Characterization of Rheological Properties and Degradation of Genipin Crosslinked Fibrin Hydrogel For Annulus Repair

Dave Khyati
CUNY City College

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ABSTRACT

In 1972 modern fibrin sealing was developed in Vienna. Since then fibrin has effectively been used as ‘biologic glue’ for a variety of applications including its use in orthopedic and trauma surgery. In this study, fibrin gels cross linked with genipin have been studied as a potential Annulus Fibrosus sealant material in case of intervertebral disc degeneration in the lower back. Since the material is aimed for in situ use, it must have mechanical properties similar to the native tissue for efficient function. For this purpose gel modulus were studied and compared to the native tissue stiffness modulus. Degradation rate and % contraction in these gels were studied to characterize its temporal properties. Gelling time of these gels was defined in this study using rheological methods. The results from the gelation experiments using G' and G'' cross-over method were not in agreement with Winter-Chambon criterion for our gels. The results obtained by Winter-Chambon criteria suggest that these gels have an adequate Gelling time for a surgical procedure to take place. The stiffness study results show that these gels could be formulated with a modulus that would fall in the range of native annular tissue. The degradation and contraction study results suggest that the gel is not readily susceptible to degradation and also the % contraction is negligible.
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1.0 INTRODUCTION

Back pain is a common health issue and causes distress to a large number of patients with 10% of those cases leading to chronic disability [1]. Pathologies of the intervertebral disc (IVD) in the lower spine are a common source of low back pain. The IVD is fibro cartilage structure that consists of an internal semi-fluid proteoglycan rich mass called the nucleus pulposus (NP) and an outer annulus fibrosus (AF) [2]. The mechanical role of the IVD is to transmit and distribute loads on the spine while allowing for flexibility in the spinal column. [2].

Degeneration of the intervertebral disc is usually attributed to structural failure combined with advanced signs of aging [3]. When fissures in the disc grow large enough to allow gross migration of the nucleus relative to the annulus, to the extent that disc periphery is affected, the disc is said to be herniated or prolapsed. This causes NP material to protrude into the AF. Most intervention procedures intended to diminish painful spinal conditions, including spinal fusion, total disc replacement and discectomy do not repair the disc or restore its original function and may limit mobility [4]. Current methods for AF repair are limited to sutures and modified sutures which do not compensate for loss of AF tissue or restore lost biomechanical properties[4]. An intact AF would prevent re-herniation of the NP. Combining NP replacement approaches with AF repair would serve to restore original function and prevent reherniation.

Previous studies have shown that a genipin cross linked fibrin hydrogel satisfies the three basic requirements for a good AF sealant: the gel modulus matches the native annulus tissue, it supports the growth of disc cells and maintains adhesion to the tissue [5]. These gels are compatible with chondrocytes in vitro and inhibit inflammatory response when implanted in rats.[5]. However these studies fail to characterize the material’s gelling properties, degradation profile and in situ contraction upon gelling. In the present study we have used varying genipin
concentrations and cell adhesion proteins collagen and fibronectin are added to the formulation to see how they affect gel biomechanical properties.

The primary objectives of the proposed study are: i) to characterize the viscoelastic properties of various fibrin-genipin gel formulations with respect to the native tissue properties using rheological analysis; ii) To investigate the gelation time of the gel and see the effect of temperature on the gelling process; and iii) to compare the contraction and degradation rates of various gel formulations.

2.0 BACKGROUND

2.1 Fibrinogen and Genipin

Fibrinogen is a high molecular weight protein. It is a precursor of fibrin which is the basic element of a blood clot. Fibrinogen transforms into stable fibrin by means of thrombin and factor XIII which is activated by thrombin [6]. Fibrin monomers crosslink to produce a stable fibrin clot that can act as a ‘biologic glue’. It has long been approved for use as commercially available fibrin sealants which are used in cardiovascular operations and various types of surgeries especially in patients with haemostatic disorders and as an aid in adhesive sealing of wounds [7]. Fibrin sealants are typically found to be biocompatible without inducing excessive inflammation, foreign body reaction, tissue necrosis or extensive fibrosis. Although fibrin is a good sealant, its poor mechanical properties prevent us from considering its use for the proposed application as an AF sealant. However, crosslinking fibrin with a properly chosen crosslinker would enhance its mechanical properties to match that of the native AF tissue. Genipin has been chosen as a crosslinker for the fibrin gels in the present study. Genipin is an agent extracted from the gardenia fruit. Several studies have reported genipin to have much lower cytotoxicity over
glutaraldehyde which is a commonly used cross-linking agent [8] Genipin has been reported to bind with biological tissues and biopolymers such as chitosan, gelatin and fibrinogen [9]. To enhance cell viability, addition of extracellular proteins such as collagen and fibronectin was also experimented. Fibronectin is known to assist in formation of fibrin clot by increasing the adhesion between cells and by favoring their growth and migration [6].

### 2.2 Rheology

Oscillatory shear techniques are commonly used to analyze the rheological behavior of viscoelastic materials. In this technique, relative contributions of viscous and elastic response of materials are measured to characterize the viscoelasticity. The timescale of these tests is defined by frequency of oscillation. A sinusoidal stress is applied to the sample over a range of frequencies and the resulting strain and phase angle is measured.

The shear elastic or storage modulus $G'$ is defined as the amplitude ratio of the component of the stress ($\sigma_0$) in phase with the strain to the strain amplitude ($\gamma_0$).

$$G' = \sigma_0 \cos(\delta) / \gamma_0$$

Where $\delta$ is the phase angle between stress and strain. Similarly shear viscous (or loss) modulus $G''$ is the amplitude ratio of the component of the stress in phase with the strain to the strain amplitude.

$$G'' = \sigma_0 \sin(\delta) / \gamma_0$$

For a perfect elastic material, the stress and the strain waveforms are in phase i.e $\delta = 0^\circ$, $G'' = 0$ and $G'$ has a finite value. Thus for a given value of $\gamma_0$, $G'$ gives a measure of the energy that is stored elastically by the system per cycle when subjected to frequency oscillation.
Similarly, for a perfect viscous material, the stress and the strain waveforms are completely out of phase i.e.

\[ \delta = 90^\circ, \ G' = 0, \text{ and } G'' \text{ is finite.} \]

For such a situation, \( G'' \) represents a measure of the energy that is dissipated during flow per cycle of oscillation for each value of \( \gamma_0 \).

