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Design and Application of a Test System for Viscoelastic Characterization of Collagen Gels

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ABSTRACT

Characterization and control of the mechanical properties of the extracellular matrix are critical to the interpretation of results of *in vitro* studies of cultured tissues and cells and for the design of functional engineered constructs. In this work a viscoelastic tensile test system and custom culture chambers were developed and characterized. The system allowed quantification of strain as well as the stresses developed during cyclic viscoelastic material testing. Finite element analysis of the culture chambers indicated that the tensile strains near the actuated ends of the gel were greater than the strains experienced by material in the center of the culture chambers. However, the strain was uniformly distributed over the central substance of the gel, validating the assumption that a homogeneous strain state existed in the central region of the chamber. Viscoelastic testing was performed on collagen gels that were created with three different collagen concentrations. Results demonstrated that there was a significant increase in the dynamic stiffness of the gels with increasing equilibrium strain, collagen concentration, and frequency of applied strain. With increasing strain rate, the phase angle, representing the energy dissipated, dropped initially and then increased at higher rates. Mechanical testing of gels at different time intervals up to 7 days after polymerization demonstrated that the material properties remained stable when appropriate environmental conditions were maintained. The ability to characterize the viscoelastic properties of gels after different periods of culture will allow the quantification of alterations in gel material properties due to changes in cell cytoskeletal organization, cell–matrix interactions, and cellular activity on the matrix. Further, the test device provides a means to apply controlled mechanical loading to growing gel cultures. Finally, the results of this study will provide guidance to the design of further experiments on this substrate.

INTRODUCTION

IN VITRO CONSTRUCTS composed of tissue components and/or cells and extracellular matrix (ECM) provide simplified systems for the study of cell–matrix interplay in health, embryonic development, and pathological states such as inflammation and tumorogenesis.[1] Cell–matrix interactions play a pivotal role in the mechanical properties of tissue constructs—the very presence of the cells in the ECM constructs can increase the stiffness of the matrix, and cells can in turn modify the matrix and alter its material properties.[2–4] This is particularly significant when studying the process of wound healing due to a massive infiltration of new microvasculature at the injured site followed by an alteration of the extracellular matrix by metalloproteinases and other proteases secreted by endothelial and inflammatory cells.[5] Cells can also alter ECM properties directly. As an example, endothelial

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cells grown in ECM constructs can contract the construct[6] and produce a provisional matrix.[7] Conversely, stretching of the ECM via an attached substrate induces cell alignment as well as other varied responses such as synthesis of prostaglandin E[8,9] associated with matrix reorientation.[3] For all these cases, the material and functional properties of the ECM and the overall construct are altered directly.

Collagen is the main constituent of many ECMs and cell-populated collagen gels have been used extensively for *in vitro* systems[10] and for model studies of healing.[11] Collagen gels are viscoelastic as demonstrated by creep, stress relaxation, hysteresis, and strain rate dependence. The viscous nature of these constructs arises from both inherent viscoelasticity of the solid phase as well as fluid movement, resulting in viscous drag between the solid and fluid phases during loading.[3] For practical applications of functional tissue-engineered constructs, it is essential to characterize and control the mechanical properties of these constructs. Numerous studies have assessed the response of cells to mechanical stimulation and many novel devices have been designed to apply static as well as dynamic forces to growing cell cultures. There have been only a few instances of extensive characterization of the ECM constructs used for study of cell mechanics and interactions based on the use of various indirect techniques such as micropipettes, markers,[11] viscometry,[10] and so on.

Mechanical stretching devices have been used for the *in vitro* study of mechanotransduction, focusing either on the cellular or the ECM responses.[12] These devices have taken a variety of approaches: deformable elastic membranes, the deformation of which is induced by vacuum, hydrostatic pressurization, or platen displacement; uniaxial,[8,9,13] biaxial,[14] or radial stretching systems; fluid shear test systems[15]; piezoelectric crystals[16]; the use of commercial materials test machines[6] and similar devices[17,18]; and rheology,[10] markers and finite element analysis,[1,11] and so on. A detailed review of the various systems is provided by Brown.[12] Almost all of these systems are designed to provide mechanical stimulation to growing cells or cultures and typically do not have the ability to quantify the applied strains and resulting forces simultaneously.[16] Other limitations include strain heterogeneity, strain anisotropy, reactive stresses induced by fluid shear at the interfaces, and fluid pressurization or acceleration.[19] Little work has been performed to characterize the frequency-dependent viscoelastic behavior of cell–ECM constructs or even of isolated ECM constructs. Many of the devices that are currently used for mechanical conditioning are inadequate for use in viscoelastic materials characterization.

Our laboratories are investigating the effects of angiogenesis on the ECM. An *in vitro* model of angiogenesis in collagen gels has been developed to isolate the effects of angiogenesis on ECM material properties.[19a] In support of this work, the objectives of this study were to design and characterize a new materials test system and culture chambers for the viscoelastic characterization of collagen gels, to analyze strain distribution in the loaded culture chambers by finite element analysis, and to examine the effects of collagen concentration, strain rate, and equilibrium strain level on the viscoelastic response of collagen gels. On the basis of the viscoelastic response of native biological soft tissues, we hypothesized that there would be a moderate increase in dynamic stiffness as a function of strain level and frequency and that the phase angle would be relatively unaffected by changes in strain level and strain rate.

MATERIALS AND METHODS

For the intended application, the test system needed to be able to quantify the applied strain and the resulting forces under a range of cyclic strain rates. Further, the test chambers needed to provide a region of homogeneous strain during uniaxial extension for characterization of the viscoelastic material properties.

