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# Indentation analysis of biphasic viscoelastic hydrogels

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ABSTRACT

Mechanical properties of soft biological materials are dependent on the responses of the two phases of which they are comprised: the solid matrix and interstitial fluid. Indentation techniques are commonly used to measure properties of such materials, but comparisons between different experimental, and analytical techniques can be difficult. Most models relating load, and time during spherical indentation are based on Hertzian contact theory, but the exact limitation of this theory for soft materials are unclear. Here, we examine the response of gelatin hydrogels to shear and indentation loading to quantify combined effects of the solid, and fluid phases. The instantaneous behavior of the hydrogels is different for each test geometry, and loading rate, but the relaxed response, measured by the relaxed modulus, is the same for all tests, within 17%. Additionally, indentation depths from 15% to 25% of the radius of the spherical indenter are found to minimize error in the estimate of relaxed modulus.



## 1. Introduction

Quantitative measurements of mechanical properties of soft materials, such as tissues, are important in understanding the material response to loads, and deformations. For example, elasticity imaging relies on differences in the elastic stiffness of healthy, and diseased tissues to produce contrast for tumor detection and diagnosis (Greenleaf et al., 2003). In addition to the elastic properties, time-varying viscoelastic properties can also be useful in imaging creep tests where an applied load is held and the material is imaged over time (Greenleaf et al., 2003). Mechanical properties are also important in tissue engineering, and cell cultures where cells are known to sense and respond to the material with which they are in contact. The viability of cells in culture is greatly influenced by the effective stiffness of their extra cellular matrix (ECM) (Augst et al., 2006). A quantitative understanding of the mechanical properties of the ECM would help in understanding the cellular response of the mechanical environment.

The complexity of biological systems makes quantitative mechanical testing of such systems difficult. Inhomogeneities, irregular geometries, and difficulty in the isolation/extraction of tissue samples are just a few of the factors that affect mechanical measurements on these materials. Simplified systems, such as hydrogels, that mimic some of the mechanical properties of biological systems are useful to study basic material behavior. Tissue engineering and cell culture studies rely on the use of scaffold materials, often hydrogels (Dubruel et al., 2007; Fischback et al., 2007), on which cells are grown. Hydrogels are often used in bioimaging studies (Hall et al., 1997; Khaled et al., 2006; Han et al., 2003) as phantoms before more complicated systems, like tissue samples with tumors, are examined.

Indentation techniques are widely applied in the characterization of biological materials, and have received considerable attention over the last several years (Chen et al., 2007; Darling et al., 2006; Darling et al., 2007; Mahaffy et al., 2000; Mattice et al., 2006; Mooney et al., 2006; Krouskop et al., 1998; Wellman et al., 1999; Samani et al., 2007). Although

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the experiment is simple, effects of the thickness of biological samples, loading and boundary conditions need to be isolated when geometry-independent properties are sought. In the analysis of indentation data of biological materials, an incompressible, elastic material model using Hertzian contact theory is often assumed (Dimitriadis et al., 2002; Hayes et al., 1972). As a result of this assumption, a single parameter is found to describe the behavior, the Young's modulus, $E$ (though sometimes, $\mu$, the shear modulus is used). For spherical indentation of a semi-infinite elastic medium, Hertz calculated the contact pressure at the surface of the medium by approximating the spherical contact surface as a paraboloid (Hertz, 1881). The approximation is valid for small indentation depths compared to the radius of the spherical indenter. At larger depths the increasing difference between the contact area of a spherical versus parabolic indenter for the same indentation depth results in an increasing bias in the estimation of the elastic modulus from the experimental load-displacement data. An acceptable limit on the depth of indentation for Hertzian theory to be valid is not well understood for poroviscoelastic materials. It is envisaged that the validity of the semi-infinite assumption depends on the radius of the indenter, and thickness and width (in-plane dimensions) of the medium.

Many soft materials cannot be adequately characterized by a single parameter such as a modulus due to their inherent viscoelastic nature. The correspondence principle, in which elastic parameters in an elastic solution are replaced by the analogous viscoelastic differential or integral functions, is often used to determine the theoretical viscoelastic solution. For viscoelastic indentation problems, the analysis often begins with the elastic Hertz solution (Lee and Radok, 1960; Oyen, 2005; Cheng et al., 2005; Mattice et al., 2006; Darling et al., 2006; Mahaffy et al., 2000), and thus the viscoelastic indentation solutions are subject to the same restrictions as the Hertz solution. Recently, creep (Oyen, 2005; Cheng et al., 2005), load relaxation (Cheng et al., 2005; Mattice et al., 2006), and microrheology tests (cyclic loading) (Mahaffy et al., 2000) with a spherical indenter geometry have been used to study the viscoelastic response of soft materials. These time-dependent or frequency-dependent tests provide a good insight into viscoelastic behavior, but add complexity since there could be possible environmental effects on the sample during the length of test time, and also require specialized equipment. For example, creep and load relaxation tests that last longer require environmental control for biological materials such as tissues or cell cultures. Similarly, microrheological tests that can be conducted over a wide range of frequencies, require sophisticated instrumentation, and synchronization to achieve accurate results. Hence, a simple, and quick test that probes the viscoelastic behavior of soft biological tissue would be ideal. The quasistatic indentation test can be conducted on a simple load frame, and in a short testing time. This inherent characteristic of the indentation test potentially eliminates the need for specialized equipment or environmental control. Additionally, when indentation load-displacement data is analyzed with an appropriate viscoelastic model, the time-dependent material behavior can be estimated.

Modulus values for soft tissues have been estimated in the literature using varying experimental techniques, and analyses. To illustrate this variation, Table 1 contains the estimated elastic modulus values from current literature of three types of human breast tissue; adipose tissue, normal glandular tissue, and infiltrating ductal carcinomas (IDC). The experimental method, and test variables, such as strain rate, prestrain, and frequency, are indicated. It can be seen that, even when similar experimental techniques are used, there can be differences of nearly an order of magnitude between measured modulus values. These discrepancies emphasize the difficulty in comparing the moduli estimated using different analyses, and test methods.

