An Experimental and Modeling Study of the Viscoelastic Behavior of Collagen Gel

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The macroscopic viscoelastic behavior of collagen gel was studied through relaxation time distribution spectrum obtained from stress relaxation tests and viscoelastic constitutive modeling. Biaxial stress relaxation tests were performed to characterize the viscoelastic behavior of collagen gel crosslinked with Genipin solution. Relaxation time distribution spectrum was obtained from the stress relaxation data by inverse Laplace transform. Peaks at the short (0.3 s–1 s), medium (3 s–90 s), and long relaxation time (>200 s) were observed in the continuous spectrum, which likely correspond to relaxation mechanisms involve fiber, interfibril, and fibril sliding. The intensity of the long-term peaks increases with higher initial stress levels indicating the engagement of collagen fibrils at higher levels of tissue strain. We have shown that the stress relaxation behavior can be well simulated using a viscoelastic model with viscous material parameters obtained directly from the relaxation time spectrum. Results from the current study suggest that the relaxation time distribution spectrum is useful in connecting the macro-level viscoelastic behavior of collagen matrices with micro-level structure changes.
[DOI: 10.1115/1.4024131]

1 Introduction

The hierarchical structure of collagen, first unveiled by Kastelic et al. [1], is believed to be responsible for the necessary elastic strength and viscoelastic responses. Viscoelasticity is important for force/energy storage, transmission, and dissipation in biological tissues [2]. Maxwell model consisting of a Hookean spring and a Newtonian dashpot model is widely used to interpret stress relaxation data. A generalized Maxwell model includes a single spring parallels with a number of Maxwell elements. As the number of Maxwell elements approaches to infinity, the time-dependent stress $\sigma(t)$ can be described by a continuous stress function [3,4]:

$$\sigma(t) = \sigma_e + \int_0^{+\infty} \sigma(\tau) \exp\left(-\frac{t}{\tau}\right) d\tau \quad (1)$$

In Eq. (1), σ_e is the equilibrium stress, and $\sigma(\tau)$ is the relaxation time distribution function with relaxation time τ.

Viscoelastic biomaterials likely contain a continuous spectrum of relaxation time constants [5]. While Eq. (1) can be well fitted with a few exponential terms, the fitting may provide little information relevant to the characterization of intrinsic material properties [6]. In the present study, the time-dependent distribution spectrum $\sigma(\tau)$ of collagen gel is obtained through numerical inverse Laplace transform. Investigating the spectrum in terms of the number of peaks, time constants, and peak intensity was found to appropriately demonstrate the main properties of viscoelastic behaviors [7,8]. The intensity of the peak reflects the amount of dissipated energy during relaxation. The number of peaks and time constants are often correlated with specific molecular architectures; as a result it can be used as an approach to understand the structural behavior of biomaterials, as well as a useful tool to distinguish materials [9,10].

In this study, the relaxation time distribution spectrum is obtained from stress relaxation data by means of inverse Laplace transform. This spectrum is employed to understand the mechanisms of stress relaxation in collagen network. Furthermore, material parameters in the viscous model are obtained through analysis of the relaxation time spectrum. We have previously studied the effects of crosslinking on the elastic properties of collagen gel [11]. Some of the experimental approaches were adopted in the present study. Genipin (GP) solution was used to induce crosslinking in collagen gel. Biaxial stress relaxation tests were performed to characterize the viscoelastic behavior of collagen gel. Viscoelasticity was also studied with the effect of initial stress levels.

2 Materials and Methods

2.1 Sample Preparation. Nutragen Type I collagen solution (6 mg/ml) was purchased from Advanced BioMatirx. Collagen was dissolved in 0.01N HCl with a *p*H value of approximately 2.0. Neutralized collagen solution was prepared by quickly mixing Nutragen collagen solution, 10X PBS (Fisher Scientific) and 0.1M NaOH (Fisher Scientific) solution with a ratio of 8:1:1 at 4 °C with a final collagen concentration of 4.8 mg/ml. The *p*H value of the solution was adjusted to between 7.2–7.4. The neutralized solution was transferred into a custom made square reservoir that sits in a Petri dish. On each side of the reservoir, a notch was cut to fit the polyethylene bars (Fisher Scientific) prethreaded with nylon sutures. The solution was incubated at 37 °C for 12 hs for gelation. During gelation, the polyethylene bars were polymerized into the collagen gel. The collagen gel was then immersed in 0.25% GP solutions for another 6 hs in the incubator for crosslinking [11]. The collagen gel was then rinsed with distilled water to remove the residual GP solutions. The dimension of the collagen gel samples are approximately $20 \times 20 \times 1$ mm.

