Mechanics of Gelatin and Elastin based hydrogels as Tissue Engineered Constructs

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Abstract

Engineered soft hydrogels have recently garnered attention for their application as scaffolds for tissue engineering, drug delivery, and as model extracellular matrices such as arteries and cartilage that experience dynamic loading. Hydrogels exhibit nonlinear, composite, viscoelastic, and anisotropic behaviors and hence, mechanical models used to describe such materials are challenging. In this study, we synthesized three dimensional gelatin and elastin hydrogels of varying compositions and crosslinking degrees and characterized their structure-property relationships. Mechanical properties were measured using monotonic compression, dynamic mechanical analysis (DMA), and quasi-static stress relaxation tests. Compressive moduli for the gels range from 1.5 to 15 kPa and these measurements correlate with the values of storage modulus obtained from DMA studies ($p<0.05$). Ongoing studies are aimed at using stress relaxation data to create mechanical spring-dashpot models to describe the viscoelastic behavior of these materials. Scanning electron microscopy was used to assess the gel microstructure. Our results demonstrate that varying the gelatin and crosslinker concentrations leads to significant ($p<0.05$) changes in pore sizes with the lowest stiffness group exhibiting considerably larger porosity. Incorporation of elastin resulted in increased stiffness, change in hydrogel microstructure and swelling properties of the hydrogels.

Keywords: hydrogels, viscoelasticity, gelatin, elastin, tissue engineered scaffolds

1. Introduction

Tissue engineering scaffolds aim to synthesize living tissues *in vitro* by creating suitable mechanical and chemical environments for tissue growth. These scaffolds typically have compositions and mechanical properties similar to that of the native tissues which predominantly contain structural proteins collagen and elastin [1]. Collagen, of which gelatin is a denatured form, is a ubiquitous protein that gives tissues stiffness and mechanical strength. In contrast, elastin is a fatigue resistant rubber-like protein present in dynamic structures, such as arteries and ligaments, which undergo repeated cyclic deformation. Collagen and elastin composite hydrogels present multiple challenges towards our understanding of the material responses due to their non linear and viscoelastic nature while being fracture resistant and able to sustain cyclic loads to serve as biological replacements. Earlier studies characterizing the mechanical properties of biocompatible hydrogels have employed various mechanical testing methods from monotonic compression and tension [2, 3], to viscoelasticity studies through dynamic mechanical analysis and stress relaxation [4–7]. These tests provide insights into the material response of soft materials for a better tuning of the tissue properties. Few studies have however focused on characterizing the mechanics of elastin and collagen based composite hydrogels aimed toward better design of scaffolds that replicate the *in vivo* cellular environments.

The aims of this study are threefold: first, to fabricate gelatin and elastin based crosslinked hydrogels of varying material stiffness to emulate native tissue environments. Crosslinking of hydrogels is necessary to maintain the integrity and long term viability of the hydrogels. Second, to study the microstructure and water uptake behavior of the hydrogels using scanning electron microscopy and swelling experiments. Third, to quantify mechanical properties through monotonic compression tests, dynamic mechanical analysis and stress relaxation experiments. Ongoing studies in our lab are also aimed at characterizing the fracture properties of hydrogels. Because the growth

and behavior of biological tissues are exquisitely sensitive to the mechanical properties of their environment, we hope such studies will help in the design of engineered hydrogels to better emulate native tissue environments [8].

2. Materials and Methods

2.1 Isolation of elastin from bovine aorta

Native aortic tissues typically contain elastin, collagen and proteoglycans. Protocols for elastin isolation from native tissues rely on its chemical inertness and insolubility in various reagents at elevated temperatures. We used the hot alkali treatment to extract elastin from bovine thoracic aorta in this study to obtain a relatively pure and insoluble elastin protein [9]. In this method, artery samples were thoroughly washed with distilled water to clean tissues, minced using a grinder and kept in a solution of 0.1 M NaCl overnight. Treated tissues were washed thrice with distilled water to remove the salt and boiled for 45 minutes in 0.1 M NaOH solution at 95°C to remove contaminant proteins, such as collagen and proteoglycans, from aorta samples. Tissues were washed twice with distilled water and dried at 60°C to obtain purified and insoluble elastin. Finally, the dried material was powdered, sieved (150 µm pore size) and stored at 4°C until use.