However, \( \delta \) value measured by a rheometer is initially termed as the ‘raw phase’. The raw phase is then corrected for the system inertia to obtain the ‘rheological’ phase angle \( \delta \).

For viscoelastic materials like the gel that we are using, the phase angle is between 0° and 90°.

The loss factor (\( \tan \delta \)) is the quotient of lost and stored deformation energy.

\[ \tan \delta = \frac{G''}{G'} \]

The viscoelastic behavior of each real material consists of a viscous and elastic portion. The sum can be shown by a vector diagram when \( G' \) is plotted on the x-axis and \( G'' \) on the y-axis. \( G^* \) is the vector sum of the two and it depicts the gel stiffness properties. It can be represented by:

\[ |G^*| = \sqrt{(G')^2 + (G'')^2}. \]

Dynamic shear measurements are used in the present study to characterize material’s stiffness properties as well as to yield information regarding the gel’s viscoelastic properties during its various stages of transformation form liquid state to a gel-like structure. It is done by:

- Dynamic frequency Sweep test

The samples are subjected to different frequency values at a constant stress and temperature and the storage (\( G' \)) and loss (\( G'' \)) modulus is recorded as a function of frequency. Whichever
modulus is dominant at a particular frequency will indicate whether the fully structured material appears to be elastic or viscous.

- **Dynamic Strain Sweep Test**

The samples are subjected to different shear stress values at a fixed frequency to determine the linear viscoelastic region of the material. As the stress increases, the corresponding shear strain also increases and the rheological response of the material is recorded. The complex modulus of the material \(G^*\) is monitored as a function of strain values. The range in which \(G^*\) values remain constant gives the linear viscoelastic region (the region where deformation is small enough for the modulus to be independent of deformation) for the material at given temperature and frequency.

- **Dynamic time sweep test**

A dynamic time sweep test is conducted to establish any variation in measurement in a given condition of temperature, frequency and stress (or strain). The variation of dynamic response (\(G'\) and \(G''\)) is attributed to changes in properties of the sample material.

### 2.3 Gel Point (GP)

Gel point (GP) is also termed as the Sol-Gel Transition Point and it is defined as the point where there is a sudden loss of flow due to abrupt change in viscoelastic properties from an initially liquid-like state to a solid-like state [11]. A number of ways have been suggested to quantify the GP. A well-known method for determining the GP is by measuring the instant at which \(G'\) and \(G''\) cross each other in an oscillatory shear experiment with constant frequency [12] [14].
Another method that has proved efficient for different types of polymers is based on the Winter-Chambon criteria wherein the GP is identified at the time instant at which \( G' \) and \( G'' \) follow power law behavior i.e. \( G' = k' \omega^n \) and \( G'' = k'' \omega^n \). [10][5][15][14][13]

Here, \( n \) denotes the relaxation exponent and is related to material structure, \( \omega \) is the angular frequency, \( k' \) and \( k'' \) are proportionality constants.

The loss tangent is given by \( \tan(\delta) = \tan\left(\frac{m\gamma}{2}\right) \)

which implies that \( \delta \) is independent of frequency at the GP but proportional to \( n \).

However, experiments on different types of polymers state that the crossover of \( G' \) and \( G'' \) coincides with the GP only in certain cases where the relaxation exponent (\( n \)) is 0.5 and the loss tangent (\( \tan(\delta) \)) is 1 [13][14][10].

The literature speaks of a gel as an infinite network polymer of chains. If one of the segments of a linear polymer in solution can crosslink with other segment on other chains, a gel will ultimately be formed. After each such intermolecular interaction, the weight average molecular weight (\( M_w \)) has increased so that the number of cross linking sites has increased. As the gel formation proceeds, more such units react and \( M_w \) increases until it becomes infinite [15]. This point is the GP. However, no experimental methods have been performed to measure GP by this method.

Summarizing the previous research we can say that the GP can be identified in the following possible ways:

- In the rheological data, the crossover of \( G' \) and \( G'' \) has been suggested as the criteria for gelation.
The intersection point of loss tangent (tan δ) at different frequencies or in other words, the point in a multifrequency test at which tan(δ) becomes independent of the frequency corresponds to the gel point of physically crosslinked systems [13].

At the GP, the weight average molecular weight of the polymer solution is infinite i.e $M_w \rightarrow \infty$ [15].

3.0 MATERIALS AND METHODS

Table 1: Table showing gel formulations tested for each study performed

<table>
<thead>
<tr>
<th>Gel Formulation</th>
<th>Gel Stiffness test</th>
<th>GP tests (G' and G'' crossover method)</th>
<th>GP tests (multi-frequency method)</th>
<th>Degradation test</th>
<th>Contraction Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200mg/ml fibrinogen</td>
<td>250mg/ml fibrinogen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0:1</td>
<td></td>
<td></td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>0.125:1</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25:1</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>0.5:1</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td>✔</td>
</tr>
<tr>
<td>0.25:1+fibronectin</td>
<td></td>
<td></td>
<td></td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>0.25:1+collagen</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td>✔</td>
</tr>
</tbody>
</table>

3.1 Gel stiffness testing

3.1.1 Gel Fabrication

Fibrinogen isolated from bovine plasma (Sigma-Aldrich) was dissolved in phosphate buffered saline (PBS) at concentrations of 200 and 250 mg/ml. The tube was vortexed and left in water bath to let the fibrinogen mix well. Thrombin isolated from bovine plasma (Sigma-Aldrich) was dissolved in PBS at concentration of 100U/ml. To cross-link the fibrin gel genipin was used. Genipin(Wako) was dissolved in Dimethyl Sulfoxide (Fisher) at a concentration of 400 mg/ml. Genipin quantities were different for different set of gels depending on what genipin-
fibrin ratio was desired. In our experiment we used ratios of 0.25:1, 0.125:1 and 0.5:1. Fibronectin and collagen too were added in some of the experiments. In fibronectin-modified groups, fibronectin (Sigma-Aldrich) was added to the fibrinogen solution with a concentration of 60μl/ml in the gel. In collagen-modified groups, collagen type I, isolated from rat tail tendon (BD Biosciences) was mixed with 10x PBS, dH2O, and NaOH and then added to the fibrinogen solution followed by mixing of thrombin and genipin solution into it to achieve a final concentration of 0.08 wt% or 0.8 mg/ml.

