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Predicting bulk mechanical properties of cellularized collagen gels using multiphoton microscopy

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Abstract

Cellularized collagen gels are a common model in tissue engineering, but the relationship between the microstructure and bulk mechanical properties is only partially understood. Multiphoton microscopy (MPM) is an ideal non-invasive tool to examine collagen microstructure, cellularity and crosslink content in these gels. In order to identify robust image parameters that characterize microstructural determinants of the bulk elastic modulus, we performed serial MPM and mechanical tests on acellular and cellularized (normal human lung fibroblasts) collagen hydrogels, before and after glutaraldehyde crosslinking. Following gel contraction over sixteen days, cellularized collagen gel content approached that of native connective tissues (~200 mg/ml). Young's modulus (E) measurements from acellular collagen gels (range 0.5-12 kPa) exhibited a power-law concentration dependence (range 3-9 mg/ml) with exponents from 2.1-2.2, similar to other semiflexible biopolymer networks such as fibrin and actin. In contrast, cellularized collagen gel stiffness (range 0.5-27 kPa) produced concentration-dependent exponents of 0.7 uncrosslinked and 1.1 crosslinked (range ~5-200 mg/ml). The variation in E of cellularized collagen hydrogels can be explained by a power-law dependence on robust image parameters: either the second harmonic generation (SHG) and two-photon fluorescence (TPF) (matrix component) skewness (R^2 = 0.75, exponents of −1.0 and −0.6, respectively); or alternately the SHG and TPF (matrix component) speckle contrast (R^2 = 0.83, exponents of −0.7 and −1.8, respectively). Image parameters based on the cellular component of TPF signal did not improve the fits. The concentration dependence of E suggests enhanced stress relaxation in cellularized versus acellular gels. SHG and TPF image skewness and speckle contrast from cellularized collagen gels can predict E by capturing mechanically relevant information on collagen fiber, cell and crosslink density.

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INTRODUCTION

Type I collagen is an important stress-bearing component of extracellular matrix and connective tissue. The organization and crosslinking of collagen monomers, fibrils, fibers and fiber networks may largely determine bulk mechanical properties of collagen-rich connective tissues. The mechanical properties of the extracellular matrix profoundly influence cell behavior, including proliferation [1], morphology [2], migration [3], and differentiation [4]. Reciprocally, cells can influence their micromechanical environment through matrix deposition [5], degradation [6], and crosslinking [7]. This dynamic interaction between cells and matrix plays an important, but only partially understood, role in wound healing, fibrosis, tumor growth, cancer metastasis, and atherosclerosis.

Previous research efforts have sought to understand and control the dynamic interactions between collagen and cells in order to manipulate cell behavior and develop tissue-engineered constructs *in vitro* with similar mechanical properties to healthy native tissue. The fibroblast-seeded collagen gel has served as a useful model to assess fibroblast response to biomechanical and biochemical stimuli [8-11]. A particularly important feature is the ability of the fibroblast to contract collagen gels, and thus concentrate collagen to levels typically found in vivo (> 100 mg/ml) [12]. Multiphoton microscopy (MPM) can image cellularized collagen gels using endogenous second harmonic generation (SHG) and two-photon fluorescence (TPF) to provide unique information about collagen content and microstructure [13], and endogenous intracellular and matrix crosslink fluorophores, respectively [14]. We have previously assessed collagen microstructure using SHG signal imaging and demonstrated the potential to characterize bulk mechanical properties in both acellular gels (4 mg/ml collagen content) [15, 16] and native tissue (tracheal mucosa of the rabbit) [17]. The bulk stiffness of cellularized gels are likely to be affected by the collagen microstructure, cell activity, and the presence of crosslinks in the extracellular matrix. It is hypothesized that robust image parameters may be derived from SHG and TPF signals which directly relate to these determinants of cellularized gel mechanics.

The objectives of this study were two-fold: 1) to determine the concentration-dependent Young's moduli of cellularized gels over a range spanning *in vivo* (10-200 mg/ml) collagen concentrations; and 2) determine if MPM image parameters can predict Young's modulus. We found power-law dependences for the Young's modulus on collagen concentration, with a smaller power for cellularized gels (relative to acellular). The skewness and speckle contrast of SHG signal and TPF signal relate to collagen concentration by an inverse power-law, corroborated by simulated images of the SHG signal possessing a similar signal pattern (e.g., with the appearance of a random fiber network). The Young's moduli of cellularized gels correlates with the SHG and TPF skewness and speckle contrast in a power-law relationship, determined by multiple linear regression on log-transformed data. Our results suggest that cellularized gels exhibit significant stress-relaxation, and that MPM can be used to predict bulk mechanical properties of cellularized collagen gels non-invasively over a wide range of collagen concentrations, including those found *in vivo*.