2.2 Preparation of crosslinked gelatin and elastin hydrogels

Porcine gelatin Type-B powder (Sigma Aldrich, India) was dissolved in double distilled water at 50° C to obtain a clear solution. Because gelatin is soluble in water, it is necessary to crosslink the material to preserve the hydrogel structure. Collagenous materials, such as gelatin, are generally crosslinked using methods which exploit the large number of functional side groups. We used glutaraldehyde (Sigma Aldrich, India, 25% stock) as a crosslinking agent by combining it with dissolved gelatin solution in this study [10]. A cylindrical mold, containing 9 wells of dimensions 16 mm diameter and 20 mm height each, was fabricated using polydimethylsiloxane (PDMS, Sylgard ®184, Dow Corning). The inner surface of each individual well of the mold was coated with petroleum jelly to prevent adhesion to the hydrogel. Two different concentrations of gelatin and one composite gelatin-elastin hydrogel were prepared in this study. Gels were poured in each well and cured at 4° C for 6 hours. To reduce dehydration of the hydrogels during crosslinking, the mold was wrapped in plastic film. Finally, the gels were soaked in 0.1 M glycine solution to block additional glutaraldehyde action and stored in distilled water overnight prior to mechanical testing [11]. Gelatin-elastin hydrogels were prepared by suspending the insoluble elastin in gelatin solution and vortexing the mixture before adding glutaraldehyde to crosslink the gels.

Table 1 Composition of hydrogels used in the study

Group	Gelatin (gm/ml)	Elastin (gm/ml)	Glutaraldehyde (%v/v)
3G	0.03	0	0.5
5G	0.05	0	1
5GE	0.05	0.05	1

2.3 Swelling characteristics of hydrogels

Hydrogel samples were lyophilized for 24 hrs and their dry weight measured. Samples were immersed in 5 ml of distilled water at room temperature and the wet weight was measured at

regular time intervals. Before weighing, the samples were gently patted dry using tissue paper and the swelling ratios calculated at each time point as

$$Swelling\ Ratio\ (\%) = \frac{W_s - W_d}{W_d} \times 100 \quad (1)$$

w_s is the weight of the swollen sample at each time period. To determine the fluid content of the hydrogels in their maximum swollen state, Equilibrium Water Content (EWC) was calculated using

$$EWC(\%) = \frac{W_e - W_d}{W_e} \times 100 \quad (2)$$

w_e is the equilibrated sample weight and w_d is the sample weight in its dry state in equation (2).

2.4 Mechanical Tests

2.4.1 Determination of modulus of the hydrogels

Monotonic compression tests were performed on a Bose Electroforce® 3200 (Bose Corp., USA) Dynamic Mechanical Analysis (DMA) instrument equipped with two parallel compression platens. Displacements were measured using a LVDT and the forces obtained using a transducer (Bose Corp., ±22.5N). The height and diameter of gels were measured from a photograph of the gels (Nikon D7000 camera) using ImageJ software [12]. Hydrogel specimens were preloaded in the DMA instrument to 5 grams and preconditioned for 30 cycles of 10% compressive strain at 0.05Hz to obtain a repeatable material response [6, 13]. Following preconditioning, samples were compressed at a rate of $0.01s^{-1}$ until either the maximum displacement limit of the instrument was reached or the gels failed. Custom written programs in MATLAB (v7.8.0.347 (R2009a)) were used to analyze the data. Compressive moduli were calculated using the stress-strain data upto 4% strain. In all, 4 samples were tested in each of the 3G, 5G and 5GE groups and an average modulus was calculated for each group.

2.4.2 Dynamic Mechanical Testing

To obtain viscoelastic properties of the hydrogels in each of the three groups, we used fresh compression samples by preloading the hydrogel to 5 gram force and subjecting them to cyclic sinusoidal loading between 0 and 10% compressive strain over a frequency range of 0.05 to 130 Hz. 4 samples were tested in each hydrogel group. Data were acquired at least 30 times the loading frequency and the traces (Figure 1) were analyzed using custom software. In this method, a discrete Fourier transform was performed on the stress and strain data for each acquired frequency block. The phase and amplitude of the relevant portion of the resulting spectra of the stress and strain were used to compute the storage modulus (E′), loss modulus (E″) and phase angle (δ) for the hydrogel [14].