In this work, we estimate the relaxation modulus of gelatin hydrogels using common experimental methods for comparison between experiments, and we examine the bias in the Hertzian theory, and its effect on the estimated material modulus. Time-dependent moduli estimated from a shear stress relaxation experiment, stress relaxation, are compared with moduli estimated from two types of indentation tests, load relaxation, and quasistatic indentation. The instantaneous moduli, and relaxed moduli estimated from each type of experiment used in this study are compared to examine the short and long time effects, respectively, of the geometry of the specimen, load application, and rate of loading. The quasistatic indentation experiment is explored in detail using a standard linear solid material model to estimate a viscoelastic, time-dependent modulus. The limitations of the Hertz solution for elastic indentation are explored to seek

**Table 1**
Example modulus measurements (average ± standard deviation) on three types of breast tissue from different studies using various indentation techniques, and analysis. The average modulus varies greatly between studies, even when the experiments are similar (e.g. frequency = 0.1 Hz).

| Type of tissue | Experiment | Experiment details | N | Elastic modulus (kPa) | Ref. |
|---|---|---|---|---|---|
| Adipose | Sinusoidal, flat punch indentation | 0.1 Hz, 5% precomp. | 40 | 18 ± 7 | (Dimitriadis et al., 2002) |
| Adipose | Flat punch indentation | Varying rates, strain = 0.01 | 26 | 5 ± 3 | (Chadwick, 2002) |
| Adipose | Sinusoidal, flat punch indentation with FEA | 0.1 Hz, preconditioned | 71 | 3 ± 1 | (Selvadurai, 2004) |
| Normal glandular | Sinusoidal, flat punch indentation | 0.1 Hz, 5% precomp. | 31 | 28 ± 14 | (Dimitriadis et al., 2002) |
| Normal glandular | Flat punch indentation | Varying rates, strain = 0.01 | 7 | 18 ± 9 | (Chadwick, 2002) |
| Normal glandular | Sinusoidal, flat punch indentation with FEA | 0.1 Hz, preconditioned | 26 | 3 ± 1 | (Selvadurai, 2004) |
| IDC | Sinusoidal, flat punch indentation | 0.1 Hz, 5% precomp. | 32 | 106 ± 32 | (Dimitriadis et al., 2002) |
| IDC | Flat punch indentation | Varying rates, strain = 0.01 | 25 | 47 ± 20 | (Chadwick, 2002) |
| Intermediate grade IDC | Sinusoidal, flat punch indentation with FEA | 0.1 Hz, preconditioned | 21 | 20 ± 4 | (Selvadurai, 2004) |

similarities between the predicted moduli from elastic, and viscoelastic models.

## 2. Experimental methods and analysis

Three experiments, shear stress relaxation, indentation load relaxation, and quasistatic indentation, are performed on gelatin hydrogel specimens. Based on the geometry (spherical indentation, and shear) and type of loading (ramp-hold, and ramp), an analytical model is determined for each of these experiments. The analytical models relate the stress or load on the material with time, resulting in time-dependent material moduli (relaxation moduli) for the shear stress relaxation, indentation load relaxation, and quasistatic indentation experiments. Best-fit model parameters are found through curve fitting to experimental data, and the relaxation moduli from each experiment are compared. In addition to experiments, simulated data from finite element analysis (FEA) of the quasistatic, spherical indentation of an elastic medium is used to determine the bias due to the inherent geometric assumption in the elastic Hertzian contact solution. The details of the preparation of materials, the experimental methods, the analytical models, and the elastic FEA are given in the following sections.

### 2.1. Gelatin preparation

Three different concentrations of gelatin hydrogels are prepared for two types of samples used in the experiments. Two batches, totaling 400 ml each, are prepared identically for the three concentrations. Type B gelatin powder (Rousselot, Dubuque, IA) is measured by weight for 4%, 6%, and 8% concentration gelatin and is mixed with the appropriate amount of deionized water, 95.9%, 93.9%, and 91.9%, respectively. The beakers with the gelatin mixture are covered, placed in a 60 °C water bath for 70 min, and stirred every 15 min. The beakers are removed from the heated water bath and are allowed to sit at room temperature (approx. 23 °C) for 5 min before 0.1% formaldehyde is added as a chemical crosslinker. The beakers are placed in a 25 °C water bath, and the mixture is stirred continuously to cool it to approximately 27 °C. The gelatin is then poured into two types of mold, covered, and allowed to gel at room temperature for 24 h before testing.

Shear, and indentation experiments require specimens with differing geometries, although samples are prepared such that the thermal history, and therefore material properties are nearly identical. The indentation samples are cylinders 44.5 mm in diameter, and 26–27 mm in height, while the smaller rheometer samples are 25 mm in diameter, and 2–5 mm in height. To remove the effect of cooling rates in samples of different volumes, the gelatin mixture is poured into plastic molds when it is near room temperature for all concentrations. The molds are covered with an acrylic plate with a polymer mold release (Pol-Ease 2300, Polytek Development Corp. Easton, PA). The plate serves to create flat top surface for the specimen, and prevents desiccation before testing and the mold release lubricates the test surface of the specimen. The indentation specimens are tested in the mold with only the top surface open to air. For the rheometer specimens, mold release is applied to all the mold surfaces to ensure that the gelatin hydrogel can be removed completely and placed into the rheometer fixture.

### 2.2. Shear stress relaxation experiment

Shear experiments are conducted on an AR-G2 rheometer (TA Instruments, New Castle, DE, U.S.A.). Gelatin specimens are tested in a parallel plate fixture, 25 mm in diameter. Cyanoacrylate (Rawn America, Spooner, WI, U.S.A.) is used to bond the gelatin hydrogel to the upper and lower fixture surfaces to prevent slippage during experimentation. At least three specimens for each gelatin concentration are used in the stress relaxation experiments. The specimens are displaced to 5% strain over a 1 s ramp, and then held for 1800 s. Gelatin hydrogels are observed to have properties that change with time (Orescanin et al., 2009), and steady-state properties are not reached after 24 h. Thus, the duration of the relaxation experiment is optimized to estimate the relaxation parameters while reducing the total testing time for all specimens. The stress-time data are collected at 61 samples/s in the beginning of the experiment, and the sampling rate decreases throughout the experiment to a final acquisition rate of 0.03 samples/s at the end of the experiment.