While preparing the gel samples, molds with cylindrical wells of diameter 5mm and a depth of 2.5 mm were used. Fibrinogen of desired quantity was pipetted into the well. A mixture of genipin and thrombin was prepared and pipetted in the well containing fibrinogen while mixing the two solutions well. The method was repeated for all of the gels. Once prepared, the mold was kept in a closed chamber with some amount of PBS to create high humidity. The gel was then allowed to set for approximately 18 hrs which would let it crosslink properly. After 18 hours, the fully cross-linked gels were carefully removed from the mold and placed in small tubes filled with a drop of PBS.

### 3.1.2 Oscillatory Shear Tests to determine Gel stiffness

The gel formulations tested for stiffness (Error! Reference source not found.) were divided into two groups of fibrin concentrations.1) 200mg/ml- 0.125:1, 0.25:1, 0.5:1 and 0.25:1+collagen 2) 250mg/ml- 0.125:1 and 0.25:1. Gels used for stiffness testing were approximately 2.5mm thick with a diameter of 5mm. The tests were performed using a TA instruments AR 2000 rheometer fitted with parallel plate geometry. It had flat plates of 8mm covered with sand paper. The testing temperature was maintained at 25°C for all tests by a temperature control system fitted to the rheometer. The gel was subjected to a controlled oscillation and its response to
varying frequencies and % strain values was recorded. The gel was allowed an equilibration time of 5 minutes followed by a dynamic frequency sweep at 1% strain from 0.032 to 32 Hz. The test concluded with a strain sweep at 0.5 Hz from 1 to 100% strain. The test protocol was adopted from Bron et al [16]. The elastic modulus (G') and viscous modulus (G'') and the phase angle (δ) were calculated at each point of the frequency and strain sweeps. ‘Rheology Advantage Data Analysis’ (TA instruments)’software was then used to plot the frequency and strain sweeps with respect to the complex modulus (G*). One gel each of 0.125:1 and 0.25:1 was subjected to repeat tests to determine variance associated with sample placement on the machine. To conduct these tests, after testing the gel once, it was taken out of the testing assembly, and tested again in the same way as a new gel is tested.

3.2 Gel point determination

3.2.1 Frequency and Strain Sweep Experiments

To determine the Gel Point (GP) of the hydrogel time sweep experiments need to be performed. However, prior to the time sweep, frequency and strain sweep experiments were carried out to decide the appropriate frequency and % strain values to be used for time sweep experiments. For this purpose, rheological measurements for 51µl of Fibrinogen dissolved at a concentration of 200mg/ml were performed at 37°C using a TA instruments AR2000 rheometer with 8mm parallel plates and the gap was set at 0.75mm. For the frequency sweep experiments, frequency was varied from 0.03 to 30 Hz at 1% strain while for the strain sweep experiments, %strain values from 0.01 to 100% were tested at 0.5 Hz frequency. No, thrombin, genipin or any agent that would cause the fibrinogen to cross-link was added since a solution with consistent viscosity was desired for these tests.
3.2.2 Time Sweep Experiments

The time sweep experiments were conducted using a Texas Instruments AR2000 Rheometer fitted with parallel plate geometry with 8mm plates. The gel was surrounded by droplets of water and the working assembly was encapsulated in plastic tray held upside down to prevent the gel from dehydrating. Temperature of 37°C was maintained for all tests. Additional tests were conducted on 0:1 and 0.25:1 gels at 25°C and 4°C to see the effect of temperature on the gelling process. The gap was maintained at 0.75mm for all samples. Different gel formulations tested for GP studies are as listed in Table 1 and the sample volume for different gel formulations is shown in Table 2.

Table 2: Gel volumes used for different gel formulations tested in GP determination study.

<table>
<thead>
<tr>
<th>Sample Volume</th>
<th>Gel Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>56µl</td>
<td>0:1 and 0.25:1+collagen</td>
</tr>
<tr>
<td>56.8µl</td>
<td>0.25:1 and 0.25:1+fibronectin gels</td>
</tr>
<tr>
<td>57.6µl</td>
<td>0.5:1</td>
</tr>
</tbody>
</table>

The GP determination tests were conducted in two different ways:

1. In the first set of experiments for GP determination a time sweep at constant shear frequency(1Hz) and %strain(3%) was performed for 30minutes to monitor the in situ gelation behavior of the hydrogel by looking at the elastic stored modulus($G'$) and viscous loss modulus($G''$) with time.

2. Alternatively the gelation time was determined by employing the Winter-Chambon Criteria. Multi frequency tests were carried out in the frequency range of 0.5-5Hz (0.5, 2,
3.5 and 5 Hz) at 3% strain for 30 minutes and the gelation time was determined based on the value of tan\(\delta\) at different frequencies. The testing protocol was adopted from Jiao et al [13].

### 3.3 Degradation Test

Samples prepared for degradation test were similar to those for stiffness measurements. Swelling ratio and degradation studies were conducted on 0:1, 0.25:1, 0.5:1, 0.25:1+fibronectin and 0.25:1+collagen gels (see Table 1) and sample volumes were same as those for GP studies(Table 2). These gels were allowed to set at 37°C. After about 18 hours, gels were removed from their molds, weighed for Day 0 weights \(W_i\) and stored in 24-well culture plates at 37 °C with 1ml PBS added to each well. The well plate was wrapped in a parafilm to avoid water loss. At predefined time points, the gel was removed, blotted gently with filter paper to remove surface water and its swollen weight was taken \(W_s\). Lyophilization of each sample was carried out using a freeze drying method to obtain the dry weight \(W_d\). The swelling ratio was calculated using the formula \(\frac{W_s - W_d}{W_d}\). The degradation percentage was calculated using the formula \(\frac{W_d - W_i}{W_i} \times 100\%\) where \(W_i\) is the weight of the gel on day 0.

### 3.4 Contraction Studies

Contraction studies were carried out on 0:1, 0.25:1, 0.5:1, 0.25:1+fibronectin and 0.25:1+collagen gels with the same sample volume as for the time sweep experiments. (Table 1)
The set up of the experiment is shown in Figure 1. The solution of the gel was placed between two glass slides with. The ends of the glass slide were held with a paper clip to hold the assembly in place. Two filter paper soaked in water were placed on either side of the gel to prevent it from drying. The entire assembly was placed under the microscope with focus of the lens adjusted to give the best view of the gel underneath. The gel was kept under observation for 60 minutes with images being captured at: 30sec, 1min, 5min, 10min, 20min, 30min and 60min.(n=4 was tested for each gel formulation). Image J software was used to measure the area of the gel at each time point.