MATERIALS AND METHODS

Acellular collagen hydrogels

Acellular collagen hydrogels were prepared as previously described at pH 6.5, creating coarse-structured gels with large fiber diameters and large pores [16]. Final gel preparations contained 0.9 ml of 3-9 mg/ml collagen, and were allowed to polymerize at room temperature for 24 hours before mechanical testing and imaging. For mechanical (indentation) testing, the samples remained in the original polymerization chambers (12-well glass bottom plate, MatTek Corporation, Ashland, MA) thus avoiding irreversible gel

deformation. After glutaraldehyde (GTA) crosslinking (room temperature, 4% GTA/PBS, 24 hours) and extensive rinses with PBS to remove unreacted GTA, the gels were again tested via indentation.

Cellularized collagen gels

Cellularized collagen gels were prepared similarly to acellular gels, except after all other components were mixed on ice and pH was adjusted to 6.5 with a small amount of sodium hydroxide, 50,000 normal human lung fibroblasts (NHLFs, passages 3-7, Lonza, Basel, Switzerland) were added per ml of a 4 mg/ml collagen solution. The NHLFs had undergone monolayer culture to 70-80% confluency under standard tissue culture conditions as previously described [18]. 0.5 ml of the collagen-NHLF solution was pipetted into wells of two 24-well plates (Corning) and allowed to polymerize at room temperature (24 °C) for one hour, creating 16 tissue contructs. After one hour, excess DMEM was added and changed several times during the first day of culture to ensure equilibration to pH 7.4. The constructs were cultured in DMEM overnight, and then released from the wells using a sterile spatula, placed in 13.5 cm-diameter Petri dishes, and covered with DMEM supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO). These floating gels were cultured in standard conditions for sixteen days, during which time gels were periodically removed from the Petri dishes using a spoon-shaped sterile spatula for imaging and mechanical testing.

In a second experiment, cellularized gels were prepared identically except the polymerization occurred at pH 9.5, which produces a collagen network with more, smaller diameter fibers [16]. These gels were cultured for 12 days in identical conditions, except for some gels DMEM media plus 10% FBS was supplemented with additional soluble factors: 2 ng/ml TGF-β2, 35 pg/ml PGE2, or 10 μM GM6001. TGF-β2 has been shown to affect alpha-smooth muscle actin [19] and tenascin expression, as well as SHG signal from a lung fibroblast-seeded collagen gel construct [8], and has been shown to enhance lung fibroblast proliferation and matrix synthesis [20]. In contrast, PGE2 has negative effects on lung fibroblast proliferation and matrix synthesis [21-23]. GM6001 is an inhibitor of MMP-1,2,3,8, and 9, and is known to inhibit lung fibroblast mediated collagen gel contraction [24].

In a third experiment, cellularized gels were prepared identically to the time-course experiment except that some gels were polymerized with 25 μg/ml DQ collagen (type I from bovine skin, fluorescein conjugate, Invitrogen, Carlsbad, CA). After one week of floating culture, as described above, the gels were imaged with a confocal fluorescence feature of the Zeiss LSM 510Meta, with excitation wavelength of 488 nm and emission filter of 500-550 nm.

GTA crosslinking of some of the cellularized gels occurred after initial imaging and mechanical testing. Gels were incubated at room temperature in 4% GTA/PBS for 24 hours, and then washed for several hours with 3-5 × 50 ml PBS. Estimated collagen concentration (c, mg/ml) was calculated by dividing initial collagen mass in the gels (2 mg) by gel volume calculated from caliper measurements of gel height (h, mm), and diameter (d, mm), according to the following relation: $c = 2/\left(\frac{\pi d^2 h}{4}\right)/1000$.

Multiphoton microscopy

A LSM 510 Meta multiphoton microscope (Zeiss, Jena, Germany) was used for all imaging experiments. All SHG and TPF signals were collected in the epi-configuration with an Achroplan 40×/0.8 NA water-immersion objective (Zeiss). Each 12-bit image contained

512×512 pixels, and each pixel was ~440×440 nm. Pixel sampling rate was 625 kHz; pixel dwell time was 1.6 μs. Cellularized collagen gels were placed on 22×50 mm No. 1 coverslips (170 μm thickness) for imaging. Multiphoton signals were produced by a circularly-polarized Chameleon laser tuned to 780 nm. Power before the objective was ~102 mW, and at the sample focus ~92 mW. The SHG signal was collected using the instrument's Meta detector, with wavelength cutoff points set at 383 nm and 405 nm, whereas the TPF signal was collected with an infrared-blocking 500-550 nm bandpass filter. At this excitation (780 nm), TPF emission at 500-550 is restricted mainly to: glutaraldehyde [15] and pyridinium-type crosslinks [25, 26] in collagen; and NADH, FAD, riboflavin and other vitamin-derived fluorophores in cells [27, 28], whereas the SHG signal is specific for collagen. SHG images with signal averaged over 16 frames were collected within the first 10 μm from the tissue surface in the region of highest SHG signal (before depth-dependent decay) to provide the best assessment of collagen microstructure. Five single image frames were collected per tissue (with lateral separation of 1.1 mm).