A third order, generalized Maxwell model is used to characterize the behavior of the gelatin hydrogel in the shear stress relaxation experiments. For an applied step in strain, the stress relaxation behavior,

$$\sigma(t) = \sigma_0 + \sigma_1 e^{-t/\tau_1} + \sigma_2 e^{-t/\tau_2} + \sigma_3 e^{-t/\tau_3} \tag{1}$$

results in a shear relaxation modulus of the form

$$G^{SR}(t) = G_0 + G_1 e^{-t/\tau_1} + G_2 e^{-t/\tau_2} + G_3 e^{-t/\tau_3} \tag{2}$$

where $G_0$ is the instantaneous modulus and $G_i$ are the modulus amplitudes associated with time constants, $\tau_i$. Although, the applied step in strain is a convenient theoretical assumption it is difficult to achieve during experimentation. The applied strain, $\epsilon_a$, occurs over a finite ramp time, $t_R$. Model parameters for a ramp load are

$$G_0 = \sigma_0/\epsilon_a \tag{3}$$

$$G_i = \sigma_i/(RCF_i\, \epsilon_a), \; i = 1 - 3 \tag{4}$$

$$RCF_i = \frac{\tau_i}{t_R}\left(e^{\frac{t_R}{\tau_i}} - 1\right) \tag{5}$$

Ramp correction factors, $RCF_i$, adjust the modulus amplitudes to account for the difference between the assumed step in displacement and the actual ramped displacement (Mattice et al., 2006; Oyen, 2005; Oyen, 2005; Oyen, 2006).

The data analysis of the stress relaxation experiment involves curve-fitting a modified data set with constant sampling rate. The original data is modified by eliminating data points from the beginning of the experiment, and interpolating between points at the end to produce a data set that has a constant sampling rate of approximately 0.5 samples/s. The constant sampling rate of the modified data set allow for accurate curve fitting for the entire duration of the experiment. The model in Eq. 1 is used to fit the stress data using Mathematica (Wolfram Research, Urbana, IL, U.S.A.). The model parameters for stress relaxation are used to determine parameters for the relaxation modulus, $G(t)$, by Eqs. (3)–(5).

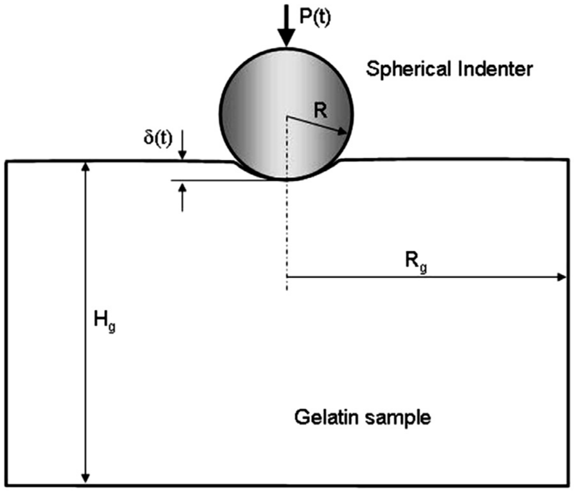



**Fig. 1.** Schematic of spherical indentation with parameters.

### 2.3. Indentation load relaxation experiment

Indentation experiments are performed on a TA.XTplus Texture Analyzer (Stable Micro Systems Ltd., Surrey, U.K.) using a 1 kg load cell and a spherical-tip indenter that is 5 mm in diameter. The vertical position of the indenter tip is calibrated to accurately determine the height of the gelatin hydrogels. Then, the specimens are centered below the indenter tip. To create a nearly frictionless contact between the spherical indenter probe, and the gelatin hydrogel, polymer mold release is applied to the spherical probe surface prior to testing. The indenter tip is lowered gradually until it contacts the surface of the specimen, which is critical to the accuracy of the experimental data. In the indentation load relaxation experiment, the indenter tip is displaced at the rate of 1 mm/s to the desired indentation depth of 1 mm, and then the position is held while the load relaxation data are captured for 1800 s. Load-time data are collected at a rate of 10 samples/s.

The analytical framework for the indentation load relaxation experiment was presented by Mattice et al. (Mattice et al., 2006), and is briefly outlined here for a spherical indenter of radius, $R$, and a ramp time, $t_R$. The indentation geometry is shown in Fig. 1. To model the indentation-load relaxation for an applied ramp-hold displacement, Mattice and coworkers assumed the following relaxation model for the indentation load,

$$P^{LR}(t) = P_0 + P_1\, e^{-t/\tau_1} + P_2\, e^{-t/\tau_2} + P_3\, e^{-t/\tau_3} \tag{6}$$

based on a modulus relaxation function, $G^{LR}$, of the form of Eq. (2). Here, $P^{LR}$ is the time-dependent load on the indenter, $P_0$ and $G_0$ are the instantaneous load and modulus parameters, and $P_i$ and $G_i$ are the load relaxation and modulus parameters associated with time constants $\tau_i$. The force response to an applied step in displacement during indentation relaxation of an incompressible material,

$$P^{step}(t) = \frac{8\sqrt{R}}{3}\delta^{3/2} 2G(t) \tag{7}$$

is used to approximate a relationship between $P_i$ and $G_i$:

$$G_0 = \frac{P_0}{2\delta_{max}^{3/2}\left(8\sqrt{R}/3\right)} \tag{8}$$

$$G_i = \frac{P_i}{2\delta_{max}^{3/2}\left(RCF_i\, 8\sqrt{R}/3\right)},\ i = 1-3 \tag{9}$$

Here, $\delta$ is the time-varying indentation depth, and $\delta_{\max}$ is the maximum value. These expressions account for the geometry of indentation, and contain the adjustments needed for the ramp load, where the ramp correction factors are the same as in Eq. (5).

After completing the experiments, the data are analyzed by performing curve-fitting using Mathematica. The initial load ramp response is removed from each data set, and the load data are shifted such that the peak load occurs at time $t = 0$ s. The assumed model from Eq. (6) is fit to the load-time data using a least squares fit algorithm to determine load parameters, $P_i$. Material parameters, $G_i$, are found using Eqs. (8) and (9), and are averaged for all samples of the same gelatin concentration.