Chamber design

Custom culture chambers (65.0 × 18.0 × 12.7 mm) were CNC machined from sheets of Acrylic and Lexan (Regional Supplies, Salt Lake City, UT)—materials that do not adhere to collagen (Fig. 1). A glass microscope slide (75 × 25 mm) formed the bottom of the chambers to enable microscopic evaluation of growing gels. Chambers were attached to the microscope slide with biocompatible silicone glue (Med 1041; Nusil, Carpinteria, CA). The gels were polymerized around a fixed horizontal anchor post (6.0 × 8.5 × 3.0 mm) and a moveable actuating post (5.0 × 17.0 × 3.0 mm), yielding a gage length of 20 mm (4:1 aspect ratio). Four holes (1.6 mm in diameter) in both the actuating and anchor posts provided a mechanical link between the polymerized collagen gel and the test system. Elongation of the gels required free space behind the actuating post. A large reservoir was incorporated into the design for this purpose. During polymerization, the reservoir was occluded with a removable block. The actuating post was held upright with two 316 stainless steel pins (Small Parts, Miami Lakes, FL) that passed through apertures in the chamber sides. These pins also ensured that a consistent specimen length was maintained between each test. The actuating post was attached to a 0.25-N load cell (accuracy, ±0.00012 N; Transducer Techniques, Temecula, CA). Specimen thickness was measured midway between the anchor bar and the flag, using digital calipers. An average of measurements taken at the edge of the chamber and in the center of the gel was used to account for meniscal effects at the edges of the gel.

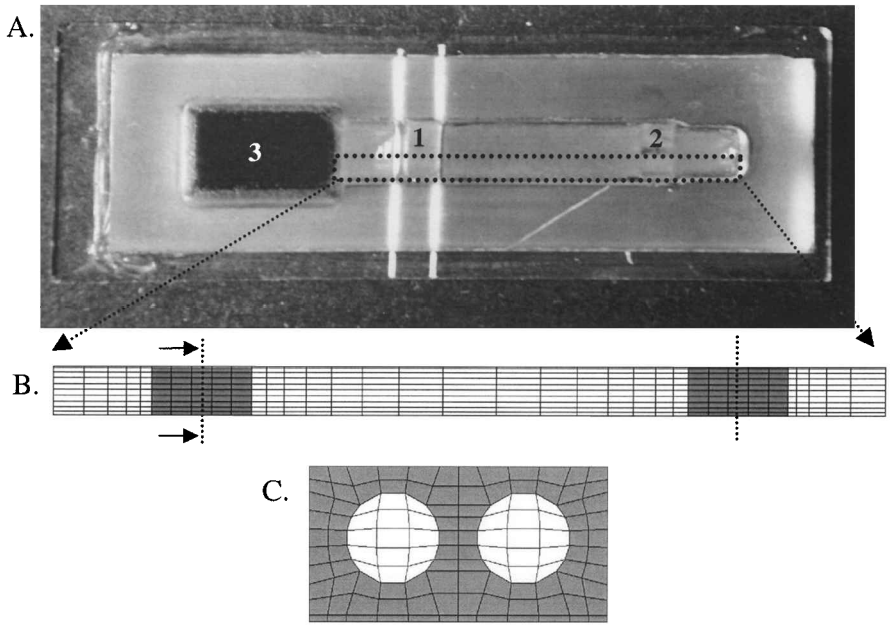

FIG. 1. **(A)** Custom Acrylite chambers with polymerized collagen I gel and culture medium. Actuating post (1) is held in position by stainless steel pins. The second post (2) is attached to the base of the chamber. The reservoir (3) contains only culture medium and provides space for stretching of tensile test region, between labels 1 and 2. The posts contained four small holes near their base to allow polymerization of the collagen gel through the posts. Dotted line indicates region of gel represented in finite element analyses. **(B)** Finite element mesh used to analyze the transfer of strain from the actuating posts to the center of the specimen. **(C)** Cross-section at the level of the posts as indicated by solid arrows in **(B).** In **(B)** and **(C),** the FE mesh used for the gel material is represented by the lighter shade and the FE mesh used for the post is represented by the darker shade.

Finite element modeling

A half-symmetry FE model of the gel and anchor posts was created, using commercial mesh generation software (XYZ Scientific Applications, Livermore, CA) to simulate the equilibrium tensile test configuration in terms of application of forces and boundary conditions (Fig. 1B). The anchor posts were modeled as rigid and the gel was represented as a hyperelastic neo-Hookean material[20] with the following strain energy function W[21]:

$$W = G(\tilde{I}_1 - 3) + \frac{K}{2}[\ln(J)]^2 \qquad (1)$$

Here, G is the shear modulus, $\tilde{I}_1$ is the first deviatoric invariant of the right Cauchy deformation tensor,[22] K is the bulk modulus of the material, and $J = V/V_0$ is the local volume ratio. The shear modulus G was chosen on the basis of experimental values for tangent equilibrium modulus E for gels polymerized with collagen (3.0 mg/mL) at 6% equilibrium strain (7314 Pa; see Fig. 6) and K, using the relationship from linear elasticity, $G = 3EK/(9K - E)$. As the volumetric (bulk) material behavior of the collagen gels was unknown, the bulk modulus K was varied over three orders of magnitude so that the ratio of G to K was 10:1, 100:1, or 1000:1. The holes in the anchor and actuating posts were included in the model and the gel was modeled as passing through the holes (Fig. 1C). Two types of boundary conditions were considered between the gel and the anchor posts. First, we examined the case of perfect bonding between the posts and the gel. Second, the case of no adhesion between the front of the anchor block and the interior of the holes and the gel material was investigated; rather, sliding contact surfaces were used to simulate the transfer of load between the gel and actuating/anchor posts on those surfaces during actuation. The nonlinear, implicitly integrated FE code NIKE3D (Lawrence Livermore National Laboratory, Livermore, CA) was used for all FE analyses.[23] The motion of the movable anchor block was discretized in quasi-time and an incremental–iterative nonlinear solution procedure was used (see, e.g., Bathe[24]).