Image analysis

All image analysis was performed with ImageJ (Wayne Rasband, NIH, Bethesda, MD). Noise-subtraction was performed on all images in the following manner: 5-10 void regions from 5-10 SHG and TPF images were traced and the mean and standard deviation of pixel intensities were quantified, and found to be consistent among the image sets. The noise threshold was set at the mean plus three times the standard deviation from these void regions. SHG and TPF images were smoothed, and TPF images were further despeckled (since they contained a higher noise floor than SHG images) using corresponding ImageJ processing tools.

Matrix and cell-derived TPF signal from images of uncrosslinked and GTA-crosslinked cellularized gels were separated by using a mask defined by a threshold. The threshold was easily determined, as the cell and matrix contributions to the TPF signal were disparate. In uncrosslinked gels, the image mean intensity was used as the threshold separating cell-derived signal (brighter than mean) and matrix-derived signal (less intense than the mean). In GTA crosslinked gels, the mask threshold was visually determined, at a level providing clear separation between cell-associated (brighter) and matrix-associated (less intense) TPF signals. Masked pixels were assigned values of zero, while unmasked pixels retained their values.

The mean signal intensity, signal image area fraction, and skewness of the pixel histogram were calculated in ImageJ. Skewness is defined by the computational formula:

$$skew = \left\{ n\Sigma X_i^3 - 3\Sigma X_i \Sigma X_i^2 + 2\left(\Sigma \frac{X_i)^3}{n}\right\} \right/ \left\{ (n-1)(n-2)\, s^3 \right\}, \quad (1)$$

in which n is the number of pixels, X_i the intensity of pixel i, and s is the standard deviation. In practical terms, skewness describes the symmetry of the pixel histogram. A right-weighted (toward more intense signal) pixel distribution produces a positive skew, whereas a left-weighted pixel distribution produces a negative skew. An equally-weighted Gaussian distribution has a skewness of zero. To show the effects on skewness of images with sparse and dense SHG and TPF signal patterns, representative images, pixel histograms and skewness values are shown (see Supplemental Data, Figure S1).

The speckle contrast was calculated using a Matlab (version 7.4.0, Natick, MA) routine in which the speckle contrast value of each central pixel was the standard deviation divided by the mean of the pixel region. A pixel region of 2401 (49×49 pixels or 1055 μm^2) was

empirically determined to produce optimal variation in the speckle contrast parameter. The mean speckle contrast of each image was calculated from the pixel values.

SHG image texture simulation

The textural features of SHG images from collagen gels were simulated using a Matlab routine. The constructed images were meant to simulate images of a randomly-oriented collagen fiber network, in order to determine the relationship between robust, gain-independent image parameters and fiber number density. Collagen fiber segments within the MPM image plane were simulated as two-dimensional elliptical Gaussian functions. The length and width of the fiber segments were distributed normally, with mean and standard deviation determined from $n = 50$ line-segment measurements from SHG images of real collagen gels. Gaussian peak intensity was directly related to the length and width, so that larger fiber segments possessed proportionally more intense signal. Furthermore, the fibers edges were defined where the signal fell to $1/e^2$ times the maximum intensity of each Gaussian function. The simulated fiber areas and intensities were determined so that SHG images from cellularized gels at day 0 of culture would have similar mean intensity and signal area fraction to the simulated image of corresponding fiber number density. Fiber orientations were distributed uniformly through 360°, and positioned at random locations within a 512×512 pixel matrix. Intersecting fibers were allowed to superimpose, creating a linear relationship between mean image intensity and fiber number density, as well as a reasonable approximation to the texture of SHG images from cellularized gels containing 4-200 mg/ml collagen.

In order to relate simulated images to SHG images from cellularized gels, simulated images were assigned collagen concentrations equal to the number of Gaussian ‘fibers’ in the simulation times a scaling factor, with units of mg/ml/fiber number. The scaling factor was determined by counting the number of fiber segments in SHG images of cellularized gels at day 0 of culture. These SHG images were thresholded at the noise-cutoff, despeckled as before to remove remaining noise, and a binary opening algorithm was performed in ImageJ to isolate adjacent fibers. Then, particle analysis was performed in ImageJ to count particles larger than 1 μm^2. It was determined that the day 0 gel images contained 178±34 fibers (μ ±s.d.), with an average gel concentration of 6.4 mg/ml. Therefore the scaling factor used for simulated images containing 200-5000 fiber segments was 0.0356 mg/ml/fiber. A second scaling factor was used to ensure similar brightness of individual Gaussian ellipses to collagen fibers SHG signal, and overall simulated image intensity roughly equal to that from SHG images of day 0 cellularized gels.