2.2 Stress Relaxation Test. Biaxial stress relaxation test was performed to characterize the viscoelastic properties of collagen gel using a planar biaxial tensile tester [12]. A roughly square-shaped sample was mounted so that it can be stretched along the x- and y in-plane directions simultaneously. Four carbon dot markers were placed at the center of the sample, and a CCD camera was used to track the position of markers from which the tissue strains in both directions can be determined throughout the deformation. The load applied to the specimen was measured and recorded using load cells during the loading and unloading processes. Biaxial tensile test was firstly performed to reach synchronization. Immediately after the sample was loaded to the target stretch with a rise time of 5 s and held at this constant stretch for 600 s. The load in both loading directions was recorded during the holding period. Stress relaxation preconditioning tests were first performed to

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Contributed by the Bioengineering Division of ASME for publication in the Journal of Biomechanical Engineering. Manuscript received April 16, 2012; final manuscript received March 25, 2013; accepted manuscript posted April 4, 2013; published online April 24, 2013. Assoc. Editor: Stephen Klisch.

achieve repeatable stress relaxation behavior. Specifically, three cycles of stress relaxation tests were performed to confirm the repeatability of the viscoelastic behavior. Stress relaxation experiments were performed at different initial strain levels. Stresses at each strain level are reported during the holding period [13].

2.3 Finite Element Modeling

2.3.1 Hyperelastic Behavior. The hyperelastic stress-strain behavior of collagen gel was modeled using the constitutive model by Gasser et al. [14]. The general form of the strain energy function is:

$$U = C_{10}(\bar{I}_1 - 3) + \frac{k_1}{2k_2}\sum_{\alpha=1}^{N}\left\{\exp\left[k_2\langle\bar{E}_\alpha\rangle^2\right] - 1\right\} + \frac{1}{D}\left(\frac{(J^{el})^2 - 1}{2} - \ln J^{el}\right) \tag{2}$$

In Eq. (2),

$$\bar{E}_\alpha = \kappa(\bar{I}_1 - 3) + (1 - 3\kappa)(\bar{I}_{4(\alpha\alpha)} - 1)$$

where

$$\kappa = (1/4)\int_0^\pi \rho(\Theta)\sin^3\Theta d\Theta$$

Material parameter C_{10} is associated with the initial stiffness of the material; κ is associated with the dispersion of the collagen fiber orientation; and γ is the mean direction of fibers. k_1 is a stresslike parameter and k_2 is a dimensionless constant related to the collagen fibers. The ratio between k_1 and k_2 defines the curvature of the stress-strain curve at the stiffening region. Interested readers are referred to Ref. [14] for more detailed explanation of the model. This constitutive model was adopted in our earlier study and was able to capture both the isotropic and anisotropic hyperelastic behaviors of collagen gel [11].

2.3.2 Time-Dependent Viscous Behavior. To model the time-dependent viscous behavior, a dimensionless relaxation modulus with a Prony series expansion is assumed to be:

$$g_R(t) = 1 - \sum_{i=1}^{N} g_i\{1 - \exp[-(t/\tau_i)]\} \tag{3}$$

where g_i and τ_i are material parameters, and N is the number of Maxwell elements. When $t \to \infty$

$$g_R(\infty) = 1 - \sum_{i=1}^{N} g_i = g_e \tag{4}$$

where g_e is the equilibrium relaxation modulus. Thus, Eq. (3) can be then written as:

$$g_R(t) = g_e + \sum_{i=1}^{N} g_i \exp[-(t/\tau_i)] \tag{5}$$

The time-dependent stress can be described as $\sigma_0 g_R(t)$:

$$\sigma(t) = \sigma_0\left\{g_e + \sum_{i=1}^{N} g_i \exp[-(t/\tau_i)]\right\} \tag{6}$$

where σ_0 is the initial stress in stress relaxation, and $\sigma_0 g_e = \sigma_e$ is the equilibrium stress when the decay time approaches infinity.