Preparation of collagen gels

Sterile rat tail collagen type I (BD Biosciences Discovery Labware, Bedford, MA) was mixed with concentrated Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) to yield a final collagen concentration of either 1.5, 3, or 4.5 mg/mL and 1× DMEM. Streptomycin (100 μg/mL, GIBCO-BRL; Invitrogen, Carlsbad, CA), penicillin (100 units/mL, GIBCO-BRL; Invitrogen) and amphotericin B (Fungizone, 0.25 μg/mL,

GIBCO-BRL; Invitrogen) were added to yield the final DMEM solution. The collagen solution was prepared in a sterile environment at 4°C, the pH was adjusted to pH 7.4 with 1 M NaOH, and 1.5 mL of this solution was pipetted into custom culture chambers. Care was taken to ensure that air bubbles were removed. The solution was polymerized at 37°C and 100% humidity in a sterile environment for 30 min. The gels were then covered with 2.5 mL of the 1× DMEM solution. The gels were incubated at 37°C in a 5% CO_2 environment. Gels were tested either on the same day of preparation or at specific time intervals after polymerization and storage in an incubator.

Twenty-two separate collagen gels and chambers were used to evaluate the effect of culture period, and six chambers each were used for the three different collagen concentrations. Chambers used for evaluation of the effect of culture period were tested either on day 1 (n = 11) or day 7 (n = 11) of culture. Chambers with different concentrations of collagen were tested on the day of polymerization.

Mechanical test device

A mechanical test device was designed and constructed, using a piezoelectric stage (Piezojena, Hopedale, MA) as a mechanical actuator to generate sinusoidal motion profiles at specified frequencies (Fig. 2). The piezo stage enabled controlled microscopic displacements at a variety of frequencies while minimizing the effects of heat and electric fields.[16] Culture chambers were mounted on the piezo stage with the long axis of the chambers aligned with the movement axis of the stage. The piezo actuator was attached to two translation stages (Newport, Irvine, CA) to allow positioning during setup. The bottom stage also allowed application of lateral (y axis) movements larger than the movement range of the piezo stage. Displacement was measured with an LVDT (Schaevitz, Hampton, VA). The load cell was mounted on a separate platform attached to a z axis positioning stage, eliminating motion-induced artifacts. The piezo stage was driven by amplified signals (12V40 amplifier, Piezosystems, Hopedale, MA) from a programmable digital function generator (Tektronix, Wilsonville, OR). Force and displacement data were collected with an A/D card and Labview software (National Instruments, Austin, TX).

Testing protocol

Before testing, each gel was placed in an acrylic test enclosure and allowed to equilibrate to ambient temperature in the enclosure (25–27°C). The CO_2 concentration in the enclosure was maintained at 5% during testing. Each chamber was mounted on the piezo stage and the actuating post was secured to the load cell interface. The load cell stage was then raised approximately 150 μm so that the actuating post did not touch the chamber base. The gel was allowed to equilibrate for 5 min and then the load cell was rezeroed. The gel was preconditioned by first stretching it to 6% strain and then allowing it to stress-relax for 20 min. The zero load length was then reestablished and the gel was stretched to 2% of the new initial length at approximately 1%/s and allowed to stress-relax until the change in force was within the noise band of the load cell signal (less than 0.0001 N/s). This typically required 20 min, with larger strain levels requiring a longer relaxation time. This was followed by cyclic sinusoidal stretching at frequencies of 0.05, 0.1, 1.0, and 5.0 Hz with an amplitude of ±0.5% strain. The entire procedure was then repeated at equilibrium strain levels of 4 and 6%. The strain amplitude of ±0.5% was chosen on the basis of preliminary testing that demonstrated a linear response of the gels to this amplitude—in other

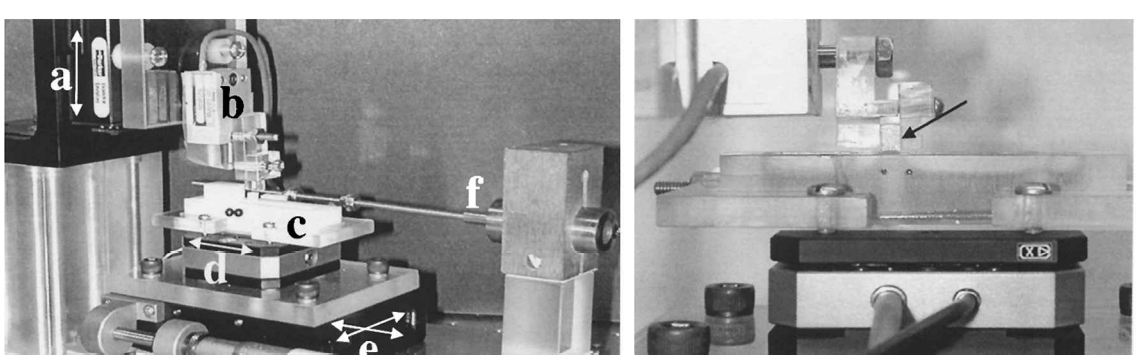

FIG. 2. *Left*: Tensile test apparatus used to perform viscoelastic testing of the collagen gels. A vertical positioning stage (a) facilitated alignment of the load cell (b) with the actuating post embedded in the polymerized gel. The chamber (c) was attached to the piezoelectric actuator (d). Side-to-side alignment and application of equilibrium tensile strains larger than the movement range of the piezoelectric actuator were applied with an x–y translation stage (e) equipped with micrometer heads. Displacement and strain were measured with an LVDT (f). White arrows indicate movement directions of translation stages and actuator. *Right:* Close-up of the interface between the load cell and actuating post (black arrow).

words, when a sinusoidal strain–time input was applied, the resulting stress–time signal was well described by a sine wave in terms of time variation and the positive and negative portions of the signal amplitude were nearly identical about the equilibrium stress level. Small sinusoidal strain oscillations about an equilibrium strain allow application of linear viscoelastic theory for determination of dynamic stiffness and phase angle, enabling assessment of the effects of strain rate on the modulus and energy dissipation characteristics of the material.

Data reduction and statistical analysis

Equilibrium stresses were calculated from the forces measured during the relaxation tests at 2, 4, and 6% and the initial cross-sectional area for all three collagen concentrations. The resulting set of equilibrium stress–strain points for each collagen concentration were fit to a third-order polynomial. The slope of this curve was determined at each strain level to obtain the equilibrium tangent modulus. The equilibrium tangent modulus represents the modulus due to the elastic part of the material response, after all viscoelastic effects have subsided.