Mechanical testing

After multiphoton imaging, NHLF-seeded collagen hydrogels were mechanically tested using a Synergie 100 testing system (MTS Systems Corporation, Eden Prarie, MN). The hydrogels were placed on a 50 mm diameter platen covered with 600-grade ultrafine waterproof sandpaper (3M), attached using double-sided tape, to prevent slipping. Before testing, gel height, h, and diameter, d, were measured using a caliper, ranging from 1-2 mm (h) and 4-16 mm (d). Each gel was compressed to 10% of the gel height with specially-constructed 0.65 mm radius (a), nonporous, cylindrical aluminum platens. Indentation occurred at a rate of 0.05 mm/s and the resulting force was measured using a 10 N load cell, while strain was recorded based on motion of the actuator, and defined as percentage of the original sample height. Due to the small ratio of a/h, stresses beneath the platen are assumed to be nonuniform, with compressive stresses at the platen center but tensile stresses at the edges, as previously described [29]. All E were calculated assuming the gels behave as thin elastic layers bonded to a rigid bottom surface [29], directly related to the ratio a/h and the

linear slope of the low strain stress-strain curve. Cellularized gels typically displayed linear stress-strain relationships through 10% strain (see Supplemental Data, Figure S2).

Rheology was performed on glutaraldehyde crosslinked acellular gels (diameter 16 mm) only using an AR-G2 rheometer (TA Instruments, New Castle, DE) with a 20 mm diameter parallel plate configuration and a 0.7 mm gap. The gels were compressed slightly (0.1 N), and tested at an oscillation frequency of 0.01 (frequency independent), to confirm linearity of E measurements correlated versus G' (slope $m = 2.25$, $R^2 = 0.90$, Supplemental Data, Figure S2). This correlation is consistent with linear elasticity theory. The ratio of $E/G' = 2.25$ corresponds to a Poisson ratio of ~0.13 which is consistent with previous reports in acellular collagen gels [30]. Uncrosslinked gels could not be tested serially in indentation and shear due to gel fragility during transportation between testing devices.

Sircol assay

After imaging and mechanical testing, collagen content of cellularized gels was assessed using the Sircol assay (Accurate Chemical and Scientific Corporation, Westbury, NY) following a manufacturer-defined protocol for assessing pepsin-soluble collagen. Collagen gels were mixed with 5 mg/ml pepsin from porcine stomach mucosa (Sigma) dissolved in 0.5 M acetic acid, and digested for 24 hours at 4°C.

Statistics

Regressions and t-tests were performed in Excel (Microsoft, Redmond, WA). Multiple linear regression was performed on log-transformed data using SigmaStat (Systat Software, San Jose, CA).

RESULTS

Free-floating cellularized gels contract without losing collagen content

During the 16 day course of free-floating culture, the fibroblast-embedded collagen gels contracted from 320±40 μl on day 0 to 14±4 μl on day 15 (Figure 1). At polymerization, the collagen content of the gels was 2 mg (the gels were formed from 500 μl of a 4 mg/ml collagen solution), but the gel volume decreased immediately upon release of the anchored gels into free-floating culture. Despite the large decrease in gel volume during the culture period, the amount of pepsin-soluble collagen within the gels did not change significantly (160±60 μg on day 0 to 150±40 μg on day 15; t-test, $p = 0.46$). Assuming that the collagen content of the gels remained constant, the estimated collagen concentration in the gels can be shown to increase, from 6±1 mg/ml on day 1 to 160±50 mg/ml on day 15 (Figure 1). These data indicate that the free-floating cellularized gels tested span a range of collagen concentrations that include sparse and dense connective tissues.

Multiphoton images reveal changes in microstructure and cellularity during contraction

In order to understand the microstructure-mechanics relationships of cellularized gels during cell-mediated matrix contraction, collagen structure was assessed from SHG images, while cellularity was assessed from TPF images. SHG (Figure 2A-C) and TPF (Figure 2D-F) images from gels show clear increases in fiber number density and cellularity, respectively, during gel contraction. Textural simulations of SHG images using a randomly-oriented collagen network (Figure 2G-I) contain normally-distributed fiber diameters (3.7±2.1 μm) and lengths (23.6±8.2 μm) based on measurements from 50 fibers. The simulations have a similar appearance to the SHG images of cellularized gels. The Gaussian signal intensity profiles of simulated fibers fit closely the SHG intensity profiles from real collagen fiber segments (see Supplemental Data, Figure S3). Particle analysis of SHG images of cellularized gels on day 0 of culture revealed 178±34 fiber segments (mean ± s.d.). Based on

this measurement, simulation images containing 200-5000 fiber segments were assigned concentration values of 7-180 mg/ml. Visual comparison of SHG images and simulations shows a rough parity of texture and collagen fiber density for similar collagen concentrations (Fig. 2A-C versus Fig. 2G-I).

Besides fiber morphology and number density, structural characteristics that may affect bulk mechanics were immediately apparent from SHG and TPF images. Cells exclude the collagen network creating void regions in the matrix (Figure 2C, arrow), and collagen fibers are packed to greater density with smaller pores as the gels contract (Figure 2C, arrowhead).