Equation (6) can be considered as the discrete form of a continuous stress relaxation function in Eq. (1). Material parameters g_i and τ_i were determined from the relaxation time distribution spectrum which was obtained from the stress relaxation experiment data through inverse Laplace transform using the CONTIN program [15]. Usually there are multiple peaks in the relaxation spectrum owing to the different stress relaxation components. The peaks in the relaxation spectrum reflect the dominant relaxation processes, and the corresponding relaxation time at each peak can be used to determine parameter τ_i. The area under each peak of the spectrum, M_i, is calculated by

$$M_i = \int_{\tau_{\min}}^{\tau_{\max}} \sigma(\tau)d\tau \tag{7}$$

which can be considered to be equivalent to the magnitude of $\sigma_0 g_i$ in Eq. (6), so that g_i can be obtained as $g_i = M_i/\sigma_0$. Due to the intrinsic assumptions on separable strain and time dependence of the stress response, the viscoelastic model is incapable to simulate the initial strain depend behavior. Thus, parameters in the viscous model need to be obtained for each initial stress/strain level.

The finite element simulation follows the same procedure as in the experiment. During the first time step, biaxial tension was applied to the sample in to simulate the biaxial tensile loading. In the second time step, displacements were held constant for 600 s, and the stress was recorded during the stress relaxation process. The material parameters in the hyperelastic constitutive model in Eq. (2) were determined by fitting the simulation results to the biaxial tensile testing data. Both the hyperelastic and viscous material parameters are included in this simulation. In the stress relaxation step, the viscous material parameters were obtained from the stress relaxation spectrum as described above. Due to the symmetric loading conditions in biaxial tensile test, a quarter of the collagen sample was modeled in ABAQUS 6.10. Shell edge load, and X- and Y-symmetry boundary conditions were applied in order to simulate biaxial tensile testing experimental settings. General-purpose shell elements (S4R) with inherent plane stress assumption were used in the finite element model. A root-mean-square (RMS) measure of the goodness of fit is calculated as:

$$\text{RMS} = \sqrt{\frac{\sum_{1}^{n}\left(\sigma_{\text{Simulation}} - \sigma_{\text{Experiment}}\right)^2}{n}} \tag{8}$$

3 Results

The stress relaxation results are presented in Fig. 1. Effect of initial stress levels on stress relaxation behavior of collagen gel was investigated. Each sample was tested at multiple initial stress levels. Multiple stress relaxation tests demonstrate an increase in the rate of stress relaxation with higher initial stresses. The continuous relaxation spectrum obtained from CONTIN analysis is plotted in Fig. 2. The intensity of the peaks as well as the area under the spectrum increases with increasing initial stress levels. Usually there are three peaks in the continuous distribution curve located at short relaxation time (0.3 s–1 s), medium relaxation time (3 s–90 s), and long relaxation time (>200 s). However, the

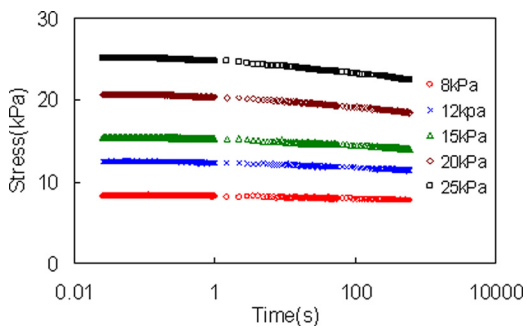

Fig. 1 Stress relaxation results of a collagen gel sample at different initial stress levels

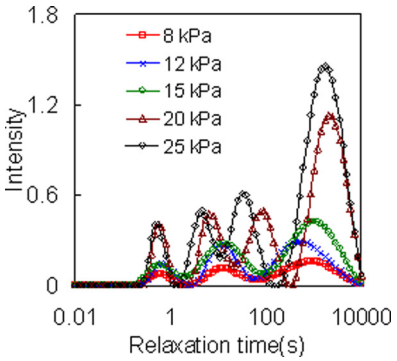

Fig. 2 Relaxation time distribution spectra obtained from biaxial stress relaxation tests of collagen gel under different initial stress levels

