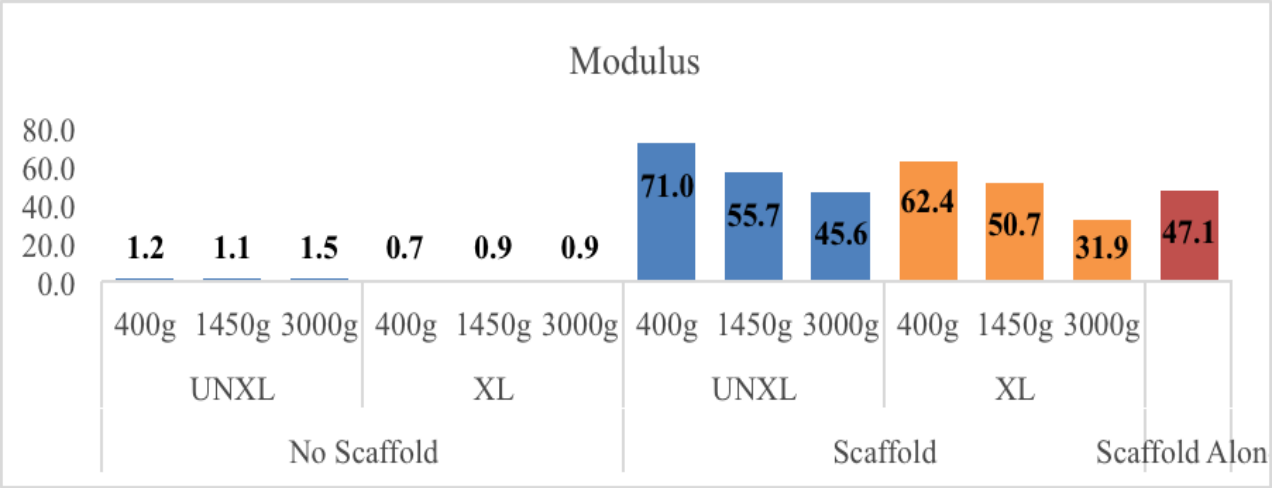


Figure 8: Estimated Strain at Break

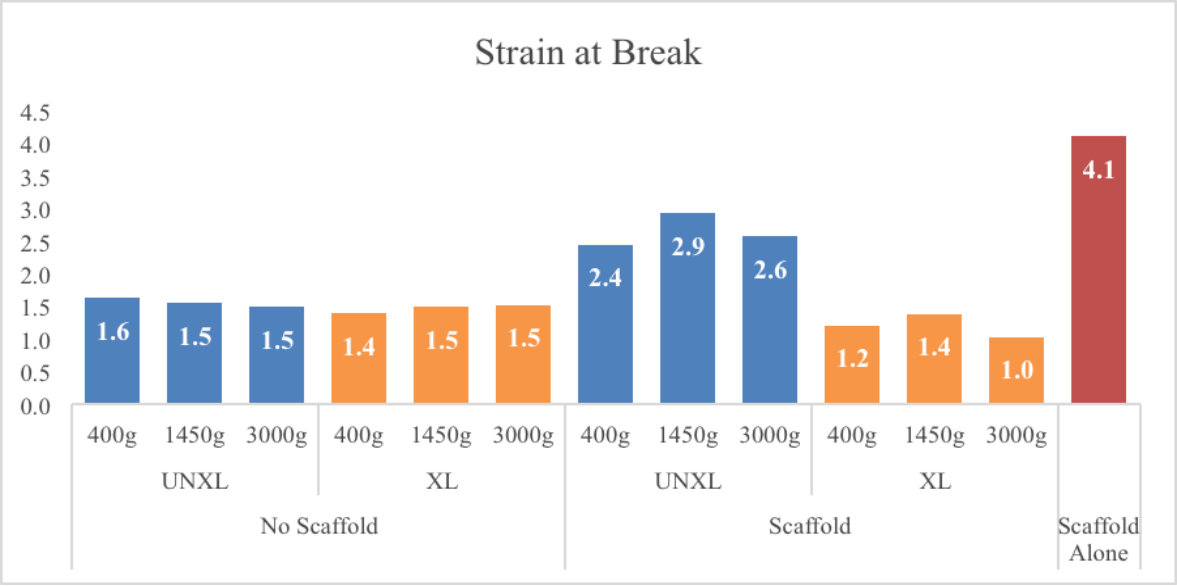


Figure 9: Estimated Energy to Break (N*mm)