4.0 RESULTS

4.1 Gel stiffness testing

Rheological testing indicated that strain amplitude of these genipin cross-linked fibrin gels could be changed by varying the fibrin concentration and genipin:fibrin ratio to get desired results. The tests performed in this study were limited to the use of 200mg/ml and 250mg/ml fibrin concentration.
Figure 2: Average Frequency sweep model showing a) dynamic shear modulus and b) phase angle for gels with varying fibrin concentrations and genipin to fibrin ratios compared to human AF. The data for human AF is adopted from an earlier study using the same experimental setup [5].

*One way ANOVA on G* results showed that 0:1 and 0.125:1 gels had significantly different G* values from Human AF (P<0.05)

Varying genipin:fibrin ratio also showed significant changes in gel stiffness properties. (Figure 2). The dynamic frequency and strain sweep results for all the above mentioned gel formulations is shown at the end of result section in (Figure 14-Figure 19). The frequency
sweep results show that genipin cross-linked fibrin gels demonstrate an increased stiffness at higher frequencies. Stiffness test measurement for 0:1 gels and AF tissue were adopted from previously done tests. [5]. Figure 2a draws a comparison between the stiffness results from all the different gel formulations with the native AF tissue. Gels with higher fibrin concentration certainly make the gel stiffer. Gels made with 250mg/ml and 0.25:1 genipin:fibrin ratio were found to have stiffness values closest to that of native tissue stiffness properties.

As far as the effect of genipin concentration is considered, it is seen that for 200mg/ml fibrin concentration, gels with 0.25:1 and 0.5:1 genipin: fibrin ratio have similar G* values even though one would expect the stiffness to be higher with higher genipin concentration. This is expected because if we look at G* values in 0:1, 0.125:1 and 0.25:1 gels, it increases as the genipin concentration is increased (Figure 2a). Also for 250mg/ml fibrin concentration, the G* value is increased when genipin:fibrin ratio changes from 0.125:1 to 0.25:1 (Figure 2a). Collagen increases the gel stiffness to some extent but it does not make a very significant change from 0.25:1 or 0.5:1 gels. Figure 2b shows the phase angle value at 1Hz for various gel formulations. For all the formulations the phase angle value is below 10 degrees which means the behavior is more elastic than viscous. Native tissue has a phase value of 18 degrees at 1Hz which is much closer to 0 degrees than 90 degrees. So it can be concluded that the native tissue too exhibits more elastic properties than viscous.

It was seen from stiffness results that these gels have a very high batch to batch variability (ranging from 10% to 40% for different gel formulations). To evaluate the variability associated with specimen placement and device handling techniques, a few repeat tests were conducted by testing the same gel two consecutive times. Figure 3 shows the results from these repeat tests.
Figure 3: Results from repeat tests for two gels each of 0.125:1 and 0.25:1 to find the source of variance in results

The data of the repeat tests show a maximum variance of 4% which is negligible compared to maximum variance in the original data set that is close to 40%. This suggests that the high variability in results is not an outcome of inconsistent sample handling or device operation and that it comes from other sources such as batch variation and gel mixing.

4.2 Gel Point determination

The working frequency was selected based on the raw phase of the data. As described in the background section, raw phase is essentially the measured phase difference between the sinusoidal torque and displacement in an oscillatory experiment prior to correction for system inertia which gives phase angle $\delta$. The frequency at which, the phase angle between the applied torque and the resulting displacement is zero would be equivalent to an elastic solid. However, these viscoelastic gels contain viscous components that dissipate energy. Hence we would consider the frequency at which the phase angle is minimum to be the best working frequency.
Figure 4: Frequency Sweep experiment results from 4 different samples of 200mg/ml Fibrinogen solution. The blue colored sinusoid indicates applied stress and the red is the resulting strain on arbitrary scales with data taken from the circled areas in the frequency sweep.

Table 3: Frequency values for three points marked under (b) area in Fig 3

<table>
<thead>
<tr>
<th>Frequency(Hz)</th>
<th>Point 1</th>
<th>Point 2</th>
<th>Point 3</th>
<th>Average of Median</th>
<th>Selected Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel 1</td>
<td>0.7536</td>
<td>0.9487</td>
<td>1.194</td>
<td>0.9487</td>
<td>1 Hz</td>
</tr>
<tr>
<td>Gel 2</td>
<td>0.7536</td>
<td>0.9487</td>
<td>1.194</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel 3</td>
<td>0.7536</td>
<td>0.9487</td>
<td>1.194</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel 4</td>
<td>0.7536</td>
<td>0.9487</td>
<td>1.194</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4 shows the phase angle at three different frequency points. At lower frequencies, the phase angle is not very high but the response is noisy (Figure 4a). As the frequency increases, the response gets clearer and in the range of 0.7-1.2 Hz frequency, the noise as well as the phase angle is at a minimum (Figure 4b). As the frequency increases beyond that range, the phase angle starts to increase and at much higher frequencies it is almost 90° out of phase (Figure 4c). Table 3 gives precise information about the frequency in the range of 0.7-1.2 Hz for all four gels.
median value for the three points in the selected range was calculated and an average of those median values was used to select 1Hz as the frequency for the gelation test time sweep.

A similar strain sweep test was used to determine the % strain value strain value for gelation tests. Oscillatory strain sweep experiments were conducted and the phase angle was observed at different % strain values. It was observed that in the range of 2.1-3.5% strain, the phase angle and the signal noise were at a minimum (Figure 5c). % strain values falling below that range produced noisy responses (Figure 5b) while % strain values beyond that range had very high phase angle (Figure 5c) which is not desirable.

Figure 5: Strain Sweep experiment results from 4 different samples of 200mg/ml Fibrinogen solution. The blue colored sinusoid indicates applied stress and the red is the resulting strain at the marked areas.
Figure 6: Dynamics of elastic, $G'$ and viscous $G''$ moduli and loss tangent tanδ (inset graph) at 1 Hz for (a) Genipin:fibrinogen=0:1 and (b) genipin:fibrinogen=0.25:1. The gelation time is determined as the time at which $G'$ and $G''$ intersect each other (i.e at which tan (δ) drops below 1) which occurred at the very beginning of the experiment.
Table 4: % strain values for three points marked under (b) area in Fig 3.