Multiphoton image parameters correlate with collagen content

Several signal and image parameters changed during the sixteen day *in vitro* culture period. Trends from the textural simulations suggest that the functional form of SHG image parameters is largely due to changes in collagen fiber concentrations (Figure 3A,C,E,G, SHG versus simulation). For example, SHG signal intensity is a linear function of collagen concentration (Figure 3A, solid line, $m = 6.8$ a.u./mg/ml, $R^2 = 0.78$), which is corroborated by the linear relationship between image intensity and concentration in the simulated images (Figure 3B, dashed line, $m = 6.9$ a.u./mg/ml, $R^2 = 1.0$). In contrast, matrix TPF image fraction also increases linearly with collagen concentration but the slope is ~7-fold greater for GTA-crosslinked gels ($R^2 = 0.95$) than for uncrosslinked gels ($R^2 = 0.84$). The cell-derived TPF signal increases with collagen concentration, but with a weaker linear relationship (Figure 3B, open circles, $R^2 = 0.53$).

SHG signal area fraction increases quickly to a plateau near 100% by ~60 mg/ml (Figure 3C, solid markers), and depends upon collagen concentration in a logarithmic fashion ($R^2 = 0.66$ for the linear fit of ln(1-*area fraction*) versus *concentration*). This relationship is confirmed by the simulated signal area fraction (Figure 3C, open markers), which reaches a plateau near 100% by ~100 mg/ml ($R^2 = 0.99$ for the linear fit of ln(1-*area fraction*) versus *concentration*). Matrix-derived TPF signal from GTA crosslinked gels occupies nearly the entire image area (Figure 3D, filled diamonds), and matrix-derived TPF signal image fraction from uncrosslinked gels increases in logarithmic form (Figure 3D, open diamonds). In contrast, cell-derived TPF signal area fraction increases linearly with collagen concentration (Figure 3D, open circles).

The signal intensity is gain-dependent and the area fraction possesses a limited range, decreasing the utility of these parameters for robust characterization of mechanically-important microstructural features of cellularized gels. However, image parameters such as skewness and speckle contrast are gain-independent and are thus more robust parameters to potentially characterize structural features that impact bulk mechanics. In contrast to the linear intensity and log area fraction dependences, we find that the skewness of the image pixel histograms relates to collagen concentration in SHG images (Figure 3E) and TPF images (Figure 3F) with a power-law dependence (SHG, exponent $n = -0.6$, $R^2 = 0.90$; simulation, $n = -0.5$, $R^2 = 0.99$; matrix + GTA TPF, exponent $n = -0.8$, $R^2 = 0.90$; matrix TPF, exponent $n = -0.4$, $R^2 = 0.47$; cell TPF, exponent $n = -0.5$, $R^2 = 0.70$). The speckle contrast of SHG images (Figure 3G, solid markers) and texture simulation images (Figure 3G, open markers) scale similarly to skewness (exponent $n = -0.6$, $R^2 = 0.90$ for SHG; $n = -1.0$, $R^2 = 0.91$ for the simulation). The speckle contrast of matrix-derived TPF signals similarly decrease with increased matrix density (Figure 3H, matrix + GTA TPF, $n = -0.3$, $R^2 = 0.93$, filled diamonds; matrix TPF, $n = -0.2$, $R^2 = 0.51$, open diamonds). In contrast, the speckle contrast of cell-derived TPF signal increases linearly with collagen concentration (Figure 3H, $R^2 = 0.89$, open circles).

E depends upon collagen crosslinking, concentration and fibroblast activity

The power-law dependence of the elastic modulus on collagen concentration has been demonstrated for crosslinked and uncrosslinked acellular collagen gels containing less than 10 mg/ml collagen content [31-34], but not for cellularized gels beyond 10 mg/ml. E from free-floating, contracting cellularized gels was compared to that of acellular collagen gels between 3-9 mg/ml, before and after GTA crosslinking of the collagen network.

The concentration dependence of E measured from uncrosslinked and GTA crosslinked acellular and cellularized gels is positive in all cases studied (Figure 4A). Crosslinked gels (Figure 4, open markers) showed increased E at every collagen concentration compared to uncrosslinked gels (Fig. 4, filled markers). Interestingly, acellular gels were stiffer on average at 7.5 and 9 mg/ml than cellularized gels at similar concentrations. A power law concentration-dependence of E produced an exponent of 2.1 uncrosslinked acellular gels (R^2 = 0.89), and 2.2 for crosslinked acellular gels (R^2 = 0.97). In contrast the exponents for cellularized gels were smaller: ~0.7 for uncrosslinked gels (R^2 = 0.82), and ~1.1 for crosslinked cellularized gels (R^2 = 0.89).

In a parallel experiment, cellularized gels polymerized with and without DQ collagen were cultured for one week and the fluorescence signal from DQ collagen (ex.488, em. 500-550) was imaged. While the control shows little fluorescence signal (Figure 4B, lower panel), the cellularized gels containing DQ collagen, which has been shown to fluoresce when cleaved from collagen fibrils by MMP activity, showed an enhanced (~7-fold) fluorescence signal (Figure 4B, upper panel).