Variation in the strain with angular frequency (ω) is given as

$$\varepsilon(t) = \varepsilon_o \ \sin(\omega t) \quad (3)$$

Stress corresponding to the strain loading is represented by

$$\sigma(t) = \sigma_o \ \sin(\omega t + \delta) \quad (4)$$

σ_o and ε_o are the amplitudes of the sinusoidal stress and strain curves respectively.

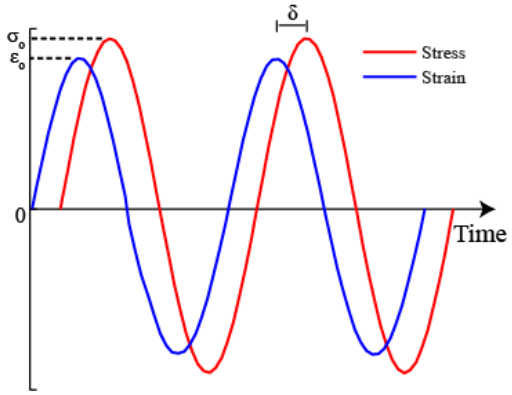

Figure 1. Schematic representation of stress-strain traces from DMA experiments used to compute the various parameters as described below.

The complex modulus, given as E* = E′ + iE″, has components

$$E' = \frac{\sigma_o}{\varepsilon_o}\cos(\delta) \text{ and } E'' = \frac{\sigma_o}{\varepsilon_o}\sin(\delta) \quad (5)$$

2.4.3 Stress Relaxation Experiments

We also performed quasi-static stress relaxation experiments by subjecting the hydrogels to a step displacement loading of 10% and a hold time of 15 minutes. Prior to this, each hydrogel was preloaded to 5 gram force as described earlier. Strains in the hydrogels were next increased to a total of 30% in two discrete steps with a hold time of 15 minutes (Figure 2). Data were acquired at 30 Hz for the first five minutes after each strain increment followed by 3Hz for the next five minutes. The remaining data were acquired at 1Hz to capture the total decay in forces during the hold period. Such experiments are useful to arrive at suitable viscoelastic models, obtained using a combination of springs and dashpots, to describe the mechanics of hydrogels.

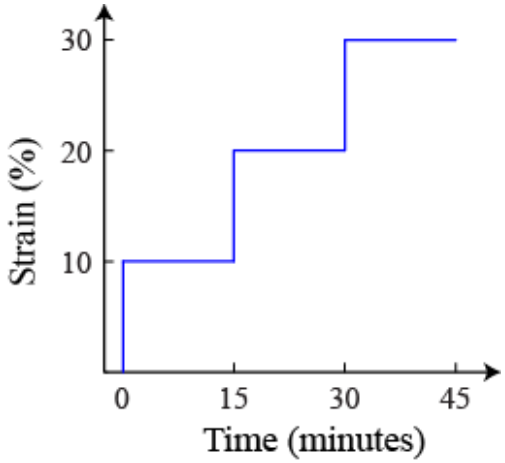

Figure 2. Schematic plot of a stress relaxation experiment obtained by imposing a constant step strain to the sample for a finite period

2.5 Microstructure of the hydrogels

Transverse sections of hydrogel samples were lyophilized for 20 hours and observed using an environmental scanning electron microscope (ESEM, Quanta 200). Image J was used to calculate the pore sizes of the hydrogels [12].