### 2.4. Quasistatic indentation experiment

Quasistatic indentation is also performed using TA.XTplus Texture Analyzer with the same initial steps to determine contact as the indentation load relaxation experiment. A single indentation cycle is performed to a depth of 2 mm for the quasistatic indentation experiments. Experiments are conducted with an indenter tip speed of 0.01 mm/s, and load-displacement data are collected at 2 samples/s using Texture Exponent software (Stable Micro Systems Ltd.). For each gelatin concentration, at least 4 samples are tested.

Following the approach of Mattice et al. (Mattice et al., 2006), we used one-dimensional viscoelastic theory to describe the viscoelastic response due to spherical indentation loading using the three-element, standard linear solid (SLS) model. The SLS model is composed of a Voigt element (spring and dashpot in parallel) in series with a free spring. Boltzmann's superposition principle, usually written in terms of stress, and strain is modified to relate load, and displacement of indentation based on the geometry of the experiment. Based on Hertzian contact mechanics, the load as a function of time for an indentation displacement history, $\delta(t)$, is shown to be

$$P^{QS}(t) = \frac{16\sqrt{R}}{3}\int_0^t G(t-\tau)\, d\left(\delta^{3/2}(\tau)\right) \tag{10}$$

for an indenter of radius, R. The shear relaxation modulus for the SLS model of an incompressible material was derived and found to be,

$$G^{QS}(t) = G_0\left[\left(\frac{G_0}{G_0+G_1}\right)e^{-\frac{(G_0+G_1)t}{\eta}} + \frac{G_1}{G_0+G_1}\right] \tag{11}$$

where $G_0$ is the shear stiffness of the free spring, and $G_1$ and $\eta$ are the shear stiffness, and shear viscosity of the spring and dashpot, respectively, in the Voigt element.

Here we evaluate the integral in Eq. (10) similar to the work of Lee and Radok (Lee and Radok, 1960) using a constant-velocity indentation, $\delta(t) = vt$, and the relaxation modulus in Eq. (11) to determine the time-dependent indentation load,

$$P^{QS}(t) = \frac{8G_0\sqrt{Rv^3}}{G_0+G_1}\left[\frac{2G_1}{3}t^{3/2} + \frac{G_0\eta}{G_0+G_1}t^{1/2} - \frac{G_0\eta^{3/2}}{(G_0+G_1)^{3/2}}\frac{\sqrt{\pi}}{2}e^{-\frac{(G_0+G_1)t}{\eta}}Erfi\left(\sqrt{\frac{(G_0+G_1)t}{\eta}}\right)\right], \tag{12}$$

where $v$ is the velocity of indentation.

The indentation-load response based on the viscoelastic SLS model, Eq. (12), is used to fit each set of load-displacement data. The full indentation depth of 2 mm is not used in curve fitting. Instead, the data are cut off at various depths (0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 1.0 and 1.5 mm), and curve fitting is performed eight times for a single experimental specimen. The information gathered from the best fit parameters at the different indentation depths is used to gain insight into the growing bias at larger indentation depths due to limitations in the Hertzian theory. The parameters are averaged for all samples of the same gelatin concentration at each indentation depth.

### 2.5. Finite element analysis of quasistatic indentation experiment

The quasistatic indentation of an elastic incompressible material is simulated using FEA, and the predicted results are compared with those obtained from an analytical solution assuming a Hertzian contact. The material parameters used in the FEA for the incompressible material are elastic modulus, $E_m = 7395$ Pa, and Poisson's ratio $\nu = 0.5$. FEA is performed in ABAQUS (ABAQUS manual, 2007) FEA software using CAX4R, 4-node axisymmetric finite element with a reduced integration method. Investigations on the indentation of biological materials (Mooney et al., 2006; Olberding and Suh, 2006) as well as porous materials such as soils (Selvadurai, 2004) recommend the radius of the material to be greater than about 10 times the contact radius at the maximum depth of indentation for a semi-infinite assumption to be valid. Hence, in this study, the axisymmetric model is chosen with a radius of 500 mm, and a height of 500 mm to simulate the semi-infinite incompressible medium. The radius of the spherical indenter is assumed to be 2.5 mm, same as that used in the indentation experiments. The spherical indenter is modeled as a rigid analytical surface, and a frictionless contact is assumed between the indenter, and the material interface. The axisymmetric boundary condition is enforced along the central axis, and the surfaces have boundary conditions corresponding to a semi-infinite medium. The center of the spherical probe is displaced along the cylinder axis (z-axis) at the same rate of indentation that is applied during the quasistatic indentation experiments. The indenter is displaced to a depth of 1.5 mm into the material in 0.01 mm increments, to simulate load-displacement data from 0 to 1.5 mm indentation. The Hertz solution for elastic indentation,

$$P(t) = \frac{16\,G\,\sqrt{R}}{3}\delta^{3/2} \tag{13}$$

is used to curve fit the FEA data in MATLAB to predict the elastic shear modulus, G, as a function of indentation depth. The predicted values of G are compared with the value input into the FEA, $G_m = E_m/3 = 2465$ Pa.

## 3. Results

The three analytical viscoelastic models (Eqs. (1), (6), and (12)) are independently fit to the respective experimental data from stress relaxation, indentation load relaxation, and quasistatic indentation experiments, as previously described. The chosen models are sufficient to predict the behavior of the gelatin hydrogels in all three concentrations, and are valid for the entire duration of the experiments. An example of stress relaxation data along with the corresponding best fit model is shown in Fig. 2(a) for hydrogels with different gelatin concentrations. Representative load-time data for the indentation load relaxation experiments are shown in Fig. 2(b). Representative load-displacement data for indentation up to $\delta = 0.4$ mm in the quasistatic experiments are shown in Fig. 2(c) with the corresponding best fits.

The relaxation moduli determined from the different experiments are compared and contrasted to understand the material behavior under the various loading conditions. The parameters estimated from the experimental data lead to time-varying functions for relaxation modulus for the three experiments. The analytical models for each experiment are different, with its respective set of parameters. Thus, the relaxation functions for each experiment are also different. Example relaxation functions for the three models are compared in Fig. 3 for 8% gelatin hydrogel. It is seen in Fig. 3(a) that the instantaneous modulus at time t = 0 is different for each experiment, but the relaxed moduli nearly converge at long times, in Fig. 3(b). Two values that can be easily compared between the three experimental techniques are the zero time (instantaneous), and long time (relaxed) moduli of the hydrogels. The averaged instantaneous, and relaxed modulus values are reported in Table 2 by gelatin hydrogel concentration for the three experiments.