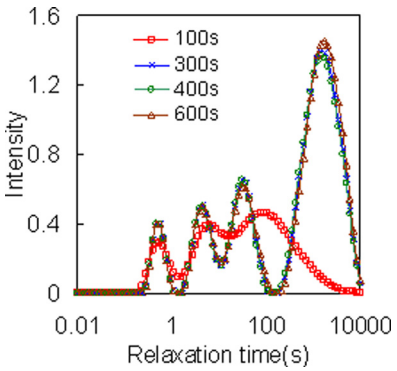

Fig. 3 Effect of relaxation time on relaxation time distribution spectrum for collagen gel at the initial stress level of 25 kPa

number of peaks can increase with higher initial stress levels, as shown in Fig. 2.

We further studied the effects of relaxation time on the results, as shown in Fig. 3. The total relaxation time used in the current study is 600 s. As we shorten the relaxation time to 300 s and 400 s, the relaxation spectra are essentially unchanged. However further shortening the relaxation time to 100 s results in noticeable changes in the general shape of the spectrum and missing of the peak between 100–1000 s. Since the relaxation time used in stress relaxation studies is usually much greater than 100 s, the present method of analysis can be considered to be approximately independent of relaxation time used.

In the present study, a hyperelastic and generalized Maxwell viscoelastic model are incorporated to model the time-dependent and time-independent mechanical response of collagen gel. Figure 4 shows the simulation results of collagen gel under biaxial tensile and stress relaxation tests. Experimental data are also shows for the purpose of comparison. Material parameters for the hyperelastic model are chosen in order to fit the biaxial tensile experimental data. In the present study, $C_{10} = 20$ kPa, $k_1 = 30{,}000$ kPa, and

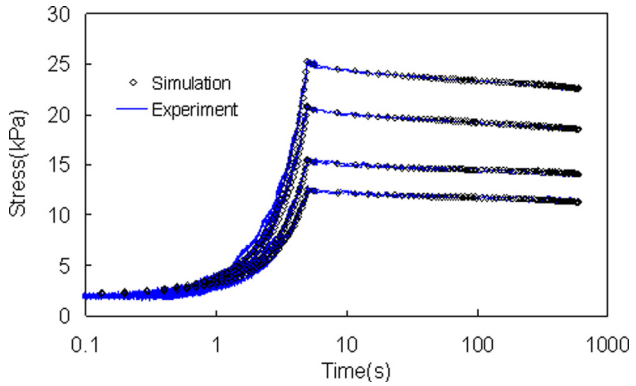

Fig. 4 Simulation results of biaxial tensile—stress relaxation tests of collagen gel at different initial stress levels. Solid lines represent the simulation results. Experiment results were shown in symbols for comparison. Material parameters in the hyperelastic model are $C_{10} = 20$ kPa, $k_1 = 30$ MPa, $k_2 = 1150$, $\gamma = 45$ deg and $\kappa = 0.333$; material parameters in the viscous model are listed in Table 1.

$k_2 = 1150$. For isotropic material, $\gamma = 45$ deg and κ was set to be 0.333. Material parameters g_i and τ_i for the viscous part were obtained as described earlier based on the stress relaxation distribution spectrum and are summarized in Table 1. For simulations of stress relaxation at different initial stress levels, the hyperelastic material parameters were kept the same for the collagen gel and the viscous material parameters are varied based on the stress relaxation spectrum.

4 Discussion

Material parameters in the general Maxwell model are commonly determined by exponential curve fitting. The data can be fit well within experimental error, yet the solutions may be significantly different. In the present study the parameters in the viscous model were obtained from analysis of relaxation time distribution spectrum. The number of terms in the Prony series is determined by the number of peaks in the spectrum. Material parameter τ_i is the relaxation time at the peak, and g_i is directly determined from the spectrum. The simulation results fit the experiment data well. More importantly, material parameters obtained from the stress relaxation spectrum reflect contributions from different stress relaxation components in collagen gel.