3. Results and Discussion

3.1 Morphology of hydrogels

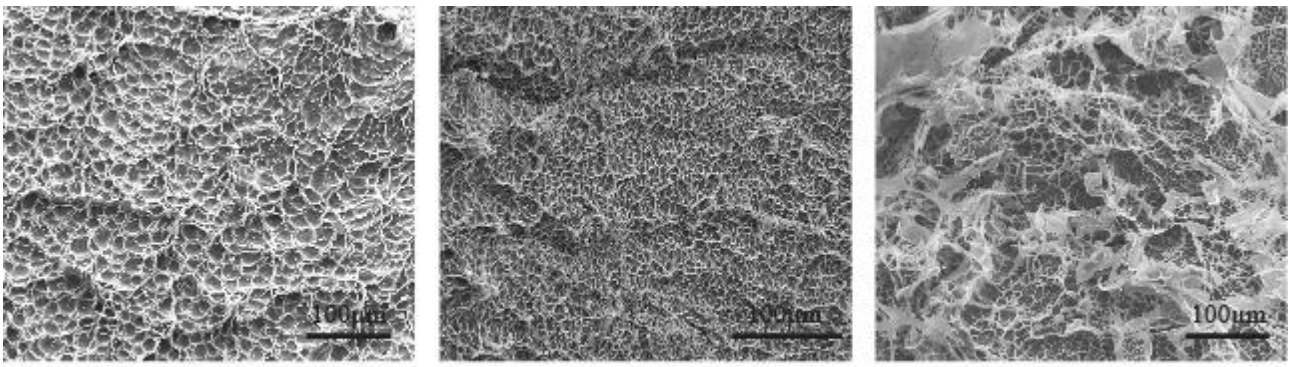

Figure 3. ESEM of gelatin and gelatin-elastin hydrogels show differing microstructures

When imaged using ESEM, hydrogels display a highly porous microstructure (Fig 3.) with variations in the degree of porosity and heterogeneity between the different groups considered in the study. The 3G and 5G hydrogels are predominantly homogeneous with differing porosities. In contrast, regions of heterogeneity exist in the 5GE gels with insoluble elastin forming sheet-like fibrous islands shown as white regions in the micrograph. To quantify the areal porosity of hydrogels, we used image processing tools in ImageJ. These results show that there are no significant differences ($p<0.05$) in the average pore size for the 5G (6.2±1.4 μm) and 5GE (6.5±1.5μm) samples. However, we note a significant difference in average pore size in comparisons of the 3G (15.8±3.4 μm) gel with the other groups respectively ($p<0.05$). Differences in the hydrogel porosity are attributed to variations in the polymer and crosslinker concentrations used in the fabrication of the 3G and 5G hydrogels. An increase in polymer concentration, while keeping other processing variables constant, may lead to a tighter packing of individual biopolymer chains and consequently a smaller pore size. Further, the amount of glutaraldehyde was doubled in the 5G gels as compared to the 3G hydrogels which may lead to a higher crosslink density and a possible reduction in the pore size. Similar results on decrease in pore size with an increase in the base polymer concentration is observed in non crosslinked gelatin hydrogels [15]. Because gel porosities affect diffusion of nutrients in the hydrogels, control of these pore dimensions will ultimately be dictated by requirements posed by different cells cultured in the hydrogels to serve as viable tissue engineered scaffolds.

3.2 Variation in the swelling characteristics of the hydrogels

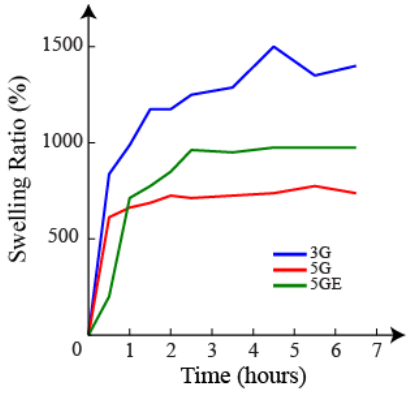

Figure 4. Swelling ratios of 5G 5GE and 3G hydrogels calculated as a function of time

Data from swelling studies (Figure 4) suggest that an increase in polymer and crosslinker concentrations leads to decrease in the swelling ratio that are in agreement with earlier work [16]. The EWC for the hydrogels also decreases with increasing polymer and crosslinker concentration with the 3G gels having the highest EWC and the 5GE gels showing the lowest EWC (Table 2). Increasing the monomer concentration leads to the formation of a higher number of network chains that reduces the diffusion of solvent through the sample and hence a corresponding decrease in hydrogel swelling behavior. Further, an increase in the crosslinker concentration causes the formation of a higher density of crosslinked chains that may consequently reduce the swelling of the hydrogels [17]. The decrease in pore size due to increasing polymer and crosslinker concentration hence results in the lowering of the EWC of the hydrogels because of reduced water absorption and retention in the hydrogel. The presence of sheet-like fibrous islands of elastin in the 5GE gels may further lead to blocking of pores in the gel that prevent water uptake and hence results in the low EWC noted in this study. However, additional samples are required to show statistically significant results.