Cyclic stress–time and strain–time data were fit to a four-parameter sine function (Sigmaplot; SPSS, Chicago, IL):

$$f = Y_0 + A \sin\left(\frac{2\pi}{b} t + \phi\right) \tag{2}$$

Here, Y_0 is the equilibrium stress or strain level, A is the amplitude, $2\pi/b$ is the frequency of oscillation (radians), and ϕ is the phase angle (radians). When fitting the stress–time data, the value of b obtained from the fit of the strain–time data was used. The dynamic stiffness M (Pa) and phase angle ϕ were calculated as[25]

$$M = \frac{A_\sigma}{A_\varepsilon}; \qquad \phi = \phi_\sigma - \phi_\varepsilon \tag{3}$$

Here, $(A_\varepsilon, \phi_\varepsilon)$ and (A_σ, ϕ_σ) are the amplitudes and phases of the strain–time and stress–time data, respectively. The dynamic stiffness M is the ratio of the amplitude of the stress response to that of the strain input, and thus rep-

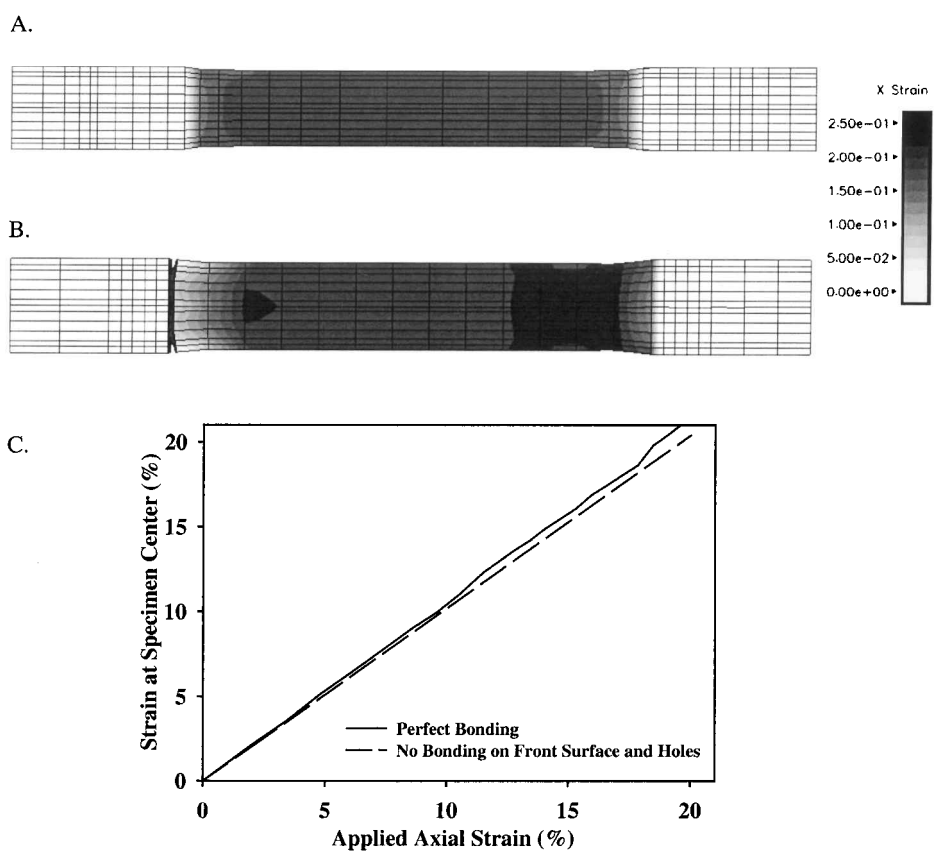

FIG. 3. Results of finite element simulations. **(A)** Axial strain distribution for the case of perfect bonding between the posts and collagen gel. **(B)** Axial strain distribution for the case in which the gel is allowed to separate from the interior face of the posts and is not bonded in the holes through the posts. **(C)** Graphs of applied axial strain versus strain in the center of the sample for the two boundary conditions considered.

resents a measurement of the viscoelastic tangent modulus of the material for a particular strain rate and amplitude. The phase angle ϕ describes the lag in time between the stress and strain sine waves and is a direct measurement of energy dissipation by the material. For a purely elastic material, $\phi = 0$, indicating that the stress–time and strain–time signals are completely in phase, whereas for a fluid, $\phi = \pi/2$, indicating that the stress response leads the strain by 90°. Viscoelastic materials will exhibit a phase angle between these two values.

Three one-factor analyses of variance (ANOVAs) were used to assess the effect of collagen concentration on equilibrium stress at each strain level. A two-factor repeated measures ANOVA was used to assess the effect of strain level (repeated measure) and collagen concentration on the equilibrium stiffness of the gels. Two-factor repeated measures ANOVAs were used to examine the effect of equilibrium strain level and excitation frequency on dynamic stiffness and phase at each time point. To evaluate the effect of culture period, a two-factor repeated measures ANOVA was performed at each strain level and effect of interactions between the factors (day and frequency) was also evaluated. A two-way ANOVA was used to evaluate the effect of concentration, frequency, and interactions between these factors. The effects of equilibrium versus dynamic calculation of modulus (E versus M, repeated measure) and collagen concentration on the modulus of the material were assessed at each strain level, using a two-factor repeated measures ANOVA. Significance was set at $p \leq 0.05$ for all comparisons. When significance was found, Tukey tests were performed between the different levels of each factor.

RESULTS

Integrity of all gels was maintained during the mechanical testing. There were no measurable variations in pH or molarity over the culture periods and evaporation of medium was negligible. In the course of the pilot tests as well as over 50 tests with these gels, there was no damage to the gels during the mechanical testing. By running a sharp no. 11 surgical blade around the edges of the gel just before testing we ensured that the gels were free of all the recesses of the chamber. During loading, gels stretched at the anchor points, but the gels did not separate from the anchors because of polymerization around the posts. Preliminary tests examining the effect of repeated testing of the same gels at similar strain levels and different frequencies did not demonstrate any effect of the testing itself.

