Effects of Crosslinking Condition on Mechanical and Biological Properties of Biomimetic Fibrous Composite Material for Arterial Medial Equivalent

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ABSTRACT

Biomechanical properties of a vascular graft scaffold can be optimized by studying the effect of genipin crosslinker. Collagen-based scaffolds were prepared from a mixture of rat tail collagen, chitosan and bovine aortic elastin and crosslinked with varying concentrations of genipin. Biomechanical testing results showed that the linear modulus of the uncrosslinked samples had the least modulus (84.75 kPa ± 26.45 kPa) while crosslinked scaffolds exhibited much higher linear moduli. Similar increase was observed for the ultimate strength. However, an un-crosslinked sample exhibited the best compatibility with arterial smooth muscle cells (SMC) because increase in concentration of genipin decreased SMC contraction. These studies show superior biomechanical properties can be achieved by balancing mechanical and biological properties of the scaffold with an appropriate crosslinking condition.

Keywords: collagen, genipin, strength, biocompatibility, smooth muscle cell

1 INTRODUCTION

Mimicking a tissue to achieve suitable biomechanical properties is a tough venture. Many researchers have demonstrated the use of synthetic as well as bio-derived materials for vascular constructs [1, 2, 3, 4]. Yet they have not been able to satisfy all of the important parameters for the design of a scaffold material. These parameters include mechanical strength, compliance, cell functionality and immediate availability, to just name a few [5]. Uncrosslinked biological constructs generally have poor mechanical properties. As burst pressure of a vascular graft is related to the modulus of elasticity of its material [6], artificial vascular tissue needs to be endowed with a sufficiently high linear modulus. However, crosslinked constructs often inhibited cell activities. To promote wound healing after surgery, the material should also allow for cell proliferation and invasion [5]. Initial study has shown that mixtures of collagen, chitosan and elastin crosslinked with a fruit – derived biochemical crosslinker, genipin, exhibited some mechanical behaviors that resembled blood vessel material [7], compared to other widely-used crosslinkers examined. This study involves the use of genipin with various concentrations to crosslink constructs made of these biomimetic fibers, to form a medial-equivalent structure in vitro. Mechanical properties are improved with the concentration increase. However, it was found that increasing its concentration hinders cell activities including proliferation, migration and contraction of scaffold, although genipin is a very suitable crosslinker with superior compatibility with arterial smooth muscle and endothelial cells [7]. The results from this study suggest that designing a suitable vascular graft material should employ an appropriate crosslinking condition striking a balance between mechanical and biological properties.

2 MATERIALS & METHODS

2.1 Collagen Based Scaffold Preparation

Chitosan glutamate (Protasan, UP G 113; Milan Panic Biomedicals Inc Solon, OH; average molecular weight < 20 kDa, 75-90% deacetylation) was dissolved in 1% acetic acid, achieving a final concentration of 10mg/mL. The elastin solution was prepared by suspending elastin (Elastin Products Co., Owensville, MI) in 0.2M Tris solution (pH 8.8) with Triton X-100 surfactant, followed by filtering and washing the elastin suspension with 0.2M Tris solution (pH 8.8) without surfactant. The filtered elastin was finally resuspended in 0.2M Tris solution (pH 8.8) at a concentration of 20mg/mL. The collagen-chitosan-elastin gel constructs were prepared from a prepolymer mixture containing 3mg/mL of collagen, 3.5mg/mL of chitosan and 7mg/mL of elastin. Denatured rat tail type I collagen (BD Biosciences Inc, San Jose, CA) in acetic acid (pH 4.0) at a concentration of 8.9mg/mL was used. In addition, 10x Hank's balanced salt solution (HBSS) containing phenol

red was used to achieve physiological salinity and 1M hydrochloric acid or 1M sodium hydroxide were added to the prepolymer mixture so that pH of the prepolymer mixture was adjusted to 7.3. Finally, the mixture was finally topped off with d. i. H_20 to keep the collagen concentration constant at 3.0 mg/mL.