Table 2. Equilibrium water content (EWC) for a hydrogel sample in each group

Group	EWC (%)
5G	**88.05**
5GE	**76.74**
3G	**93.33**

3.2 Mechanical Characterization

3.2.1 Variation in the moduli of the hydrogels

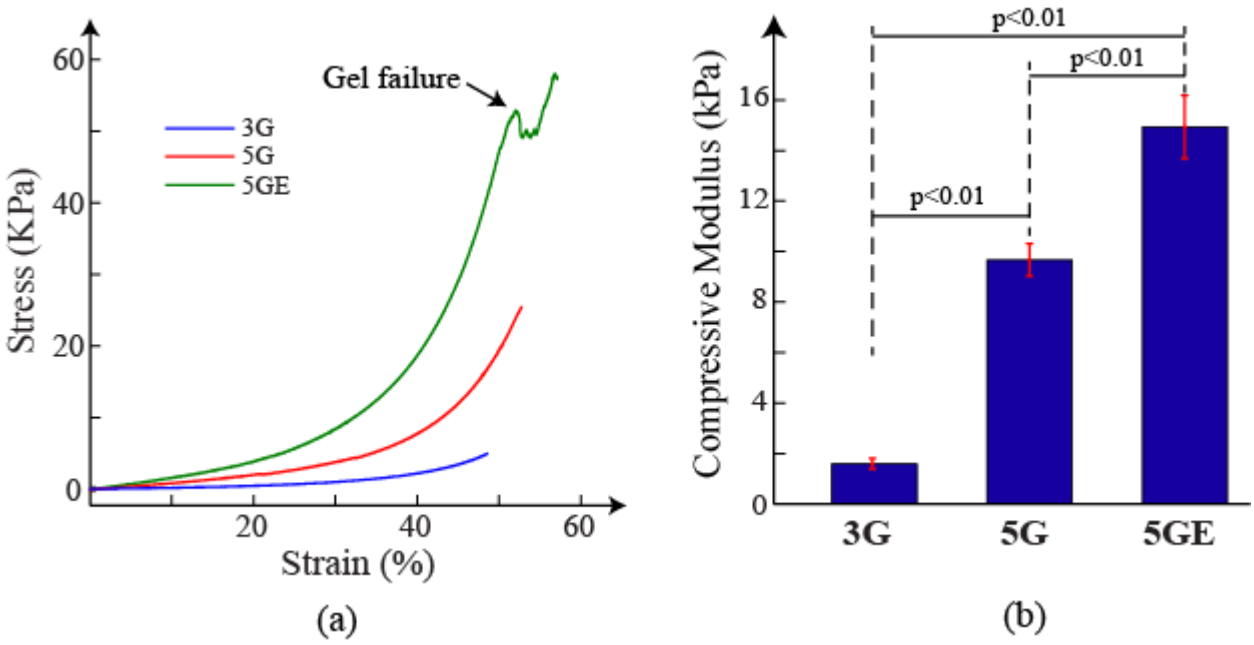

Figure 5. (a) Representative traces from monotonic compression tests on Gelatin and Gelatin - Elastin hydrogels (b) Compressive modulus for Gelatin and Gelatin - Elastin hydrogels at 4% strain

Preconditioning of gels, though not reported in most studies [2, 18], seems crucial and is an established method for reporting the stress-strain results for soft tissue materials [13]. Our results show a clear variation in the peak amplitude of load from the first to the last reported cycle during preconditioning with maximum differences visible between the first and second cycles of loading.

In contrast, data from the last five cycles show repeatable stress-strain response for all hydrogel groups in this study. Representative traces of stress-strain responses (Figure 5a) corresponding to the three hydrogel groups show that an increase in gelatin content or addition of elastin leads to an increased nonlinear material response in the observed strain range. The 5G and 5GE groups demonstrate a response similar to that observed for native tissues such as arteries and cartilage whereas the 3G gels have a highly compliant response [2, 13, 18]. Compressive moduli were calculated for all samples and are reported in Figure 5b. The higher moduli in the 5EG group (14.938±1.253 kPa) and 5G samples (9.669±0.644 kPa) correlate with an increase in the amount of material and glutaraldehyde concentrations as compared to the 3G group (1.591±0.210 kPa). However, our studies show that elastin containing hydrogels fractured at strains of about 0.54±0.02 whereas the gelatin gels showed no signs of damage at these strains.