Studying the tissue level constitutive behavior, previous studies have shown that the stress–relaxation behavior of collagen based material was well described by a function with three exponential decay terms which reflecting the short-, medium-, and long-term relaxation components in the tissue [16,17]. In studies by Komatsu et al. [16], the relaxation curves of rabbit periodontal ligament was fitted with time constants between 0.2–0.4 s, 2–4 s, and 100–400 s. Wagenseil et al. [17] reported that the stress relaxation behavior of fibroblast populated collagen s consists of three time constants between 1–10 s, 10–100 s, and >1000 s. The three exponential term decay model has also been used to describe the

Table 1 Summary of material parameters in the viscous model (Eq. 6) obtained from relaxation spectrum of collagen gel under stress relaxation at different initial stress levels (Fig. 1). The last column of the table gives the RMS between finite element simulation and experimental results.

Initial stress (kPa)	g_1	$\tau_1(s)$	g_2	$\tau_2(s)$	g_3	$\tau_3(s)$	g_4	$\tau_4(s)$	RMS
12	0.0132	0.665	0.0354	13	0.0670	511			0.128
15	0.0133	0.558	0.0427	13	0.0756	1030			0.129
20	0.0159	0.558	0.0295	6.46	0.0351	89	0.104	2070	0.0805
25	0.0133	0.558	0.0233	4.55	0.0342	31.2	0.124	1740	0.117

relaxation behavior of periodontal ligament in human [18]. According to study by Sundararaghavan et al. [19], collagen fibers are observed in GP crosslinked collagen gel. In the present study, peaks were observed in the relaxation spectra with corresponding time constants between 0.3 s–1 s, 3 s–90 s, and >200 s. This further validates the results from previous studies on using a three term exponential function to fit the viscoelastic behavior of collagen tissue.

We hypothesize that the peaks in the relaxation spectra indicate that the relaxation behavior of collagen through hierarchy can be depicted as inter fiber relaxation, inter fibril relaxation, and fibril relaxation with increased order of relaxation time constants. This is in agreement with the fact that intra fibril crosslinks is the most stable part under long-term load [20,21], and therefore the fibril relaxation is likely to be the slowest. It is important to note that initial stress/strain levels can affect the relaxation rate and thus the time constants. The relaxation spectra provide insights on the effects of mechanical stresses on the major relaxation components in collagen gel. The relaxation spectra demonstrate an increase in the intensity of peaks with higher initial stress level (Fig. 2). It is also noted that there is a more significant increase in the intensity of the long-term peak with higher initial stress levels. The covalent crosslinks between collagen molecules and microfibrils plays an important role in stabilizing the fibrils and the collagen network. Rigozzi et al. [22] employed an atomic force microscope to characterize the diameter and periodic banding (D-period) of individual collagen fibrils under macroscopic tendon extension. The D-period banding and fibril diameter were statistically unchanged for tendon strain of 5%. However significant changes were observed when the macroscopic tendon strain increases to 10%. The appreciable increase in the intensity of the long-term peak in the relaxation spectra at higher stress levels from our results further confirms the engagement of collagen fibrils at higher levels of tissue strain. Interestingly, we have observed that the number of peaks increases from three to four at higher initial stress levels. Specifically, the middle peak from the relaxation spectra splits into two peaks resulting in the increase of peak numbers. The splitting of the middle peak in the relaxation spectrum may indicate increased structural heterogeneity at the fibril level with mechanical loading.

5 Conclusions

The material parameters in the viscous model were obtained directly from analysis of relaxation time distribution spectrum, which allows including the contributions of major stress relaxation components into constitutive modeling. Relaxation time distribution spectrum obtained from inverse Laplace transform provides useful information on understanding the underlying stress relaxation mechanisms. Our study hypothesizes that relaxation at the fiber, inter-fibril, and fibril level plays important roles in the viscoelastic behavior of collagen gel. The appreciable increase in the intensity of the long-term peak in the relaxation spectra at higher stress levels may correlate with the engagement of collagen fibrils at higher levels of tissue strain. The coupled experimental-modeling approach is promising to provide fundamental understandings of the viscoelastic structure-function of collagen matrices.

Acknowledgment

This work was supported by National Science Foundation grant CMMI 0954825.

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