The final mixture was poured into moulds with care to avoid bubble formation. The scaffolds were obtained by polymerizing the prepolymer mixture in a cell culture incubator at 37°C for 10 hours. For mechanical characterization, gels were polymerized in a dog-bone shaped mould based on ASTM-D269 standard while a 24-well plate was used to prepare gels for the cytotoxicity tests. Genipin solutions of concentrations 1mM, 5mM, 10mM, 25mM and 50mM were prepared by dissolving 0.113g, 0.565g, 1.13g, 2.825g, and 5.65g in 500ml D-PBS (Dulbecco's Phosphate Buffered Saline, Invitrogen Corporation, Carlsbad, CA) respectively. After polymerization, the gels were suspended in genipin solutions for 10 hours to allow for crosslinking to occur.

2.2 Mechanical Characterization

The dog-bone shaped gel constructs were subjected to uniaxial tensile testing using an MTS Insight electromechanical testing system (MTS Systems Corp., Eden Prairie, MN) in a 1x D-PBS bath maintained at 37°C. The samples were subjected to a strain rate of 1% per second until failure and a 5N load cell (MTS Systems Corp., Eden Prairie, MN) was used to measure the stresses. The stress-strain curves were plotted for each of the samples to determine peak stress, strain at break and linear modulus. These curves were smoothed using Igor Pro 6.03A© (Wavemetrics Inc., Lake Oswego, OR). For each crosslinker concentration, 10 samples were tested. Data collected was statistically analyzed using Student's *t*-test. The level of significance was set at $\alpha = 0.05$ for 95% statistical significance. Error bars on all the histogram charts represent the standard error of the mean (SEM) based on the total number of the samples.

2.3 Biological Characterization

Viability

Bovine pulmonary arterial smooth muscle cells (BPASMC) were cultured in a T-75 cell culture flask, starting approximately five days before gel preparation, until confluence was reached. The BPASMC were then seeded in 12-well plates (cell concentration = 235,700 cells per mL per well for approximately 50% confluence) covered with and additional 1ml of cell culture medium (Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum, 2% L-Glutamine, and 1% Penicillin/Streptomycin) and incubated at 37˚C and 5% CO_2 a day before gel preparation. After the gels were polymerized and crosslinked, the gels were thoroughly washed five times in D-PBS and transferred into the 12-well plates seeded with BPASMC; this was referred to as Day 0. The next day (Day 1), a LIVE/DEAD assay (kit obtained from Invitrogen Corporation, Carlsbad, CA) was performed to determine cell viability. After aspirating the medium and five washes with D-PBS, 500µl of LIVE/DEAD staining solution (obtained by mixing 20µl of 2mM Ethidium homodimer-1 stock solution followed by 5µl of 4mM calcein AM stock solution in 10 mL of D-PBS) was added to each well. Plates were then incubated at room temperature for 40 minutes. The staining solution was then aspirated and the gels were washed five times with D-PBS and covered with cell culture medium. The cells were then viewed under a fluorescent microscope, six photographs per well were taken (three red-dead, three green-alive). Photographs were analyzed using ImageJ software (NIH, Bethesda, MD) to determine cell counts. In these tests, gels were crosslinked with 0mM (no crosslinking), 1mM, 5mM, 10mM and 25mM genipin respectively.

Contraction

BPASMC were cultured in the same manner as for viability testing. Gels were cast into 24-well plate wells and allowed to polymerize 1 hour in a cell culture incubator at 37˚C. They were then crosslinked with 0mM (no crosslinking), 1mM, 5mM, 10mM and 25mM genipin. For 5 hours. Gels were then washed five times with D-PBS. Gels were then seeded with 200µl of BPASMC suspension (589,250 cells per ml), incubated at 37˚C and 5% CO_2 for one hour to allow for cell adhesion. Gels were then covered with 1ml of cell culture medium and were detached from the culture plate wells by running a P200 pipette tip around the circumference of the gel. BPASMC seeded gels were then cultured at 37˚C and 5% CO_2 for 72 hours, with medium being changed every 24 hours. Photos were taken at the 72 hour mark using a digital camera place approximately 40cm above the 24-well culture plate containing the gels. Photos were then analyzed in ImageJ software to determine gel area in order to evaluate gel contraction.