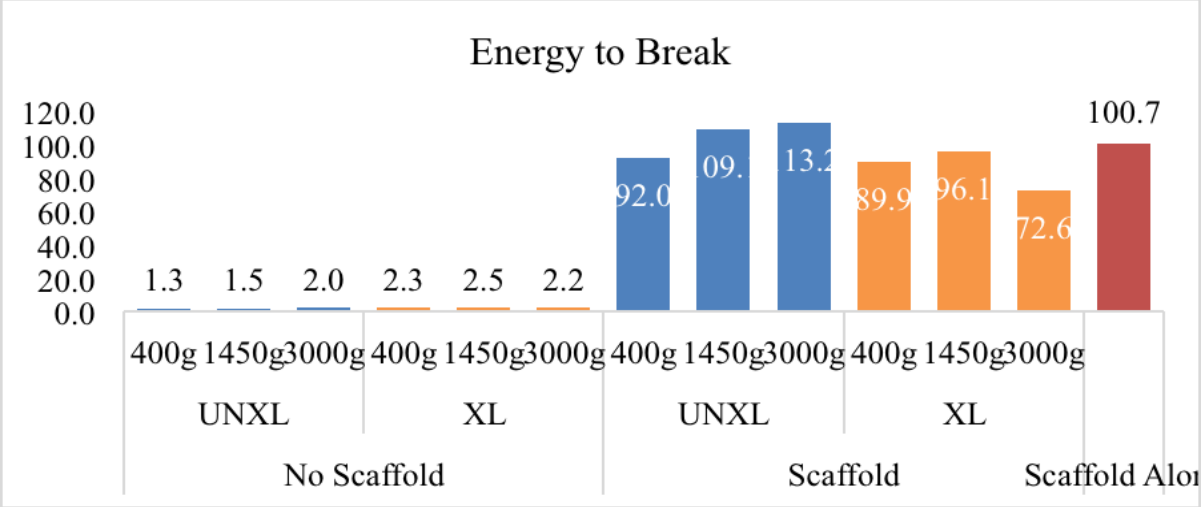


Table 1: ANOVA Model Results

	Trypsin Degradation	**Modulus**	**Strain at Break**	**Energy to Break**
Scaffold (Yes/No)	<.0001*	<.0001*	<.0001*	<.0001*
Centrifuge Rate (400, 1450, 3000)	<.0001*	0.0008*	0.2116	0.5871
Crosslinking (XL or UNXL)	<.0001*	0.0969	<.0001*	0.0736
Scaffold*Centrifuge Rate	<.0001*	0.0007*	0.2777	0.5974
Centrifuge Rate * Crosslinking	<.0001*	0.8140	0.9735	0.2512
Scaffold*Crosslinking	0.2290	0.1307	<.0001*	0.0544
Scaffold*Centrifuge Rate*Crosslinking	0.0064*	0.8352	0.4000	0.2831

*Statistically significant at 0.05 level

Table 2: Baseline Scaffold Mechanical Properties

	Mean	Standard Deviation
Modulus (MPa)	47.0	1.44
Strain at Break (mm/mm)	4.1	0.37
Energy to Break (N*mm)	100.7	6.72

Table 3: Pairwise Comparisons of Percent Remaining for Scaffold, Crosslinking, Centrifuge Rates

Comparison	Estimated Difference (% Remaining)	Adj P
No Scaffold at 400g: UNXL vs XL	-60%	<.0001*
No Scaffold at 1450g: UNXL vs XL	-89%	<.0001*
No Scaffold at 3000g: UNXL vs XL	-30%	<.0001*
Scaffold at 400g: UNXL vs XL	-83%	<.0001*
Scaffold at 1450g: UNXL vs XL	-77%	<.0001*
Scaffold at 3000g: UNXL vs XL	-33%	<.0001*
No Scaffold UNXL: 400g vs 1450g	36%	<.0001*
No Scaffold UNXL: 400g vs 3000g	36%	<.0001*
No Scaffold UNXL: 1450g vs 3000g	0%	1
No Scaffold XL: 400g vs 1450g	6%	0.9887
No Scaffold XL: 400g vs 3000g	66%	<.0001*
No Scaffold XL: 1450g vs 3000g	59%	<.0001*
Scaffold UNXL: 400g vs 1450g	-7%	0.9637
Scaffold UNXL: 400g vs 3000g	-32%	<.0001*
Scaffold UNXL: 1450g vs 300g	-25%	0.0001*
Scaffold XL: 400g vs 1450g	-1%	1
Scaffold XL: 400g vs 3000g	18%	0.0235*
Scaffold XL: 1450g vs 3000g	19%	0.0096*
UNXL at 400g: No Scaffold vs Scaffold	20%	0.0044*
UNXL at 1450g: No Scaffold vs Scaffold	-22%	0.001*
UNXL at 3000g: No Scaffold vs Scaffold	-47%	<.0001*
XL at 400g: No Scaffold vs Scaffold	-3%	1
XL at 1450g: No Scaffold vs Scaffold	-11%	0.6658
XL at 3000g: No Scaffold vs Scaffold	-51%	<.0001*

Table 4: Comparison of Mean Modulus by Scaffold and Centrifuge Rates

Comparison	Estimated Difference (MPa)	Adj P
400g: No Scaffold vs Scaffold	-65.70	<.0001
1450g: No Scaffold vs Scaffold	-52.21	<.0001
3000g: No Scaffold vs Scaffold	-37.60	<.0001
No Scaffold: 1450g vs 3000g	-0.20	1
No Scaffold: 400g vs 1450g	-0.01	1
No Scaffold: 400g vs 3000g	-0.21	1
Scaffold: 1450g vs 3000g	14.41	0.0403
Scaffold: 400g vs 1450g	13.47	0.0600
Scaffold: 400g vs 3000g	27.88	<.0001

Table 5: Comparison of Mean Strain at Break by Scaffold and Crosslinking

Comparison	**Estimated Difference (mm/mm)**	**Adj P**
UNXL: No Scaffold vs Scaffold	-1.09	<.0001*
No Scaffold: UNXL vs XL	0.09	0.9116
Scaffold: UNXL vs XL	1.44	<.0001*
XL: No Scaffold vs Scaffold	0.26	0.2193

Table 6: Estimated Mean Energy to Break for Scaffold (Yes/No)

Scaffold	Estimated Mean (N*mm)	SE	P-value*
No Scaffold	2.0	3.55	
Scaffold	95.5	3.45	
Difference (No Scaffold-Scaffold)	-93.5	4.95	<0.0001

*P-value for t-test of difference in means