<table>
<thead>
<tr>
<th>% Strain</th>
<th>Point 1</th>
<th>Point 2</th>
<th>Point 3</th>
<th>Median</th>
<th>Average</th>
<th>Selected % Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel 1</td>
<td>2.1876</td>
<td>2.7701</td>
<td>3.5298</td>
<td>2.7701</td>
<td>2.7148</td>
<td>3 %</td>
</tr>
<tr>
<td>Gel 2</td>
<td>2.1746</td>
<td>2.7419</td>
<td>3.5103</td>
<td>2.7419</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel 3</td>
<td>2.0856</td>
<td>2.7407</td>
<td>3.3307</td>
<td>2.7407</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel 4</td>
<td>2.1017</td>
<td>2.6056</td>
<td>3.3059</td>
<td>2.6056</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4 gives the % strain values for the four samples in the range of 2.1-3.5%. The median of three points in each sample was calculated and the average of those median points was used to select 3% strain as the value to be used for gelation testing.

Table 5: Values of G’ and G” at different time points starting from when the test begins to the point when G’ and G” cross each other for (a) 0:1 Gels and (b) 0.25:1 Gels. These values were recorded only for a few samples and do not necessarily represent the complete data set. Crossover values for all the samples could not recorded since it occurred too early.

**Table 5a-0:1 Gels**

<table>
<thead>
<tr>
<th>Time(s)</th>
<th>G’(Pa)</th>
<th>G”(Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5620</td>
<td>19.49</td>
<td>26.16</td>
</tr>
<tr>
<td>13.187</td>
<td>29.28</td>
<td>23.92</td>
</tr>
<tr>
<td>19.734</td>
<td>32.07</td>
<td>24.09</td>
</tr>
</tbody>
</table>

**Table 5b-0.25:1 gels**

<table>
<thead>
<tr>
<th>Time(s)</th>
<th>G’(Pa)</th>
<th>G”(Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5620</td>
<td>6.861</td>
<td>10.04</td>
</tr>
<tr>
<td>13.187</td>
<td>7.906</td>
<td>0.4847</td>
</tr>
<tr>
<td>19.734</td>
<td>7.497</td>
<td>0.3446</td>
</tr>
</tbody>
</table>

To define the GP, time sweep experiments were conducted and the crossover of G’ and G” was monitored for genipin:fibrinogen ratios of 0:1 and 0.25:1 (Figure 6a and Figure 6b respectively). At the beginning, G’ is lower than G” which was expected since the samples were still in liquid state and thus viscous properties dominated. As the solution began to turn into a gel-like state due to formation of cross-links, both moduli increased. However, the rate of increase of G’ was higher than G” since the elastic properties began to dominate. This difference in rates leads to G’ and G” crossover. The time required to achieve this crossover is the gelation time. As seen in Figure 6a, the crossover occurs quite early in time. Table 5 gives precise G’ and G”
values so that time for the crossover can be observed. It is seen that for 0:1 as well as 0.25:1 gels, the crossover occurs somewhere between 6.5-13 sec from the start of the test (Table 5). The sample loading time was recorded to be approximately 25 sec. Add to it the time recorded on the graph, the total gelling time would be around 31.5-38 sec.

As mentioned earlier, the gelation time was also evaluated from the plots of loss tangent (\(\tan \delta\)) versus time at multiple frequencies to validate the results from \(G'\) and \(G''\) crossover experiments since the literature states that the cross-over method of determining the GP is valid only for some network polymers and wrong for others [14]. It was studied that the method works only for polymers that have a relaxation exponent \(n=0.5\) and \(\tan \delta=1\). ‘n’ was evaluated for our gels by an inbuilt model in the Data Analysis software and it was found to be greater than 1 for all our gels which explains the reason for not being able to get a practically useful value of GP by the cross-over method. Another method was based on using the Winter-Chambon criterion which works on the hypothesis that \(\tan (\delta)\) is frequency independent at the GP. shows a representation of the behavior of loss tangent \(\tan (\delta)\) versus time at different constant frequencies for pure fibrin as well as 0.25:1, 0.5:1, 0.25:1+fibronectin and 0.25:1+collagen gels. One can see that \(\tan (\delta)\) lines for different frequencies coincide at the gel point. Beyond this point, \(\tan (\delta)\) decreases more gradually as a result of formation of elastic gel. It is observed that pure fibrin gels have the lowest gel time.
Figure 7: A summary of loss tangent $\tan(\delta)$ versus time for a) 0:1 gels b) 0.25:1 gels c) 0.5:1 gels d) 0.25:1+fibronectin and e) 0.25:1+collagen gels at different frequencies: 0.5Hz, 2Hz, 3.5hz and 5Hz. It should be noted that these figures are only a rough representation of the complete set. The complete figure set is shown in Figure 22 through Figure 26.
However the GP does not remain constant even for the same gel formulation when repeated tests are done. *Figure 22 through Figure 26 gives a detail description of the variability by listing the plots from repeat tests for each gel formulation. Four gels for each gel formulation were tested. Based on that, the Gel time range for all the formulations was defined* *(Table 6)*

![Figure 8: Bar graph representation of gelling time for each gel formulation based on the data in Figure 22 to Figure 26. * One way ANOVA results showed that 0:1 gels were significantly different from all (P<0.05)*](image)

*Table 6: Gelling time ±SD for different gel formulations: 0:1, 0.25:1, 0.5:1, 0.25:1+fibronectin and 0.25:1+collagen at 37°C*

<table>
<thead>
<tr>
<th>Gel Formulation</th>
<th>Gelling Time (secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:1</td>
<td>201.7±52.5</td>
</tr>
<tr>
<td>0.25:1</td>
<td>860.7±60.7</td>
</tr>
<tr>
<td>0.5:1</td>
<td>685.7±100.3</td>
</tr>
<tr>
<td>0.25:1+fibronectin</td>
<td>741.7±130.2</td>
</tr>
<tr>
<td>0.25:1+collagen</td>
<td>905.5±50.9</td>
</tr>
</tbody>
</table>
Figure 9: Tan (δ) results for 0.25:1 gels at multiple frequencies for a) 37°C b) 25°C and c) 4°C

In order to evaluate the effect of temperature on gelling time, 0.25:1 gels were tested for 37°C, 25°C and 4°C. Figure 9 shows the gelling time for each temperature. For the gels at 37°C, tan (δ) shows a rapid drop as well as starts to converge close to 800s (Figure 9a). For the gels at 25°C, tan (δ) convergence begins around 1200sec (Figure 9b) which can be defined as the GP. However for the 4°C, no convergence or dropping of tan (δ) values is observed within the time
range of the test (Figure 9c) which suggests that it takes more than 30mins for the GP to occur in this case.