3 RESULTS

3.1 Uniaxial Tensile Tests

Figures 1(a), (b) & (c) demonstrate the quantitative results from uniaxial tensile testing performed on the collagen-chitosan-elastin constructs crosslinked with varying concentrations of genipin. The figure shows that any crosslinking is advantageous. Uncrosslinked samples have peak stress (failure strength or stress) of 18.01 kPa ± 6.48 kPa and modulus of 84.75 kPa ± 26.45 kPa. Crosslinking with 1mM genipin increased the peak stress to 47.1 kPa ± 15.38 kPa while the linear modulus of the scaffold also increased to 294.68 kPa ± 75.12 kPa. As crosslinker concentration was increased from 5mM genipin to 50mM genipin, peak stress increased from 49.17 kPa ± 16.01 kPa

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to 64.04 kPa ± 6.61 kPa while the modulus increased from 365.41 kPa ± 148.57 kPa to 493.06 kPa ± 109.77 kPa. An uncrosslinked sample failed at 31.35% strain while crosslinking with varying concentrations lowered the strain at break from 26.3% to 20.5%.

3.2 Cytological Tests

From the LIVE/DEAD assay test results of day 1, the number of live (green) and dead (red) cells were counted. From this data, the percentage of number of cells alive was calculated. The relationship is depicted in figure 2. Though it was found that the viability decreased from 98.37% for an uncrosslinked sample to 94.17% for a sample crosslinked with 25mM genipin, no significant differences of viability among the samples have been found.

Gel contraction was calculated by comparing gel area at the 72-hour mark to the area of the well that it was originally cast into. The contraction data is presented in figure 3. Only the uncrosslinked gels and the 1mM genipin crosslinked gels exhibited contraction, while gels that were crosslinked with higher concentration of genipin did not exhibit any contraction. The 0mM genipin (no crosslinking) and 1mM genipin crosslinked gels contracted by 18.75% and 13.34%, respectively.

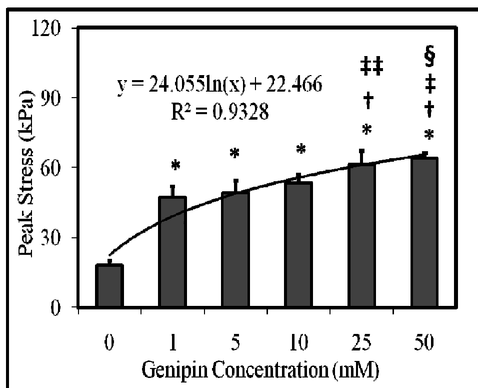

Figure 1(a)

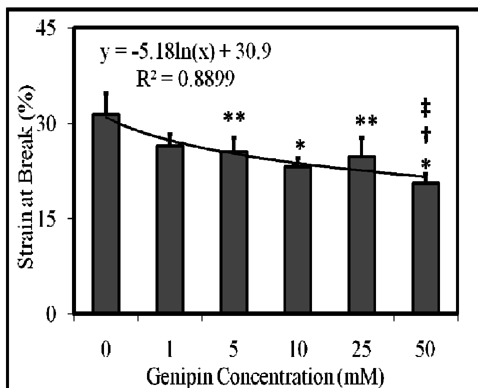

Figure 1(b)

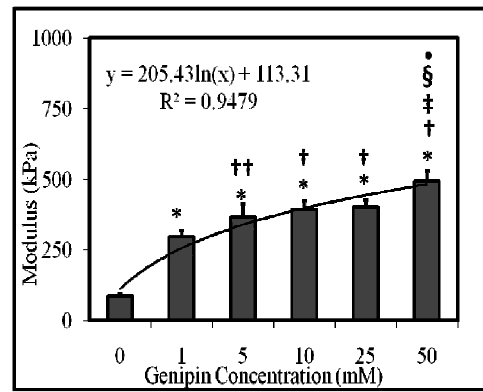

Figure 1(c)

Legend for Figure 1 (a), (b) & (c): Effect of concentration of genipin on mechanical properties of collagen-chitosan-elastin scaffolds - (a) Peak Stress (b) Strain at Break & (c) Linear Modulus (For statistical significance, $p < 0.05$ in Student's *t*-test, * - statistically significant difference in comparison to no crosslinking condition (0mM genipin), † - statistically significant difference in comparison to 1mM genipin, ‡ - statistically significant difference in comparison to 5mM genipin, § - statistically significant difference in comparison to 10mM genipin and (Round dot) - statistically significant difference in comparison to 25mM genipin. ††, ‡‡ - Indicate statistical difference in comparison to 1mM genipin and 5 mM genipin respectively for $0.05 < p < 0.1$. The error bars indicate the standard error mean.