Fig. 4 illustrates the difference between the elastic Hertzian solution (based on a parabolic indenter), and an FEA simulation of spherical indentation on an elastic medium. As evident from this Fig. 4, the experimental data up to indentation depths of $\delta/R = 0.15$ should be used in estimating the modulus as there would be a larger estimation error beyond this when using the Hertzian solution. The bias due to the geometric approximation of the Hertz solution is plotted in Fig. 5 for comparison with similar viscoelastic data.

The quasistatic indentation experiment is analyzed at multiple indentation depths to examine the effect of depth on the predicted material modulus. To compare trends in the results from different concentrations of hydrogels with different stiffness, the relaxed modulus from the quasistatic, $G_\infty^{QS}$ (see Eq. (11)), experiment is normalized by the relaxed modulus from the shear stress relaxation experiment, $G_\infty^{RS}$. The shear stress relaxation experiment is assumed to be the most reliable measure of the relaxed modulus of the hydrogels. Thus, a normalized modulus of 1 indicates perfect agreement between the two tests. Additionally, the indentation depth is normalized by the radius of the indenter to allow generalization of the results to all indenter sizes. The

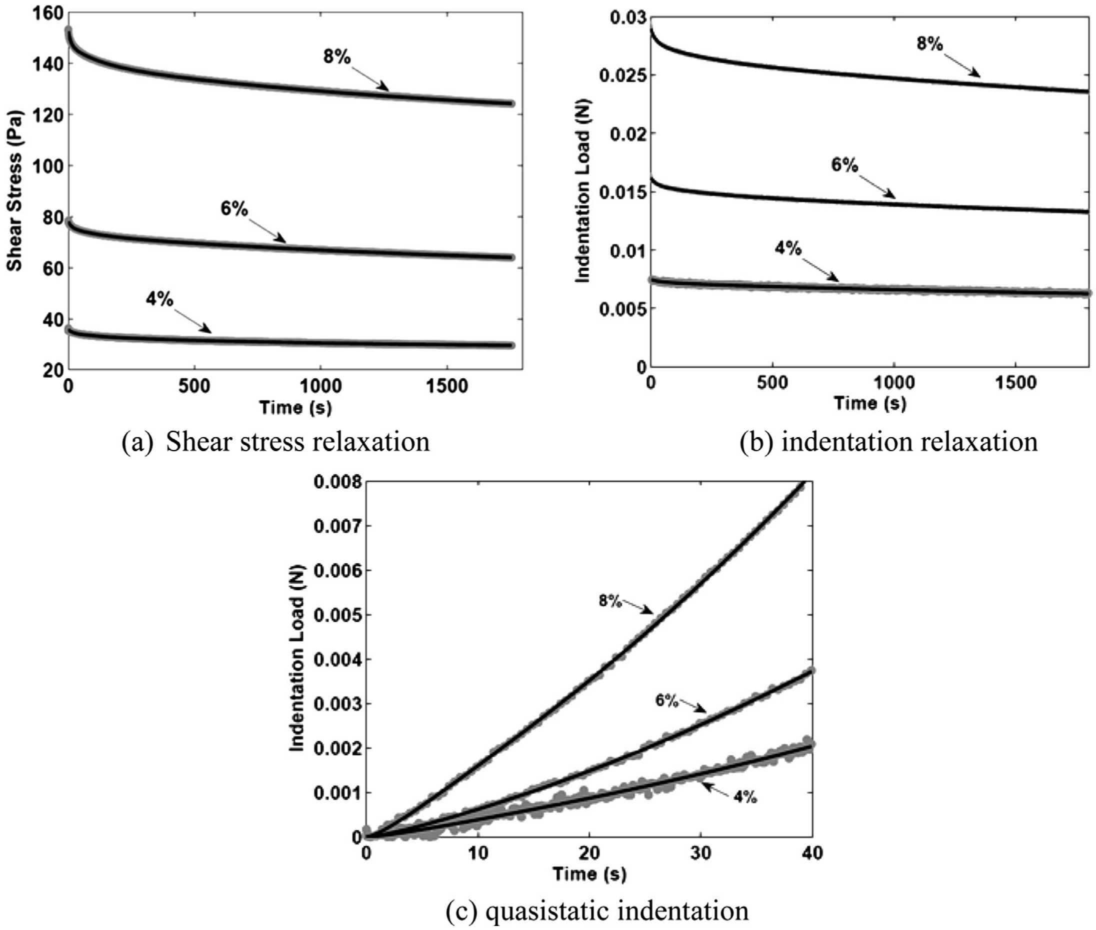



**Fig. 2.** A single, representative raw data set from each type of experiment with corresponding model fits for 4, 6, and 8% gelatin.

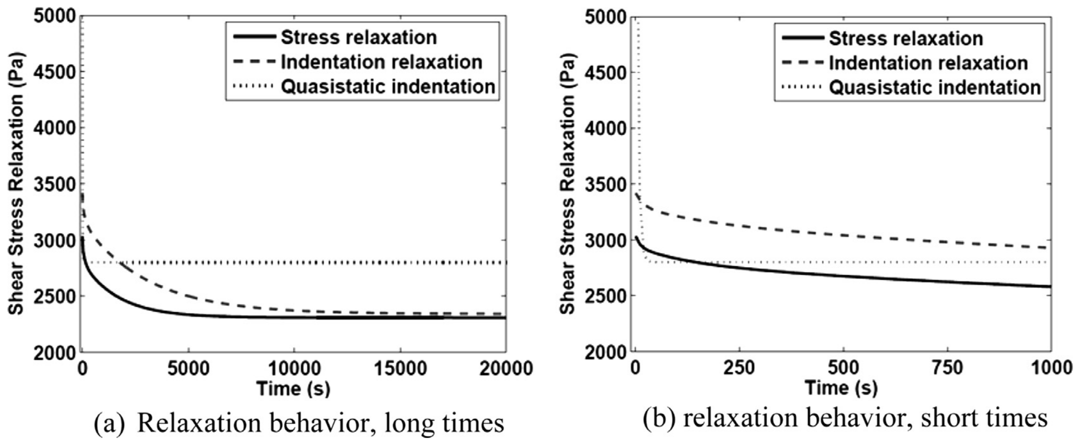



**Fig. 3.** Examples of the relaxation modulus for each of the three time-dependent experiments conducted on 8% gelatin hyrdogel, where (a) shows the short-time relaxation behavior especially for the quasistatic test which relaxes very quickly, and (b) shows the long time behavior with relaxed modulus plateaus.

normalized relaxed modulus, obtained from curve fitting experimental data to different depths, is plotted as a function of the normalized indentation depth for the three gelatin hydrogel concentration in Fig. 5(a–c).