Figure 10: Tan (δ) values plotted against time for 0.25:1 gels at a) single frequency of 1Hz and b) multiple frequencies: 0.5Hz, 2Hz, 3.5Hz and 5Hz.

Pure fibrin gels clearly have the lowest gelling time while 0.25:1 and the gels with collagen added take the maximum time to gel (Table 6). Having performed both methods for GP determination, a comparison between the two was drawn. Figure 10 shows tan (δ) values for single frequency (Figure 10a) as well as multi-frequency test (Figure 10b) for 0.25:1 gels. It is seen that tan (δ) value begins to drop around the same time in both cases and falls within the range defined for the gel in

*Figure 27 to Figure 31* show divergence in viscosity at the sol-gel transition. The complex viscosity of pure fibrin gels was compared to the same gel with addition of genipin at different concentrations and by adding collagen and fibronectin to it. The point at which viscosity value stabilizes would ideally be where the gel has fully crosslinked i.e it is completely gel-like. The results show that viscosity stabilizes as early as 500sec for pure fibrin gels while the stabilization
for other gel formulations in some cases occurs close to the end of 30 minute test while for some cases it doesn’t occur within the time frame of the test.

4.3 Degradation Study

Figure 11: Equilibrium swelling ratio of 0:1, 0.25:1, 0.5:1, 0.25:1+fibronectin and 0.25:1+collagen gels in PBS at 37°C.

Figure 11 shows the swelling ratio measured for all the gel formulations. Fibrin gels swelled up infinitely after day 4. As a result of that they also tend to degrade completely at day 7 and that is why they are not shown beyond that point in Figure 11. Swelling ratio of all other gel formulations behaves consistently over the 21-day time point.
Figure 12: % water content representing degradation of 0:1, 0.25:1, 0.5:1, 0.25:1+fibronecin and 0.25:1+collagen gels in PBS at 37°C measured at day 1, 5, 7, 10, 14 and 21. The value of fibrin gels approaching 1 for day 7 indicated that the specimen had completely degraded and was all water.

Figure 12 shows the degradation profile or the water content of the tested gel formulations over 21 days. Apart from pure fibrin gels, other gel formulations did not show significant difference in their degradation profile over the 21 day time point. The water content of all gel formulations except pure fibrin gels that degraded completely by day 7 did not show a significant change over a course of 21 days. A negligible change in swelling ratio values for all gel formulations except pure fibrin gels also indicate that the gels do not absorb much water overtime and hence takes longer to degrade. A two-way ANOVA was completed for this data, and there was significance between some data points, but these were not consistent across all time points for any one data set. This was also true for the swelling ratios.
4.4 Gel Contraction

Gel contraction studies were done to evaluate the percentage contraction of the gel. *Figure 13* shows the %contraction for each gel formulation. Based on that data, *Table 7* was formulated summarizing percentage contraction in each gel.

*Table 7:* % contraction calculated for each gel formulation based on Fig10.

<table>
<thead>
<tr>
<th>Gel Formulation</th>
<th>% contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:1</td>
<td>5±1.82</td>
</tr>
<tr>
<td>0.25:1</td>
<td>2±4.16</td>
</tr>
<tr>
<td>0.5:1</td>
<td>10±2.06</td>
</tr>
<tr>
<td>0.25:1+fibronectin</td>
<td>3±0.73</td>
</tr>
<tr>
<td>0.25:1+collagen</td>
<td>6±1.38</td>
</tr>
</tbody>
</table>

*Figure 13:* % contraction in 0:1, 0.25:1, 0.5:1, 0.25:1+fibronectin, 0.25:1+collagen measured over a time range of 60 minutes.

0.25:1 gel contracts the least with contraction close to 2% while the gel with 0.5:1 contracts the most with 10% contraction. After 0.5:1 gels, gels with collagen added to them contract the most.
Fibrin gels have % contraction similar to that of the 0.25:1+collagen gels while 0.25:1+fibronectin have a contraction pattern similar to 0.25:1 gels. A Two way ANOVA was completed for this result and it was seen that there was no significant difference between the contraction of all gel formulations. Also, there was no significant contraction seen for any of the formulations. (P>0.05)

The method used for measuring the area of the image to calculate the contraction, was prone to human error. Hence repeat tests were done to quantify the maximum possible error in measurement. Error in the range of ±0.1-4% was observed. Therefore, the error % must be considered while deciding the % contraction of the gel.
5.0 COMPLETE EXPERIMENTAL RESULTS:

**Figure 14:** Gels with fibrin concentration 200mg/ml and genipin:fibrin ratio 0.125:1 tested at 25°C for Dynamic frequency sweep at 1% strain for a) $|G^*|$ and b) $\delta$(degrees) and dynamic strain sweep at 0.5Hz frequency for c) $|G^*|$ and d) $\delta$(degrees)
Figure 15: Gels with fibrin concentration 200mg/ml and genipin:fibrin ratio 0.25:1 tested at 25°C for Dynamic frequency sweep at 1% strain for a) $|G^*|$ and b) $\delta$(degrees) and dynamic strain sweep at 0.5Hz frequency for c) $|G^*|$ and d) $\delta$(degrees)
Figure 16: Gels with fibrin concentration 200mg/ml and genipin:fibrin ratio 0.5:1 tested at 25°C for Dynamic frequency sweep at 1% strain for a) $|G^*|$ and b) $\delta$(degrees) and dynamic strain sweep at 0.5Hz frequency for c) $|G^*|$ and d) $\delta$(degrees)
Figure 17: Gels with fibrin concentration 200mg/ml and genipin:fibrin ratio 0.25:1+collagen tested at 25°C for Dynamic frequency sweep at 1% strain for (a) $|G^*|$ and (b) $\delta$ (degrees) and dynamic strain sweep at 0.5Hz frequency for (c) $|G^*|$ and (d) $\delta$ (degrees)
Figure 18: Gels with fibrin concentration 250mg/ml and genipin:fibrin ratio 0.125:1 tested at 25°C for Dynamic frequency sweep at 1% strain for a) $|G^*|$ and b) $\delta$(degrees) and dynamic strain sweep at 0.5Hz frequency for c) $|G^*|$ and d) $\delta$(degrees)
Figure 19: Gels with fibrin concentration 250mg/ml and genipin:fibrin ratio 0.25:1 at 25°C for Dynamic frequency sweep at 1% strain for (a) $|G^*|$ and (b) $\delta$(degrees) and dynamic strain sweep at 0.5Hz frequency for (c) $|G^*|$ and (d) $\delta$(degrees)
Figure 20: Time sweep test results for GP determination in 0:1 gels for 1Hz frequency and 3% strain at 37°C for n=4 a), c), e), g) $G', G''$ vs time. b), d), f), h) tan (δ) vs time. The crossover point occurs very early in time so it cannot be captured on the graph.
Figure 21: Time sweep test results for GP determination in 0.25:1 gels for 1Hz frequency and 3% strain at 37°C for n=4 a), c), e), g) $G', G''$ vs time. b), d), f), h) $\tan(\delta)$ vs time. The crossover point occurs very early in time so it cannot be captured on the graph.
Figure 22: Multifrequency Time sweep test results for GP determination in 0:1 gels for 3% strain and frequency of 0.5, 2, 3.5 and 5 Hz at 37°C for n=4. The point marked in the figure is where tanδ for different frequencies converge or the GP.
Figure 23: Multifrequency Time sweep test results for GP determination in 0.25:1 gels for 3% strain and frequency of 0.5, 2, 3.5 and 5 Hz at 37°C for n=4. The point marked is in the figure is where tanδ for different frequencies converge or the GP.
Figure 24: Multifrequency Time sweep test results for GP determination in 0.5:1 gels for 3% strain and frequency of 0.5, 2, 3.5 and 5 Hz at 37°C for n=4. The point marked in the figure is where tanδ for different frequencies converge or the GP.
Figure 25: Multi-frequency Time sweep test results for GP determination in 0.25:1+fibronectin gels for 3% strain and frequency of 0.5, 2, 3.5 and 5 Hz at 37°C for n=4. The point marked in the figure is where tanδ for different frequencies converge or the GP.
Figure 26: Multifrequency Time sweep test results for GP determination in 0.25:1+collagen gels for 3% strain and frequency of 0.5, 2, 3.5 and 5 Hz at 37°C for n=4. The point marked is in the figure is where tanδ for different frequencies converge or the GP.
Figure 27: Dynamic viscosity measurement for 0:1 gels at multiple frequencies: 0.5, 2, 3.5 and 5Hz for n=4. The marked value represents the point beyond which viscosity value reaches steady state.
Figure 28: Dynamic viscosity measurement for 0.25:1 gels at multiple frequencies: 0.5, 2, 3.5 and 5 Hz for n=4. The marked value represents the point beyond which viscosity value reaches steady state.
Figure 29: Dynamic viscosity measurement for 0.5:1 gels at multiple frequencies: 0.5, 2, 3.5 and 5 Hz for n=4. The marked value represents the point beyond which viscosity value reaches steady state.
Figure 30: Dynamic viscosity measurement for 0.25:1+fibronectin gels at multiple frequencies: 0.5, 2, 3.5 and 5 Hz for n=4. The marked value represents the point beyond which viscosity value reaches steady state.
Figure 31: Dynamic viscosity measurement for 0.25:1+collagen gels at multiple frequencies: 0.5, 2, 3.5 and 5 Hz for n=4. The marked value represents the point beyond which viscosity value reaches steady state.
Figure 32: % contraction in 0:1 gels, 0.5:1 gels, 0.25:1 gels and 0.25:1+fibronectin gels and 0.25:1+collagen gels at time points of 1, 5, 10, 30 and 60 minutes.
6.0 DISCUSSION

As described in previous sections, the current study was divided into 4 different parts with each section testing a unique property of the genipin cross-linked fibrin gels. Shear tests characterized gel stiffness properties, GP determination tests defined the gelling time. The degradation test quantified the temporal stability of the gel’s hydration and dimensions in high humidity environment. All of these properties are essential in describing genipin crosslinked fibrin gel’s potential success for its intended use as an injectable material for AF repair. Gels with varying genipin concentrations were used for this purpose and the effect of addition of collagen and fibronectin was also observed. Our shear test results showed that genipin was required to achieve a modulus close to the native tissue. Also, these gels can be tuned to have a modulus suitable to match native human AF tissues by varying genipin:fibrin ratio and fibrin concentrations. Results from the gelation test demonstrated that fibrin reached the gelation point in a few minutes after it was loaded onto the test machine but that addition of genipin increased the gelling time to values considered more useful for mixing and injecting into a patient but still short enough so that it would be very solid by the time a surgical procedure was completed. It was also seen that gelling time shows a very evident temperature dependency occurring more rapidly at body temperature than room temperature. Another important conclusion was that the G' and G" crossover method of GP determination does not hold true for our gels. Rather the Winter-Chambon criterion was more useful for our genipin crosslinked fibrin gels since it gives GP values which could be validated by the observations while handling the gel. The degradation test results lead us to believe that our gels do not completely degrade over a period of 21 days except for the fibrin gels which degrdaded completely on the 7th day. Lastly the contraction studies suggest that there is minimum contraction taking place as the gel turns from a liquid to a fully crosslinked gel.
The stiffness results from rheological testing indicate that a desired value of modulus can be achieved by varying the concentrations based on the specific purpose. However, it is essential to assess these behaviors across a broad range of loading conditions. Increasing the fibrin concentrations causes a linear increase in modulus of the gel. However, increasing the genipin concentration does not lead to a linear increase in modulus although it does increase the modulus in some cases. As discussed earlier, these tests had high variability. It was seen that the variability arises usually when a new batch of gels was made. All gels made in a single batch showed similar results. Although this variability is noted in all gel formulations, it was the most prominent for 0.125:1 gels (Figure 14). A variety of reasons could be assumed for this source of variation. Pipette mixing was used as the standard technique which is subject to high degree of variability. Fibrin being a biological material is also susceptible to variations. Genipin solution once made can ideally be used for 14 days. However, it might make a difference to use freshly made genipin as opposed to the genipin that has been stored for a week. To overcome these sources of errors the following could be considered in future: using syringe mixing to ensure proper mixing and delivery of equal amounts each time and to use freshly made genipin for every new batch of gels.