3.2.2 Viscoelastic characterization of hydrogels

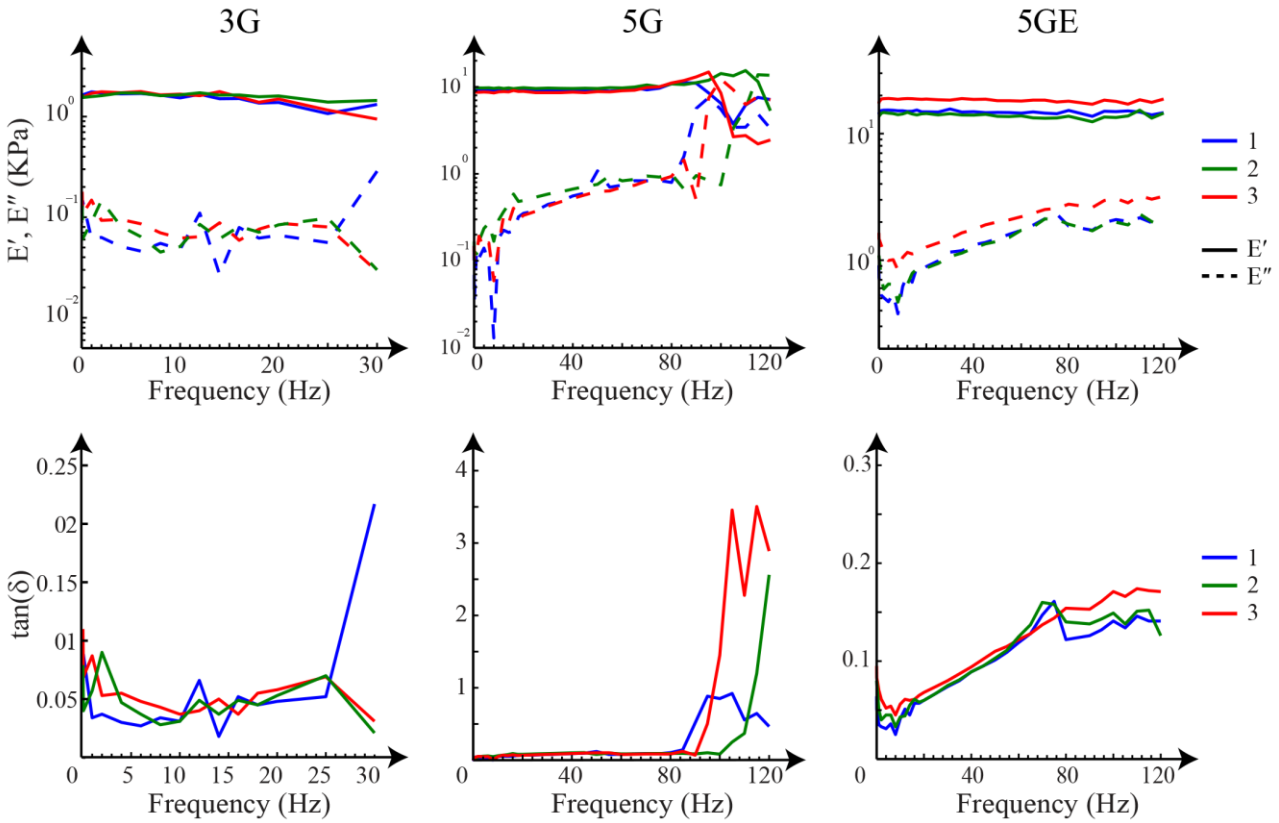

Figure 6. Variation in E′, E″ and tan δ with frequency for each of the hydrogels

Table 3 Mean values of tan δ, E′ and E″ for 3G gels upto 25Hz, 5G gels upto 80Hz and 5GE gels upto 6Hz (n=3)

	3G Group	5G Group	5GE Group
Compressive Modulus (kPa)	1.591±0.210	9.669±0.644	14.938±1.253
Storage Modulus (kPa)	1.594±0.032	9.341±0.428	15.872±2.214
Loss Modulus (kPa)	0.082±0.011	0.456±0.067	-
tan δ	0.051±0.006	0.048±0.005	-

Gelatin gels show a mostly flat response in E′ and E″ with variation in the frequency (Figure 6). However, we note unusual behavior in the 3G gels above a frequency of about 30 Hz that lead to negative values in tan δ. We do not hence report results for 3G samples above this frequency.