The effect of indentation depth on the normalized modulus from the quasistatic indentation experiment is similar for all three concentrations of gelatin hydrogel. At small indentation depths ($\delta/R < 0.16$), there is much scatter in the normalized modulus, both at a single indentation depth (indicated by large error bars), and across the different depths (indicated by scatter in the average value at different depths). At a normalized depth of $\delta/R = 0.16$, the error bar size stabilizes

**Table 2**
The instantaneous modulus, and the relaxed modulus from the different tests on 4, 6, and 8% gelatin hydrogels. Instantaneous modulus values vary greatly, however the relaxed modulus values from different tests show some agreement within a single concentration, with the modulus from quasistatic indentation approximately 20% higher. N is the number of samples tested, and all modulus units are in Pa. ∗Note: Quasistatic indentation parameters correspond to an indentation depth of $\delta = 0.4$ mm.

| Gelatin concentration | Test | N | G(t = 0) Average ± std. dev. | G (t → ∞) Average ± std. dev. |
|---|---|---|---|---|
| 4% | (1) Shear stress relaxation | 4 | 752 ± 61 | 563 ± 29 |
| | (2) Indentation load relaxation | 3 | 870 ± 45 | 616 ± 5 |
| | (3) Quasistatic indentation∗ | 4 | 2673 ± 463 | 677 ± 19 |
| 6% | (1) Shear stress relaxation | 4 | 1504 ± 94 | 1144 ± 95 |
| | (2) Indentation load relaxation | 2 | 1878 ± 52 | 1335 ± 47 |
| | (3) Quasistatic indentation∗ | 4 | 4341 ± 1841 | 1458 ± 29 |
| 8% | (1) Shear stress relaxation | 3 | 3037 ± 127 | 2340 ± 81 |
| | (2) Indentation load relaxation | 4 | 3425 ± 63 | 2395 ± 88 |
| | (3) Quasistatic indentation∗ | 4 | 10038 ± 1994 | 2868 ± 88 |

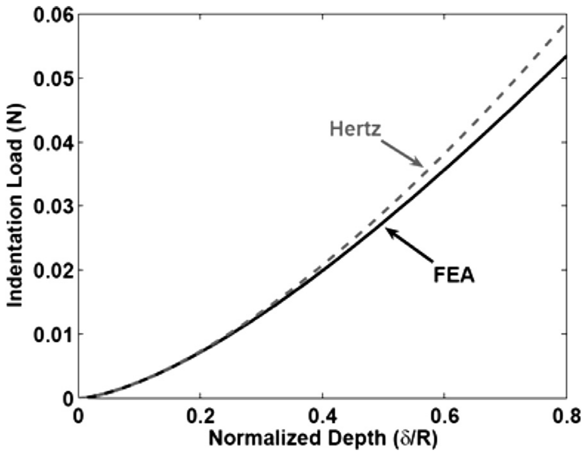



**Fig. 4.** Difference between the elastic Hertzian solution, and elastic FEA simulations of spherical indentation. The parabolic indenter assumed in Hertzian theory results in higher contact forces compared to the spherical indenter used in FEA at large indentation depths.

to the smallest random error value, and the bias is approximately within the amount of that random error. For comparison, the estimated elastic shear modulus, G, from curve fitting to FEA data is included to show the trend of decreasing modulus estimates with increasing indentation depth.