In *in vitro* culture, the effects of cell activity, MMP activity, and matrix secretion on bulk mechanics may depend upon culture time, cell concentration, and the presence of soluble factors. Without explicitly measuring these independent factors, it is nevertheless desired to see whether cell behavior may affect SHG and TPF image parameters. In a parallel experiment cellularized gels were incubated for 12 days in the presence of TGF-β2, GM6001, and PGE2 contracted to 8±1%, 76 ±6%, and 87±7% of their original volume (mean±s.d.). Normalized SHG image intensity varied linearly with normalized collagen concentration from these gels (Figure 4C, m = 1.07, R^2 = 0.94), similarly to the linear relationship from cellularized gels cultured for 0-15 days (m = 0.84, R^2 = 0.76, derived from Figure 3A, filled markers). The linearity of SHG image intensity with collagen concentration would be expected if there were no drastic alteration in the collagen network other than an increase in collagen fiber concentration. Additionally, normalized E versus collagen concentration of the soluble-factor exposed cellularized gels possesses a similar power-law dependence as the cellularized gels collected between days 0-15 (soluble factor-treated gels, Figure 4D, m = 0.83, R^2 = 0.92; time-course gels, Figure 4A, m = 0.73, R^2 = 0.82). It should be noted that the absolute magnitude of the SHG image intensity and E from the soluble factor-treated gels was different than the time-course gels, owing to the higher polymerization pH conditions of the soluble-factor-treated gels.

Skewness and speckle contrast of SHG and TPF signals predict *E* of cellularized gels

Multiphoton image parameters that serve as robust predictors of cellularized gel E must be gain-independent and sensitive to changes in collagen concentration, network microstructure, crosslinking, and changes in cellularity. We reasoned that the skewness and speckle contrast of SHG and TPF signals may possess the desired gain-independence and structural sensitivity. To determine the strength of these image parameters in predicting E we performed multiple regression of log-transformed E values on log-transformed SHG, cell-derived TPF, and matrix-derived TPF skewness (*skew*) and speckle contrast (*SC*) parameters from crosslinked and uncrosslinked cellularized gels (Figure 5). The cell-derived

TPF parameters were found to covary with SHG parameters, being unable to explain additional variation in *E*. We found that the relationship $E \sim skew_{SHG}^{-1.0} skew_{TPF,matrix}^{-0.6}$ provided a best fit which explained the most variation in *E* (Figure 5A, $R^2 = 0.80$). Similarly, the relationship $E \sim SC_{SHG}^{-0.7} SC_{TPF,matrix}^{-1.8}$ provided a best fit which explained the most variation in *E* (Figure 5B, $R^2 = 0.83$). Observation of the linear and log-log plots of the multiple regressions shows that the nonlinear model using the skewness parameters tends to overestimate *E* of gels with sparse matrix (days 0-3, Figure 5A, inset).

DISCUSSION

Understanding the dynamic relationship between microstructure and bulk mechanics in developing engineered tissues is necessary to assess properties critical for successful development and integration upon implantation. SHG and TPF signals from cellularized collagen gels have been used to track structural changes and cellularity during tissue culture [14-16, 18, 35, 36]. The ability of the SHG signal to distinguish collagen microstructure and the TPF signal to distinguish cross-links and cells suggests bulk mechanics of cellularized collagen gels may be inferred from MPM images, but few studies have attempted to correlate bulk tissue mechanics and MPM image parameters in cellularized tissues. One challenge to constructing image parameter correlations with bulk tissue properties is to identify appropriate image parameters which are sensitive to microstructural determinants of mechanics, yet independent of instrument gain and other microscope parameters. In this study we identify the SHG and TPF skewness and speckle contrast as gain-independent parameters which have predictive power for *E* (Figure 5).

We also characterized a power-law dependence of *E* on collagen concentration with a lesser power for cellularized (0.7-1.1) than acellular (2.1) gels, smaller *E* for uncrosslinked than crosslinked gels; and smaller *E* for cellularized gels than acellular gels at concentratons of 6-9 mg/ml. The power law exponents were 2.1 and 2.2 for acellular uncrosslinked and crosslinked gels, respectively; but only 0.7 and 1.1 for cellularized uncrosslinked and crosslinked gels (Figure 4). Our indentation testing results in acellular collagen gels are in closer agreement with semiflexible network (exponent 1.4-2.2) [33, 34, 37, 38], or foam lattice theoretical scaling (exponent 2) [39, 40] for networks unable to relax stress during the mechanical testing period, than with a composite model (exponent 1) [12] or rigid rod (exponent 1-2) model of the gels [41, 42]. Other researchers have reported exponents of 2.1 for fibrin [32], 2.1-2.5 for actin [32, 43], and 2.1-2.8 for acellular collagen gels of 0.5-5 mg/ml [31, 44].