Further, we note a high increase in the tan δ in the 5G group above 80 Hz. These effects may be due to possible resonance and cavitation occurring in the hydrogels that results in micro-damage to the samples [19]. Results from our study show that the storage moduli for the gelatin-elastin hydrogels have the highest values. The gelatin gels had lower storage moduli with 5G gels having greater values than the 3G gels. These storage moduli results also showed good correlation with the moduli obtained from monotonic compression experiments ($p<0.05$) (Table 3). However, DMA tests showed no significant difference in the tan δ values between the tested groups. Since 5GE gels show increase in the tan δ and E″ and a decrease in E′ with frequency, we calculated an average E′ over a small range of frequencies for these gels. There is a drop in the tan δ and loss modulus at 85 Hz for these gels, which might also be due to the resonance effects described above.

We hypothesized that the addition of elastin would result in a more elastic behavior. However data suggests that there is no significant difference in the response. This is likely due to the use of insoluble elastin which does not form a homogenous hydrogel. These are, to the best of our knowledge, the only available results from DMA experiments on crosslinked gelatin and gelatin-elastin hydrogels. Future experiments aim to use soluble elastin to delineate the mechanical properties and assess differences in the fracture properties of hydrogels using single edge notch samples. Biopolymers like elastin are present in animals that have a closed circulatory system and see minor variations in temperature. Because elastin and collagen containing tissues such as arteries and cartilage undergo variation in their loading frequencies and rates, we performed a frequency sweep instead of a temperature sweep in this study to obtain physiologically relevant results.

3.1.3 Stress Relaxation of the hydrogels

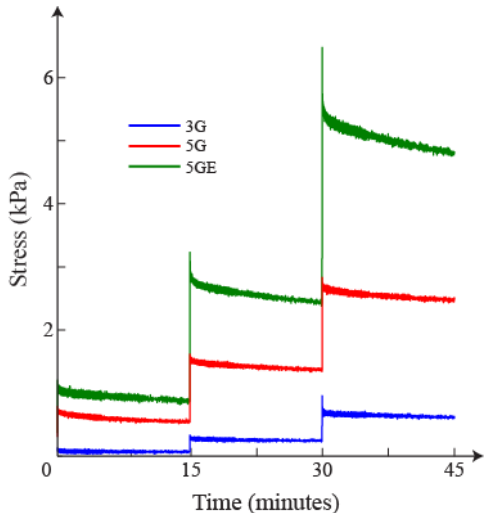

Figure 7. Representative traces from stress relaxation tests on gelatin and gelatin - elastin hydrogels

Stress relaxation data (Figure 7) indicate no significant difference in the amount of relaxation for each of the tested groups ($p<0.05$). This is in agreement with the results from DMA analysis which show no significant difference in the viscoelasticity between the gel groups. The hydrogel samples are covalently cross linked and hence the stress relaxation occurs mainly through migration of water through the gel [4]. It is possible that choosing a wider range of gel compositions may result in significant differences in the stress relaxation properties owing to larger differences in fluid migration through the gel. Although we hypothesized the addition of elastin would result in less relaxation of the gels, our data indicate otherwise that may likely be attributed to the presence of heterogeneous distribution of insoluble elastin through the sample. Future studies are aimed at using soluble elastin which has been shown to interact better with gelatin [18] and hence might show a

larger difference in the stress relaxation behavior due to formation of a homogenous network. The shape of the observed stress relaxation curves show similarity to those reported in other studies [5, 6] though variations have been observed among other studies [4–6]. Ongoing studies are aimed at modeling this response of the hydrogels and quantifying the relaxation times.

4. Conclusions

Our data on gelatin and elastin-gelatin composite hydrogels show that a variation in the monomer and crosslinker concentrations allow for tuning of the hydrogel stiffness which is essential in the fabrication of tissue engineered constructs. Microstructural analysis reveals differences in porosities of hydrogels which affects the permeability and mass transport properties through the gels. This is important in the case of nutrient transport through hydrogels when used for cell culture. The water uptake of hydrogels is also dependent upon monomer and crosslinker concentration. Addition of elastin leads to a decrease in the water content as well as the swelling ratios of the hydrogels. DMA and stress relaxation studies show no significant differences in viscoelastic nature of the gelatin hydrogels. Because incorporation of insoluble elastin did not have a significant effect on the viscoelastic properties of gels, we hope to use soluble elastin in future studies. We hope that such studies on soft materials will be useful in fabrication of tissue engineered constructs capable of closely mimicking the *in vivo* mechanical environment of the tissue.

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