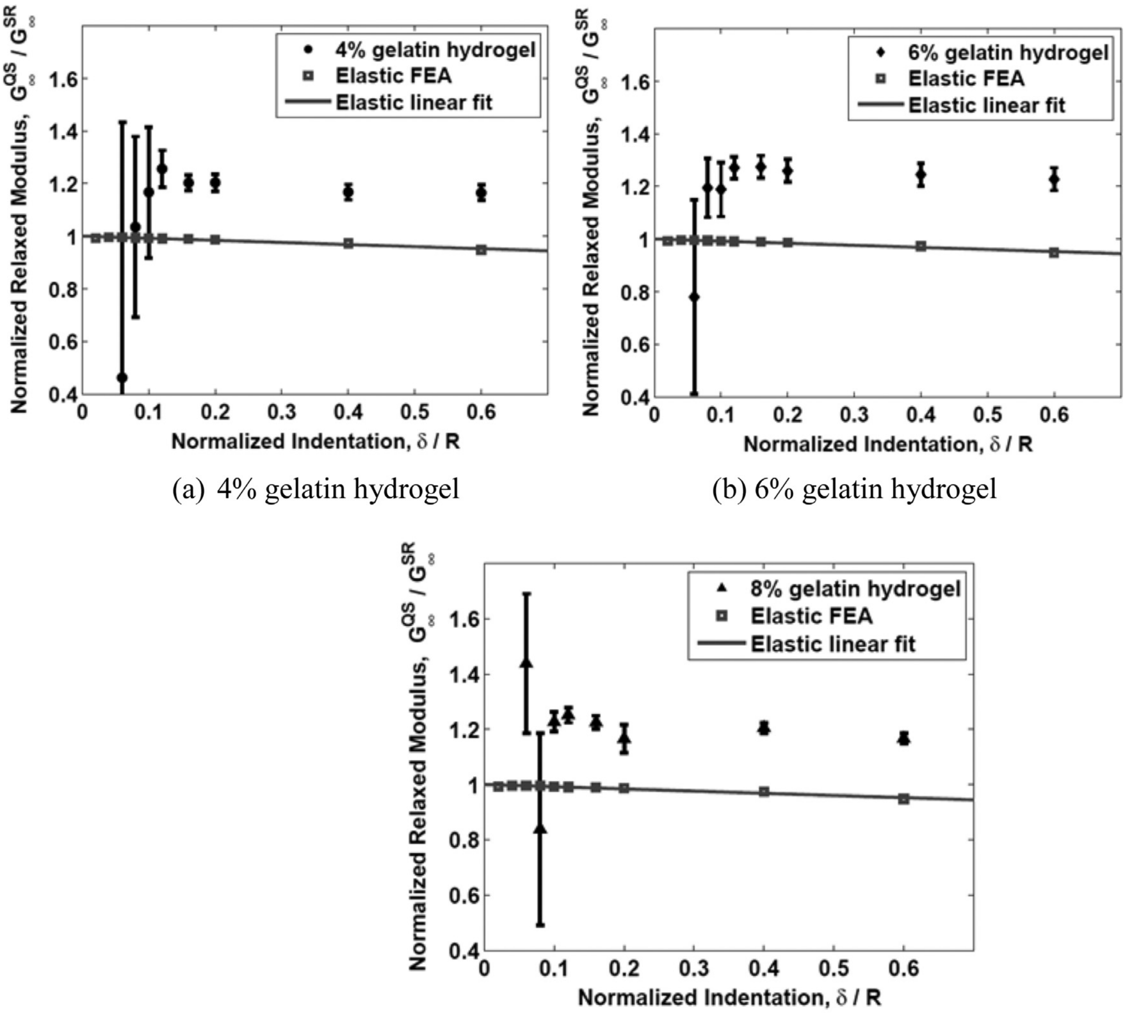
## 4. Discussion

Two types of loading of gelatin hydrogels are examined, pure torsional shear, and spherical indentation, to find material properties that can be compared between individual tests. Pure shear testing is chosen because in biphasic hydrogels it is assumed that shear loading isolates the solid matrix behavior of the material (Hayes and Bodine, 1978). A test that probes the matrix material properties alone allows separate examination of an additional viscoelastic mechanism caused by the flow of interstitial fluid under other loading conditions. Gelatin hydrogels loaded in indentation demonstrate an initial stiffness that is higher than that observed when they are loaded in shear. The greater initial stiffness observed in indentation experiments is seen in the instantaneous modulus values, G(t = 0), listed in Table 2. For each gelatin hydrogel concentration, the instantaneous moduli extracted from the two indentation experiments are higher than those obtained from the shear experiments. The indentation loading produces both shear and hydrostatic strains within the hydrogel, and therefore it probes a combined stiffness of the two phases arising from the biphasic behavior.

Unlike the instantaneous modulus, the relaxed modulus of a hydrogel is a property that depends only on the solid matrix material behavior, and not on the experiment. When a load is initially applied to a biphasic hydrogel, the interstitial fluid may be pressurized locally causing the material to appear stiffer. As time passes, the fluid slowly redistributes throughout the hydrogel, and some fluid may even exude out of the hydrogel. While the fluid pressure decays, the viscoelastic solid matrix also relaxes. After the initial loading, the material eventually reaches a relaxed state. The stiffness of the material in a relaxed state, or relaxed modulus, can be considered as the elastic stiffness after any poroviscoelastic effects have decayed.

Comparisons of the relaxed moduli extracted from the shear stress relaxation experiment with those obtained from the indentation load relaxation and quasistatic indentation tests show good agreement, as seen in Table 2. The differences between the shear stress relaxation and load relaxation experiments is less than 17%, while the difference between the shear stress relaxation, and quasistatic indentation experiments is larger, with the modulus estimated from quasistatic indentation experiments being 20–28% above those estimated from shear stress relaxation experiments. The error in the relaxed modulus predicted from the quasistatic experimental data is attributed to a simplified material behavior assumed when choosing the material model, and possible effects of varied applied strains in the different experiments. The material model used for quasistatic indentation is a three-element standard linear solid, but the other experiments require a higher order model to accurately capture the material behavior. Unfortunately, due to the complexity of performing curve fitting with a function containing multiple exponential decays, curve fitting with a more accurate, higher order model was not performed in this study.

The quasistatic test yields a relaxed modulus that is proportional to that of the shear relaxation test. The relaxed modulus of hydrogels therefore is a value that can be compared directly between the three experiments studied here. With the ultimate goal of studying tissues or cell cultures, the relaxed (long-time or low frequency) material response




(a) 4% gelatin hydrogel

(b) 6% gelatin hydrogel

(c) 8% gelatin hydrogel

**Fig. 5.** Bias and random error in the normalized relaxed modulus. The relaxed modulus from a quasistatic indentation test, $G^{QS}_{\infty}$, is normalized by the relaxed modulus from the shear stress relaxation test, $G^{SR}_{\infty}$, in order to compare trends between the different concentration of gelatin hydrogel. The average values of $G^{SR}_{\infty}$ for 4, 6, and 8% gelatin hydrogel are 563 Pa, 1144 Pa, and 2340 Pa, respectively. Error bars are ±1 standard error.

may be the most relevant to the study of cell behavior, since most cellular processes that occur naturally are governed by statistical mechanics and occur over minutes or hours.

In quasistatic indentation testing, the depth of indentation relative to the size of the indenter affects the modulus estimation. Estimates of the time-dependent modulus made at small indentation depths ($\delta/R < 0.15$) result in a large scatter between the relaxed modulus measurements for each concentration of gelatin hydrogel, as shown by the large error bars in Fig. 5. The random error in the modulus at these small indentation depths comes from the uncertainty in the contact point, and from the instrumentation noise, which can be high when compared to the measured force for these small indentation depths. Dimitriadis, and coworkers found a similar trend of a large distribution in the estimated modulus values at small indentation depths during the nanoindentation of thin elastic films (Dimitriadis et al., 2002). With increased indentation depth ($\delta/R > 0.15$), the scatter in the estimated relaxed modulus is reduced when compared to scatter in the modulus estimates at smaller depths, indicating less sensitivity of the modulus predictions to the noise in the force measurement at larger indentation depths.