Concurrent processes of matrix synthesis, degradation, and remodeling may explain the generally-reduced *E* of cellularized gels. SHG images show evidence of matrix defects near cells (Figure 2C); and TPF images show increasing fibroblast prevalence with culture time (Figure 2F and 3D, open circles) which are associated with collagenase activity in collagen gels (Figure 4B). It may not be concluded that cellularized gel *E* is wholly dependent on collagen concentration, despite the strong correlation of these two parameters. Time-dependent cell activity (collagenases, proliferation, and collagen remodeling) might lead to a reduction in *E*. Yet, for the experiments described, cell-associated TPF parameters did not improve the multiple regression of *E* on image parameters, which were best using SHG and matrix-derived TPF skewness and speckle contrast parameters. Also, cellularized gels incubated for equal time (12 days) but with soluble factors known to enhance matrix remodeling (TGF-β2), inhibit matrix remodeling (PGE2), and inhibit MMPs (GM6001) were shown to possess similarly linear SHG image intensity and weak *E* dependencies on collagen concentration (Figure 4C,D). Finally, Sircol assay measurements of collagen content from cellularized gels reveal minimal change in total collagen mass during 16 days

floating culture. We conclude that fibroblast-mediated matrix remodeling and enzymatic degradation may still play a role in determining cellularized tissue mechanics with the fibroblast, collagen concentrations, and soluble factors studied, but that collagen fiber density and crosslink content primarily determine indentation mechanics of the cellularized gels.

In cellularized collagen gels, the SHG signal arises from the noncentrosymmetric, polarizable molecular structure of collagen fibrils, whereas the TPF signal originates from FAD, NADH, and flavins, as well as collagen crosslinks [14, 25-27, 45]. Collagen second harmonic signal, cell autofluorescence, and crosslink autofluorescence yield information about the distribution and structure of these tissue components. Specifically, high skewness in the image intensity histogram indicates a non-normal, right-tailed distribution of pixel intensities, consistent with mostly dim collagen fibers within the SHG and TPF images, and very few bright fibers (see Supplemental Data, Figure S1). A low skewness indicates a symmetrical distribution, with more equal weight in left (dim fibers) and right (bright fibers) tails. As images become more populated a more symmetrical distribution of pixel intensities is observed, reflecting the texture of a dense collagen network with unimodal morphological distribution. The speckle contrast is highest for images with high local variability, such as images of sparse cells or collagen fibers surrounded by void regions. Speckle contrast is reduced in images with less contrast, such as a uniformly filled image of a dense collagen network. Skewness and speckle contrast image parameters, while insensitive to instrument gain, are sensitive to small changes in the amount and distribution of signal-generating material, and are therefore sensitive to the same microstructural parameters of cellularized gels which determine bulk mechanics.

Finally, E has a stronger inverse correlation with SHG skewness compared to matrix-derived TPF skewness (Figure 5A, exponent of −1.0 compared to −0.6). Conversely E is more sensitive to changes in TPF matrix-derived speckle contrast than SHG speckle contrast (Figure 5B exponent of −0.7 compared to −1.8). The matrix-derived TPF speckle contrast reflects the spatial organization of collagen fibers and crosslinks. Speckle contrast, as an indicator of local standard deviation, may be more sensitive to changes in signal spatial organization than skewness. It is important to note that the speckle contrast parameters provided a better fit to the cellularized gel mechanical data, although the model overestimated E from day 15 uncrosslinked gels (Figure 5B). It is possible that cell-based matrix degradation was strongest for these cells, and that the nonlinear model using SHG and matrix-derived TPF parameters is not sensitive to collagenase degradation of the collagen network. The influence of cells and crosslinks on E is also suggested by the reduced E in cellularized versus acellular gels, and increased E in crosslinked gels (Figure 4). These data as well as previous studies showing a large effect of both cells [6, 7] and crosslinking [7, 15, 46] on collagen gel stiffness corroborate the need for robust image parameters which directly relate to structural, cellular, and crosslink content patterns in cellularized gels, in order to predict gel bulk mechanics.

CONCLUSIONS

In summary, microstructural parameters change systematically during cell-mediated gel contraction: pores become smaller, fiber bundles become larger, and cells occupy holes in a dense three-dimensional collagen network. Cellularized gels tend to be weaker than acellular gels at similar concentrations, and exhibit weaker concentration-dependence consistent with increased relaxation of tension within strained network elements. For cellularized collagen gels, SHG and TPF image parameters such as skewness and speckle contrast change with collagen fiber, crosslink and cell densities in a predictable manner. Hence, cellularized gel E