Finite element simulations

Regardless of the chosen bulk modulus or boundary conditions, the finite element simulations demonstrated that a highly uniform uniaxial strain field was always present in the central 80% of the tensile test region of the sample (Fig. 3A and B). The bulk modulus had a minimal effect on the strain at the center of the sample and thus results are not shown. The boundary condition between the posts and the gel also had minimal influence on the relationship between applied actuator strain and the strain experienced by the sample in the center, although it did alter the distribution of axial strain near the posts (Fig. 3B). In both cases there was a strong linear relationship ($R^2 = 0.999$ in both cases).

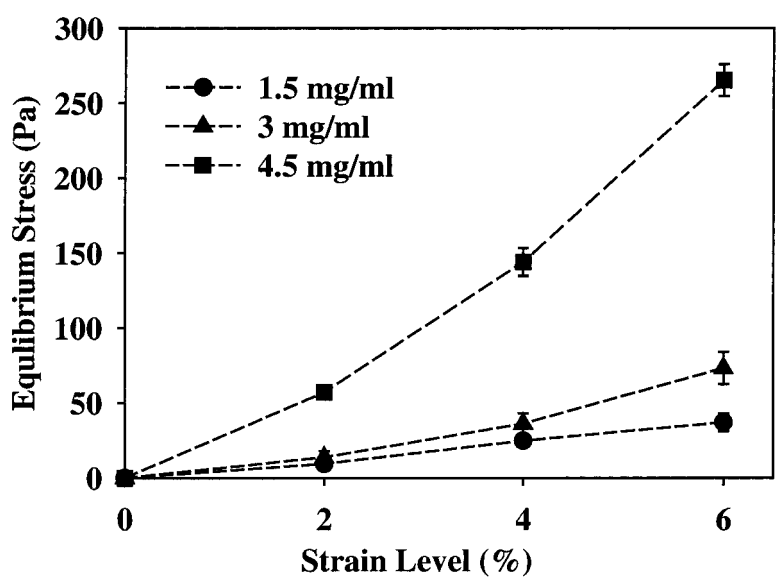

FIG. 4. Equilibrium stress–strain behavior of collagen gels with different concentrations of collagen, day 0 tests (means ± SEM).

Equilibrium stress–strain behavior

The shape of the equilibrium stress–strain curves from tests of the gels with different concentrations of collagen was nearly linear for the lowest collagen concentration and became mildly upward concave for the highest collagen concentration (Fig. 4). The average equilibrium tangent modulus E calculated from the static equilibrium stress–strain data for a collagen concentration of 3.0 mg/mL at 6% strain was 1538.5 ± 130.5 Pa. There was a significant effect of collagen concentration on the equilibrium stress at all three strain levels ($p < 0.001$ in all three cases). Tukey tests between levels revealed that there were significant differences between the 3.0- and 4.5-mg/mL collagen concentrations at all three strain levels ($p < 0.001$ in all cases) but not between the 1.5- and 3.0-mg/mL collagen concentrations.

Effect of collagen concentration on viscoelastic properties

There was a significant increase in dynamic stiffness with increasing collagen concentrations ($p < 0.001$ for 2, 4, and 6% strain) and with increasing frequency ($p = 0.015$, 2%; and $p = 0.045$, 4 and 6%), with interaction between frequency and concentration at 4 and 6% ($p = 0.712$, 2%; and $p = 0.028$, 4 and 6%) (Fig. 5). The stiffening effect was more marked with increasing concentrations of collagen than with increasing strain levels. There was also a significant effect of concentration on the phase shift at each strain level ($p = 0.003$, 4 and 6%) except at 2% strain ($p = 0.681$) and with increasing frequencies ($p = 0.015$, 2%; and $p = 0.045$, 4 and 6%), with a significant interaction between concentration and frequency at 4 and 6% ($p = 0.028$ for 4 and 6%) (Fig. 6). At lower strain levels and frequencies, the phase difference was higher for gels with lower collagen concentration, and the difference becomes less pronounced with increasing strain levels and frequency. There was a large decrease in the phase shift for all concentrations at 0.1 Hz and then an increase again at higher frequencies to values close to those at 0.05 Hz. At this trough at 0.1 Hz there was also a reversal of the concentration effect, with the 4.5% gels showing larger phase shifts than the 1.5% gels.

Effect of equilibrium strain level and strain rate on viscoelastic properties

When plotted against excitation frequency, the dynamic stiffness showed a mild positive slope on a log–log scale. There was a significant increase in dynamic stiffness with equilibrium strain level for both day 1 and day 7 groups ($p < 0.001$), indicating that the rate-dependent modulus of the material is influenced by its position on the equilibrium stress–strain curve. There was a significant effect of strain rate on phase shift ($p = 0.002$ for day 1, $p = 0.005$ for day 7), but there was no effect of strain level on the phase delay.