For the design of injectable sealant material, an important property is to have a well defined solidification rate or the gelation time. The gelation time needs to be balanced to be short enough to shorten the waiting time for the patient and prevent extrusion of the hydrogel from the injection site but on the other hand it should be of sufficient duration to allow for a proper surgical procedure to take place. Despite the relevance of this property that is related to the structure and composition of the gel formulations as well as their processing conditions not much work has been done in this area. A majority of previous studies to quantify the GP for gels utilize
the G' and G'' cross-over method.[17][10][18]. However some work suggests that G' and G''
crossover method is not a standard method to characterize the GP for all
polymers[14][5][15][13]. It is found that crossover of G' and G'' coincides with the GP only in
certain cases, where the relaxation exponent (n) is 0.5 and tan δ is 1 [13]. We calculated the
value of n for our gels by applying a default model to calculate n based on the plot in Data
Analysis software. It was found to be closer to 1 for all the gel formulations that we used. So
clearly, the G' and G'' crossover was not the best method to characterize GP for our gels.
Moreover, as seen in Figure 6 the Gelling time observed was on the order of a few seconds
which perhaps is too quick for a surgical procedure to take place. However when we repeat the
tests using multi-frequency tests, the GP values measured are practically more relevant and
useful.
From
Table 6 we see that the gel time ranges between 600-900 seconds for all the gel formulations
(except for pure fibrin gels) which is long enough for a surgical procedure to be completed but at
the same time it is quick enough not to cause the gel to squeeze out of the defect. However, it is
interesting to observe that addition of genipin to pure fibrin gels slows down the gelling process.
It is interesting because a previous study on genipin crosslinked chitosan gels indicated that
genipin actually speeds up the gelling process [11] which is not true when genipin is used to
crosslink fibrin gels. It could be hypothesized that addition of genipin triggers some sort of
mechanism which doesn’t allow thrombin to crosslink fibrin as quickly as it does in a pure fibrin
gel. While 0.5:1 genipin to fibrin ratios had shorter gel times than the 0.25:1 ratio, this concept
may still hold in a non-linear manner. However, stiffness and degradation was always improved
with the addition of genipin suggest there was sufficient mechanical integrity and that these
formulations were acceptable. Gel time was highly dependent on temperature. Higher temperature caused the gel to form cross-links more quickly compared to lower temperatures at which interaction between the materials was slowed and at 4°C there was little evidence of gelation. It is not possible to have a precise value for the gelling time because each time the test was conducted, some variance resulted from a variety of factors: measurement techniques like mixing of gel solution and variability in fibrin and genipin from biological factors. Hence it is always ideal to characterize the gelling time as a range of values instead of a precise value. The viscosity measurement confirms that pure fibrin gels crosslink much faster than other formulations which crosslink in a time range of 1000-1500 secs with little variation amongst different groups.

Water content was studied in AF in a study that reveals that a healthy disc has an AF with 70-80% water content which decreases as the disc matures. However if the disc is degenerating, the water content in the AF increases with increasing degree of degeneration[19]. The degradation studies on our gels show that these gels have a water content of approximately 85-90% which is much higher than the water content of AF but these gels could still be efficiently used as a defect filling material since its water content does not change much over a 21-day timepoint. This is useful because the degradation period should be long enough to allow extracellular matrix to regenerate and fill the defect. However, fibrin alone had a value of Qw approaching infinity and water content approaching 1 at day 7 indicating the specimen had completely degraded. This makes us conclude that genipin is important to prolong the degradation time. Contraction results show that the amount of contraction in the gel is close to 10%. However the error in measurement can be upto 4%. Therefore the overall contraction was small and not anticipated to present a problem during testing or injection. However for actual
clinical procedure i.e injection into IVD defects, it would be advisable to use an extra 5% gel volume for injection to account for this small amount of contraction upon setting.

The present study although successful in characterizing the stiffness, gelling, degradation as well as contraction properties of the gel, some studies particularly the stiffness results had high variance which might need some further testing after fixing the source of variance to get better results. It may be considered to test the gel formulations again for stiffness by using syringe mixing as a method of gel mixing in future. Variance resulting from biological materials such as fibrin and genipin cannot be controlled. Also mixing of high viscous materials adds an additional source of variance. The relatively quick gel time (Ranging from 600 to 900sec for different gel formulations) makes it possible to use it to fill defects with injection techniques. Since the results indicate that the gel does not degrade much over a course of 21 days it might be useful to test them on a longer time scale to see if they degrade when kept longer.

From the results of stiffness studies, GP studies as well as degradation tests, it is seen that gels without genipin had poor mechanical properties, very short gel time (around 200 sec) which would cause it to gel up even before the gel could be injected properly and it also degraded completely by day7. These results indicate that addition of genipin highly improves its mechanical properties as well as delays the gel time and degradation period to a desired time. However a very significant difference was not observed between all the currently tested formulations for the performed studies which suggest that all the formulations have acceptable gel time and degradation properties except pure fibrin gels. In case of actual surgical procedure, taking into account the time range of 1000-1500 secs obtained for viscosity measurement of different formulations, a time of 1500 secs (25 mins) could be defined as safe to resume patient mobility. This time frame is good since it would be enough for a surgical procedure to take place
but not too long to restrict the patient for long hours. Therefore the gel time of all formulations except for pure fibrin gels is adequate. From the results of contraction studies it is seen that there is a small amount of contraction in the gels upon setting. To account for that, an additional 5% gel volume would be recommended for injection into the defect. The present study does not give a clear idea about what gel formulation would work best for in vitro use. Fatigue tests, adhesion tests as well as cell viability tests would help in understanding the different gel formulations better. Some tests to characterize tensile properties could also be considered in future.

**7.0 CONCLUSION**

Through this study we have systematically studied the rheological properties of genipin cross-linked fibrin gels. It was an interesting finding that in case of fibrin crosslinked gels, addition of genipin actually slows down the gelling process while in most cases initiators like genipin can quicken gelation times. Rheology measurements on these gels allowed us to assess its viscoelastic properties and it was learned that the stiffness of the gel is controlled by fibrin concentration and genipin:fibrin ratio. Although the present study is useful in characterizing many important properties of these gels, it cannot by itself be used to predict which gel formulation would work best for clinical purposes. In vivo biocompatibility and in situ mechanical testing needs to be done before that decision can be made.
8.0 Bibliography