The bias in the estimate of modulus using Hertzian-based solutions is due to the geometric assumption that the indenter is parabolic instead of spherical. For an elastic medium, the estimated shear modulus via curve fitting decreases with increasing indentation, as seen by the trend line for the elastic FEA data in Fig. 5. While a linear trend is not necessarily expected for the bias in estimated modulus, for the range of depths considered, it seems appropriate. The viscoelastic solution for quasistatic indentation is based on the elastic Hertz solution, so a similar geometric assumption is inherent in the SLS indentation solution. As expected, the increasing indentation depths in the viscoelastic materials also cause a decreasing estimate of the relaxed modulus. The decreasing trend is clearly seen at normalized indentation depths greater than 0.15, when the random error in the measurement has decreased. The slope of the visible bias (beyond $\delta/R = 0.15$) is close to that of the elastic FEA trend line, supporting geometric dependence, and material independence of the bias.

Knowing that the estimate of the relaxed modulus is subject to random error at small indentation depths, and a bias at larger depths, the two types of error must be balanced to arrive at a valid material modulus. Here, the optimal normalized indentation depth is chosen to be $\delta/R = 0.16$ (or $\delta = 0.4$ mm). For the hydrogels in this study (shear modulus in the range from 600 Pa to 2400 Pa), an acceptable range for normalized indentation lies approximately between $\delta/R = 0.15$ and $\delta/R = 0.25$. In this range of normalized indentation depths, the bias due to the geometric assumption of the Hertzian elastic solution gives an error approximately less than 8%.

In general, the error in the estimate of modulus grows with decreasing material stiffness, and therefore decreasing gelatin hydrogel concentration. The trend of increasing error can be seen in Fig. 5(a–c) by the size of the error bars for each gelatin hydrogel concentration. For a given indentation depth during quasistatic indentation experiments, the softer hydrogels produce much smaller forces than the stiffer hydrogels. The resolution of the 1 kg load cell used in indentation experiments is 0.01 g or $10^{-4}$ N. For small indentation depth ($\delta < 0.2$ mm) on 4% gelatin hydrogel, the force resolution was 10% of the peak force. As a result, the sampling rate for the quasistatic indentation on 4% gelatin hydrogel specimens was increased to 10 samples/s to improve the reliability of curve fitting especially at small indentation depths.

The most reliable methods for estimating viscoelastic material properties generally involve uniform stress (or strain) that is held constant over time, but with biological materials an idealized test is not always possible. Of the experimental methods examined here, shear stress relaxation is the ideal choice, as it utilizes both uniform strain, and long test times to produce the best estimate of the material behavior. In addition, the pure shear application of strain eliminates the effect of the interstitial fluid on the measured properties. Thus, the time- dependent modulus describes the solid matrix behavior. The indentation load relaxation experiment is a second choice after the shear test, as it does not apply uniform strains, but does examine the material behavior over a long period of time. Indentation relaxation is best when the geometry of a material does not allow a stress relaxation test. When shorter test times are necessary, and the geometry does not permit other testing methods, the quasistatic indentation test is preferred. For example, often 3D cell cultures have small geometries, are confined to well plates, and the gel properties change with time if the test environment (e.g. temperature) is not controlled. For the 3D cell culture application, the quasistatic indentation test is advantageous over the indentation load-relaxation, and the shear stress relaxation tests.

The main limitation of the two theoretical models for indentation described in this work is the assumption that the specimen is semi-infinite in height and width. The indentation specimens were 44.5 mm in diameter and 26–27 mm high, which was nearly semi-infinite for the indentation depths considered. In the work by Dimitriadis et al. (Dimitriadis et al., 2002), a thickness correction term for spherical indentation of an elastic medium was developed. Although the specimen height is very close to being semi-infinite according to Dimitriadis, the force response is corrected in the analysis of the quasistatic indentation data to get a more accurate estimate of modulus. In another study using a punch indenter, a minimum ratio of indenter to container diameter of 1:10 was established to eliminate edge effects (Mooney et al., 2006). The ratio of 5 mm-diameter indenter to the 44.5 mm-diameter container is close to that limit, but the indenter in this study is spherical in shape. With spherical indentation, a smaller contact area occurs at indentation depths less than the indenter radius, so the edges of the specimen should not affect the experimentally measured force. To study specimens that are significantly smaller than those in this work, for example hydrogels for cell cultures, geometric corrections would be necessary. The Dimitriadis correction accounts for a certain range of sample heights, and another correction by Chadwick (Chadwick, 2002) can be applied for very thin specimens. However, to the best of our knowledge no correction for indentation data has been found to account for radial boundaries when the indenter, and sample are of the same order of magnitude in size. To utilize quasistatic indentation tests to measure the modulus of small cell culture hydrogels, further investigation into geometry corrections is necessary.

## 5. Conclusions

The geometry of loading applied to a hydrogel has a dramatic effect on the response of the two phases within the hydrogel. By applying a shear load to a specimen, the solid matrix response can be isolated, since no fluid flow is induced. Other types of loading, including indentation, probe a combined response of both phases to varying degrees. A clear consequence of the build-up of interstitial fluid pressure is a higher instantaneous modulus value measured for indentation tests over that of shear tests. The instantaneous modulus is therefore not a good measure of material behavior since the values are test dependent. A better quantity to assess the true behavior of hydrogels is the relaxed modulus. The relaxed modulus from a load relaxation test is shown to agree within 17% of the relaxed modulus from a shear stress relaxation test. Relaxed modulus measurements from quasistatic indentation tests are found to be consistently higher than those from shear relaxation experiments. The relaxed modulus is only dependent on the elastic behavior of the matrix, not on the rate or geometry of applied load.

A reliable estimate of relaxed modulus from quasistatic indentation requires the viscoelastic analysis of small indentation depths relative to the radius of the indenter. Beyond approximately 15% of the indenter radius, increasing indentation depths cause a growing bias in the modulus estimate. At the same time, noise in the measured force and uncertainty in contact lead to larger errors in the modulus estimate at small depths. Therefore, to avoid significant random errors and minimize the error due to the bias, an appropriate indentation depth must be chosen for analysis. In this study for gelatin hydrogel ranging from 600 Pa to 2400 Pa in stiffness (relaxed modulus), and a load resolution of 0.01 g, the acceptable normalized indentation depth was $\delta/R = 0.15 - 0.25$.

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