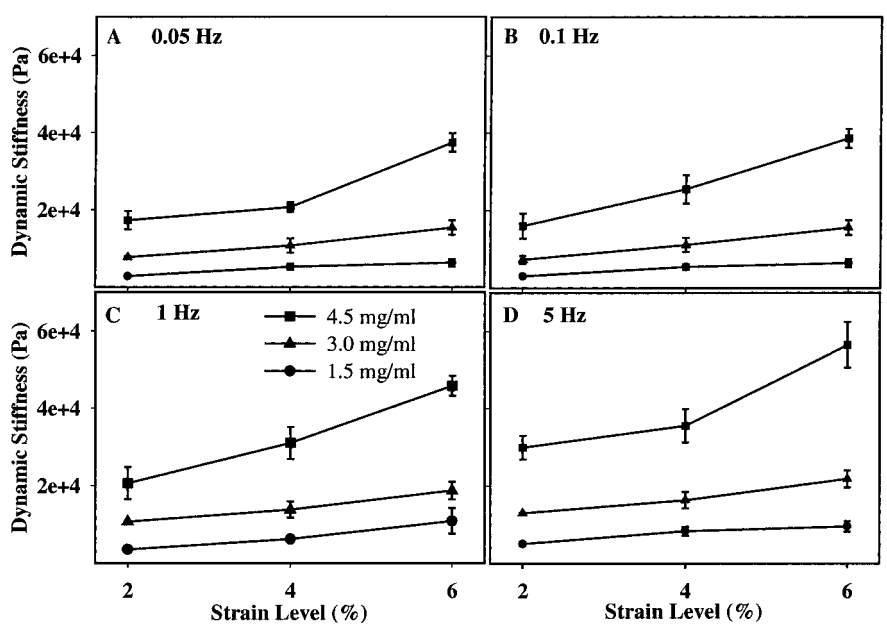

FIG. 5. Dynamic stiffness of gels with different concentrations of collagen: day 0 tests at **(A)** 0.05, **(B)** 0.1, **(C)** 1.0, and **(D)** 5.0 Hz (means ± SEM).

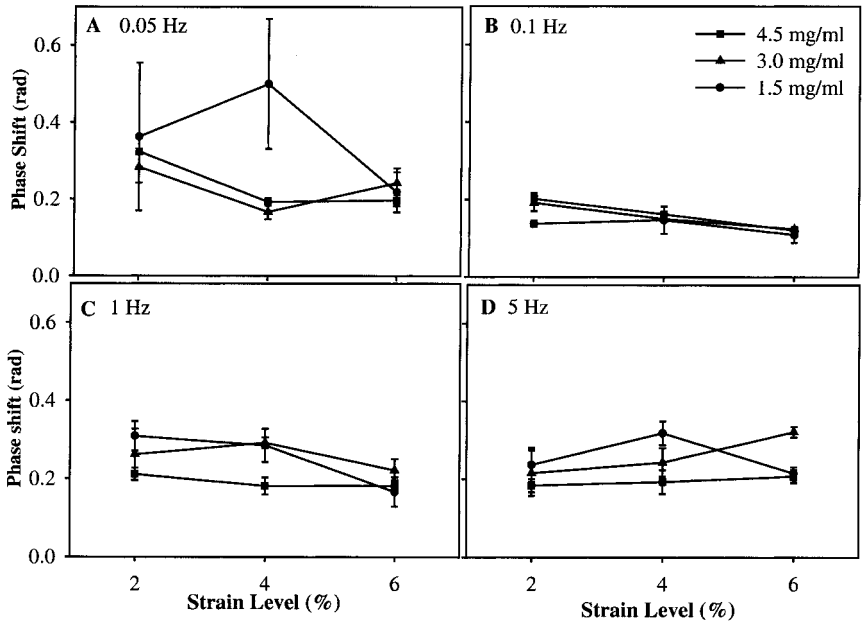

FIG. 6. Phase shift of gels with different concentrations of collagen: day 0 tests at **(A)** 0.05, **(B)** 0.1, **(C)** 1.0, and **(D)** 5.0 Hz (means ± SEM).

Dynamic stiffness versus equilibrium tangent modulus

The modulus values calculated from the equilibrium stress response of the gels were considerably lower than the dynamic stiffness values determined from the cyclic viscoelastic test data. For instance, at a collagen concentration of 3 mg/mL, 6% prestrain, and strain rate of 5 Hz, the dynamic stiffness was 21.84 ± 0.21 kPa, and the equilibrium modulus was 1.53 ± 0.01 kPa. Thus, more than 90% of the material response at 5 Hz was due to the viscoelastic part of the material behavior (Fig. 7).

Effect of test day on viscoelastic properties

There was no significant effect of postpolymerization test day on dynamic stiffness in repeated dynamic testing about equilibrium strain levels of 2, 4, and 6% (p = 0.346, 0.155, and 0.249, respectively) or on phase shift (p = 0.531, 0.939, and 0.855, respectively) (Fig. 8).

DISCUSSION

The objectives of this study were to design and evaluate custom culture chambers and a dynamic mechanical test system for measurement of the viscoelastic material properties of collagen gel constructs, and to determine the effects of collagen concentration and period of culture on the viscoelastic properties of the gels. There was a significant effect of frequency and equilibrium strain level on the dynamic stiffness and phase angle of the gels, and the dynamic stiffness increased nonlinearly with increasing concentrations of collagen. The results of this study demonstrate that, under appropriate conditions, the material properties of native collagen gels can be maintained in culture with only minimal changes up to at least 7 days.

The uniaxial tensile test system offers distinct advantages for evaluation of the material properties of ECM constructs as well as for tissue or cell mechanics. The major advantage of the present system is the ability to apply arbitrary strain waveforms, both static and dynamic, at different rates as well as the quantification of the associated stresses. The close approximation that we achieved to a routine tensile test specimen with this chamber and the actuating mechanism also ensures that we minimized errors arising due to variables introduced by shear[13] involved in gripping the substrates and strain heterogeneity. Collagen gels are also known to be stiffer at the point of anchorage because of sticking of the collagen to the substrates.[17] To avoid difficulties associated with adherence of the collagen to the culture chamber sides and bottoms, materials that do not adhere to collagen were used in the chamber design—actuation was achieved via polymerization of the gels around the anchor bars as opposed to relying on adhesion.