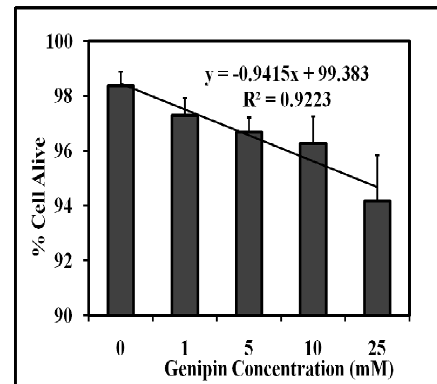

Figure 2: Effect of concentration of genipin on BPASMC viability in collagen-chitosan-elastin scaffolds. The error bars indicate the standard error mean.

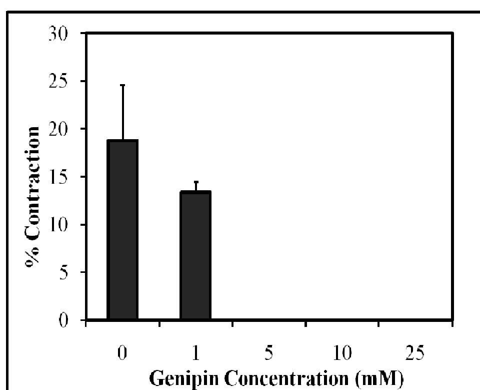

Figure 3: Effect of concentration of genipin on collagen-chitosan-elastin scaffold contraction by BPASMC. The error bars indicate the standard error mean.

4 DISCUSSION

We have characterized the effects of crosslinking on mechanical properties, cytotoxicity and contraction of collagen-chitosan-elastin gels. It can be clearly seen that peak stress and modulus have been significantly improved with crosslinking. Also, increase in crosslinker concentration also increased the same. There is significant difference in the properties provided by lower and higher crosslinker concentrations. The lower strain at break (elongation at break) is indicated by the fact that a 10-hour period of polymerization improves strength while making the construct more brittle [7,8]. Viability testing shows the genipin concentration did not significantly change cell viability. This is in good agreement with previous study by Sundararaghavan et al [9], who have also reported a decrease in viable fibroblasts as genipin concentration is increased from 0mM to 10mM. However, gel contraction by SMC was considerably reduced by increase genipin concentration. For all the gels that were crosslinked, only the gels treated with the lowest genipin concentration (1mM) were subject to contraction. In contrast to other crosslinkers like EDAC and formaldehyde, genipin imparts superior biomechanical properties [6].

Optimization of the biomechanical properties can be achieved by comparing mechanical and biological data for the range of crosslinker concentrations which can then be translated into vascular graft properties. In our case, it is seen that there is no significant difference between the mechanical properties imparted by the three lower crosslinker concentrations. These properties are substantially superior to those of an uncrosslinked scaffold. Higher crosslinker concentrations render the scaffolds stiffer than those crosslinked with lower concentrations. Increase the crosslinker concentration also inhibited SMC functions. Therefore, to strike a balance between mechanical and biological properties, it would be sensible to choose the lowest crosslinker concentration, 1mM, since this crosslinker concentration improves mechanical properties significantly when compared to uncrosslinked gels, supports the highest cell viability out of all the crosslinked gels, and is also the only concentration of crosslinker that supports gel contraction.

5 CONCLUSION

Uncrosslinked collagen-chitosan-elastin scaffolds are weak while crosslinking with genipin strengthens these scaffolds. Increasing the concentration of genipin drastically improved mechanical properties suggesting that for stronger vascular constructs crosslinking is necessary. Any amount of crosslinking reduced cell viability and crosslinking with genipin concentration above 1mM inhibited gel contraction. Results from this study have indicated the necessity of balancing the mechanical and the biological properties of the vascular construct to endow the vascular graft with superior biomechanical properties. These can be achieved by choosing a low crosslinker concentration which imparts superior mechanical properties when compared to an uncrosslinked construct while also maintaining high cell viability and functionality.

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