The FE simulations showed that the amount of strain applied to the gel is related to the boundary conditions at the interface between the posts and the collagen gel. When perfect bonding was assumed, the strain at the center of the sample was nearly identical to that applied by the actuating post. When this condition was relaxed to

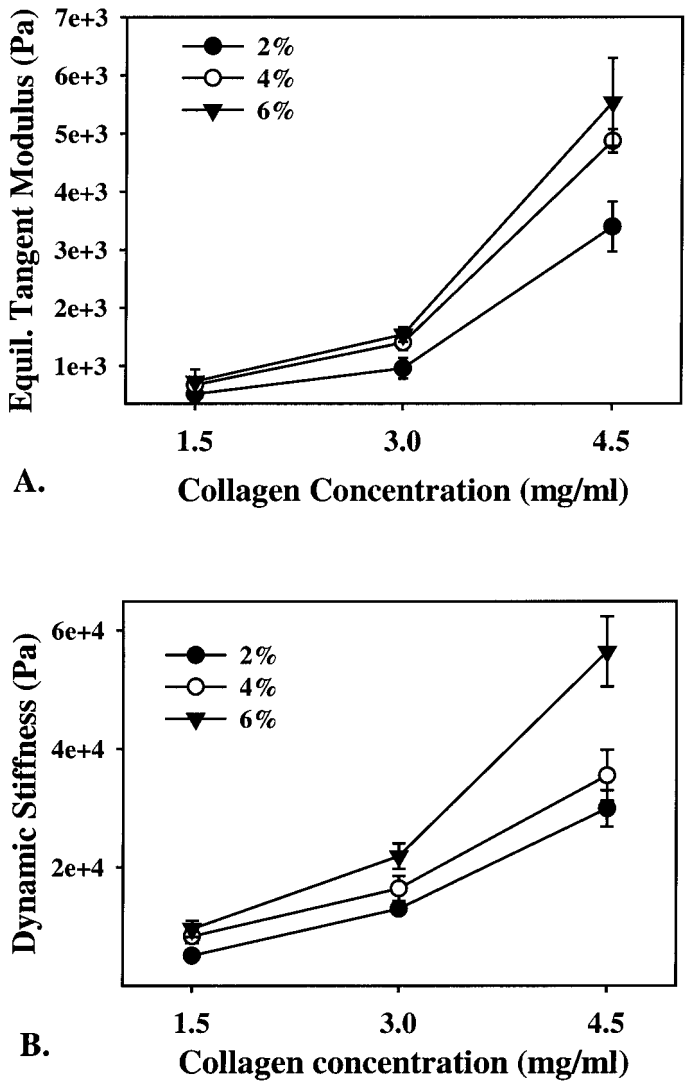

FIG. 7. Effect of collagen concentration on equilibrium tangent modulus and dynamic stiffness: day 0 tests at 5 Hz excitation (means ± SEM).

allow for transfer via contact only between the gel and posts, the strain at the center of the specimen was still close to the applied axial strain, but the distribution of strain near the posts showed higher gradients. This result is to be expected because of stress concentrations near the posts. Regardless of the amount of strain transferred, the strain field in the central three-fourths of the gage length was homogeneous and linearly related to the applied actuator strain, and this is the most important characteristic required for a tensile test configuration. Observations of the amount of separation between the gels and the posts during the experiments suggest that the true boundary conditions between the posts and the gel lie somewhere between perfect bonding and transfer solely via contact. In the modeling, we assumed isotropic hyperelastic material behavior for the gel to model the equilibrium stress–strain behavior of the material. Although the gels are clearly viscoelastic and consist of both fluid and solid phases, the assumed material behavior provides a reasonable approximation to the equilibrium material behavior for the purposes of determining the homogeneity of the strain field under conditions of small sinusoidal perturbations about an equilibrium strain configuration.

The equilibrium stress–strain behavior of the gels demonstrated a linear relationship at the lower collagen concentrations (1.5 and 3.0 mg/mL), with the relationship showing more upward concavity at the highest collagen concentration of 4.5 mg/mL. This change in the shape of the curve is likely due to an increased propensity for the collagen fibrils to cross-link at the higher collagen concentration, resulting in more recruitment of random fibers along the stretching direction during extension of the

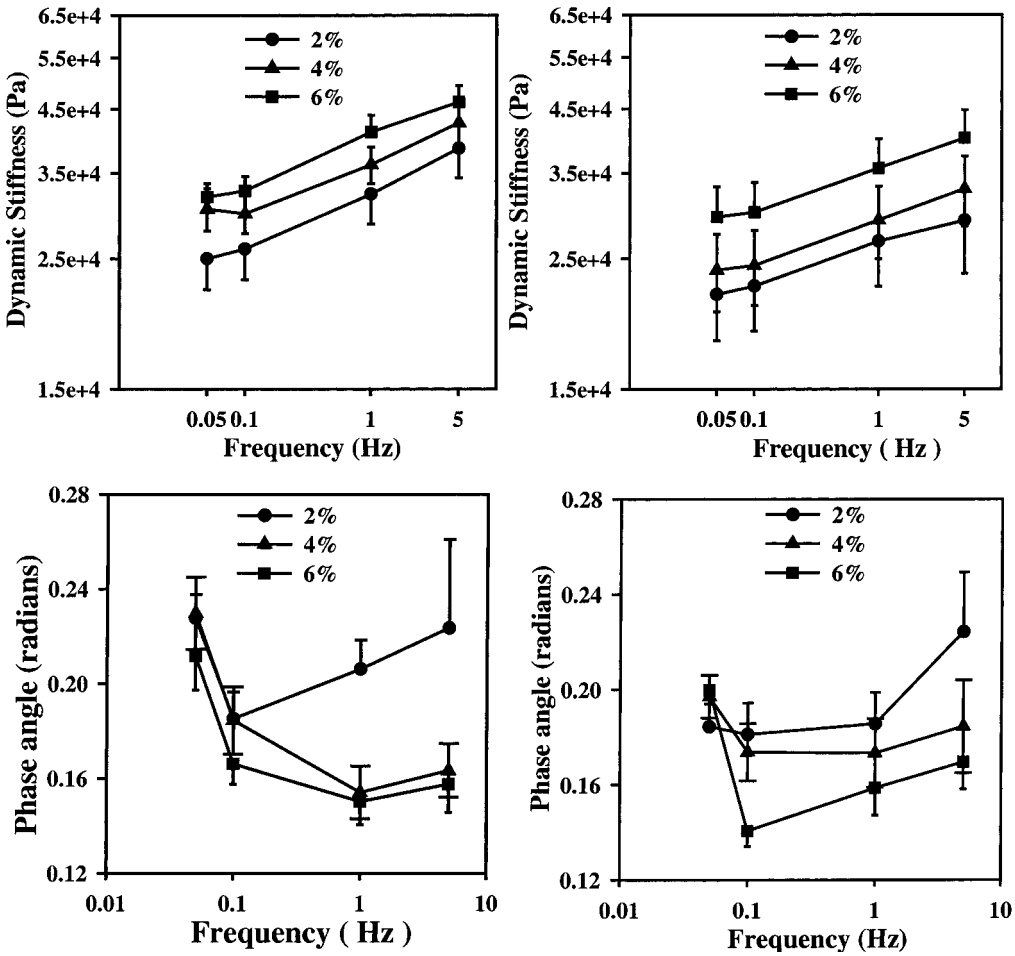

FIG. 8. *Top*: Log–log plots of dynamic stiffness as a function of frequency for day 1 (*left*) and Day 7 (*right*) gels. There was an increase in stiffness with increasing equilibrium strain levels, but there was no effect of test day. *Bottom*: Semilog plots of phase angle as a function of frequency for day 1 (*left*) and day 7 (*right*) gels. The phase angles showed a drop at 0.1 Hz and then again increased at 1 and 5 Hz. All data are for a collagen concentration of 3.0 mg/mL.

gel.[26–28] A similar trend was observed for the changes in dynamic stiffness and equilibrium tangent modulus with increasing collagen concentration. The dramatic difference between the equilibrium tangent modulus and the dynamic modulus demonstrates the contribution from the rate-dependent viscous part of the constructs.

The collagen gels exhibited a moderate strain rate-dependent dynamic stiffness and were more viscous at low and higher strain rates, with the change in frequency dependence occurring at about 0.1 Hz. The frequency dependent trends in both dynamic modulus and phase shift in our study are consistent with those reported by Wakatsuki *et al.* for cell-populated matrices.[18] Our reported values for dynamic stiffness are higher than values previously reported for the complex moduli of these gels. The differences are likely due to the much higher collagen concentrations used in the present work. Additional issues that could play a role include the differences in test temperature and the specific methods used to compute the modulus. When comparing the present results with other data in the literature (e.g., Refs. 11 and 18), it is important to note that the present data were collected at 25–27°C. At a temperature of 37°C, the temperature most often associated with incubators, the stresses can be predicted to be lower solely on the basis of the rheological behavior of collagen solutions.[27]

The properties of collagen are influenced by temperature, pH, osmolarity of the nutrient solution, fiber diameter and associated cells and extracellular constituents. It has been demonstrated that fiber diameter can influence collagen mechanical properties.[29] Fiber diameter, however, has been shown to be unaffected by changes in pH changes over a range of temperatures, and it has also been suggested that the viscoelastic properties of collagen depend more on the fiber lengths rather than their diameters.[29] This, in combination with the results of this study, demonstrates that the rise in stiffness of the gels with strain level and strain rate is not merely due to variations

in environmental conditions causing an increase in cross-linking. Another factor that needs to be considered is the theory of aging of collagen fibers with time and the increase in stability of collagen fibers even after completion of polymerization mainly due to hydrophobic interactions. The only available rheological data were acquired after a period of only 3 h of aging.[30]

Tests of the collagen gels were carried out after a particular period of culture, rather than serially. This was intentional, as serial mechanical testing of gels containing cells or cellular constructs would likely influence cellular catabolic and anabolic activities. The dramatic effects of mechanical loading on cell and tissue metabolism have been demonstrated repeatedly (e.g., Refs. 3–5, 17, and 31–33). Although the present system can be readily adapted for repeated testing inside an incubator, our intended application is for the measurement of changes in ECM properties due to growth. Attempts to remove the gels from the incubator, perform mechanical testing, and then return the gels to culture resulted in contamination of the gels. The strategy of testing the gels once after a fixed period of culture avoids both of these difficulties.

Dynamic linear viscoelastic analysis is a useful tool for investigation of strain- and rate-dependent material properties.[34] By subjecting a material to small sinusoidal perturbations about an equilibrium strain at different frequencies, the linear viscoelastic material properties of the gel can be quantified. Changes in dynamic stiffness with frequency are representative of the effect of strain rate on material modulus and changes in damping (phase shift) are representative of changes in hysteresis or energy dissipation. In the present data analysis, it is assumed that the strains in the gel are homogeneous, that the material is isotropic, and that the assumptions of linear viscoelastic theory apply for perturbations about a particular equilibrium strain level. The latter implies that when a sinusoidal strain–time input was applied, the resulting stress-time signal was well described by a sine wave in terms of time variation and it was symmetric about the equilibrium stress level. It should be noted that the system is in no way restricted to linear viscoelastic materials characterization, and that other dynamic strain profiles and magnitudes can be applied readily with the piezoelectric actuator. Such an approach could be used to characterize the nonlinear viscoelastic response of the material, using more advanced viscoelastic theories to describe the resulting data (e.g., Refs. 35–38).

This study developed a tensile test machine that allows viscoelastic characterization of ECM constructs and expands on the capabilities of other systems reported in the literature by allowing the simultaneous quantification of both applied dynamic strains and resulting stresses. The system was used to evaluate the viscoelastic material properties of collagen type I constructs as a representative extracellular matrix system. The data generated from this work can be used to formulate a model of the viscoelastic nature of these collagen gels, which can subsequently be used to describe their constitutive behavior. This model can then be extended to encompass variations induced by culturing of cells in such constructs. Our system acquires special importance when we consider the studies establishing the relationship of mechanical stimuli to cell growth and differentiation.[17,39–41] This work will also benefit other areas of tissue mechanics, where culture of cells in a physiologically significant and mechanically dynamic environment is desired.[8,9,32,33,39] We are presently working to evaluate the effects of microvasculature growth on gel material properties. When combined with the results of the present study, these data will enable the formulation of a model to predict the constitutive behavior of such ECM constructs, and the formulation of functional engineered constructs.

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