can largely be predicted by variation in SHG and matrix-derived TPF speckle contrast, which depend upon collagen and crosslink spatial patterns.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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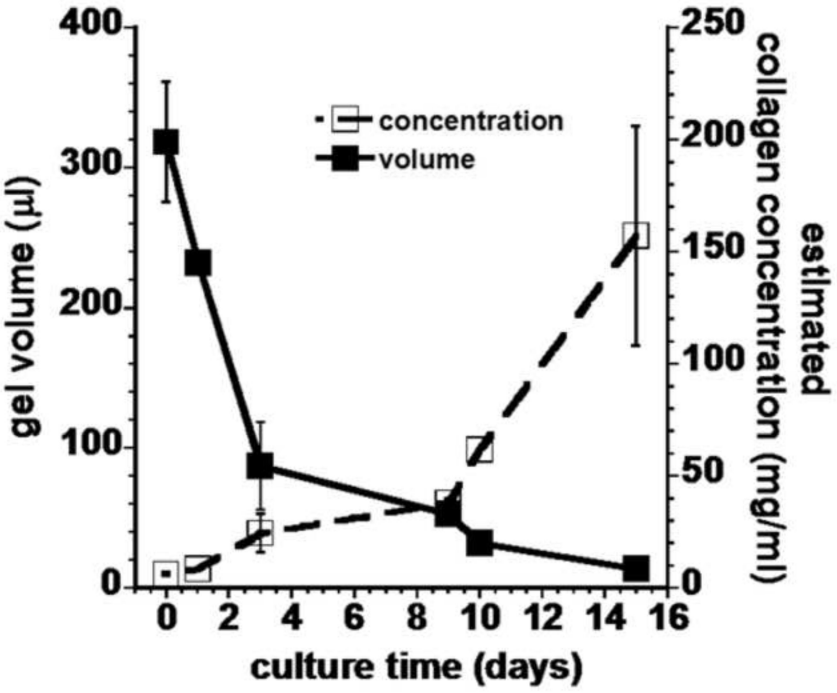

Figure 1.
Gel volume (□) and estimated concentration (□) for $n = 16$ cellularized gels collected during sixteen days floating culture.

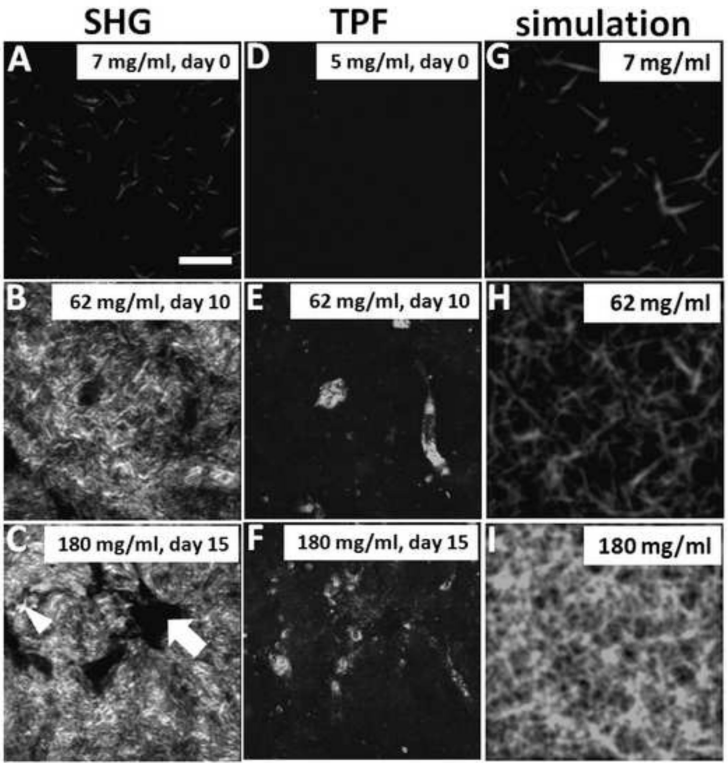

Figure 2.

Images of SHG signal (A-C), TPF signal (D-F) from cellularized gels, and simulated signal from a randomly-oriented fiber network of similar texture to the SHG images (G-I). The SHG and TPF images are from cellularized gels at three stages of contraction during floating culture, and the simulated images are of roughly corresponding collagen fiber density. Estimated collagen concentration for each gel/image is indicated, and the days of culture. Bar represents 50 μm.

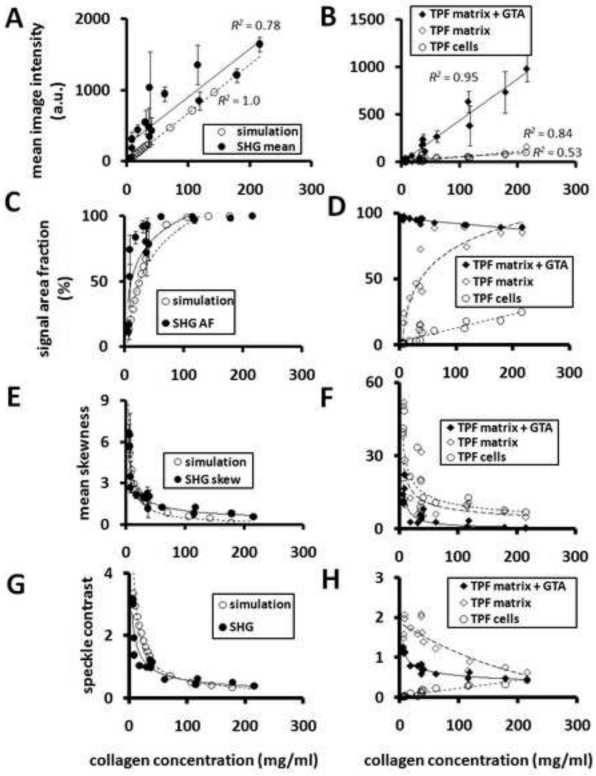

Figure 3.

Mean image intensity versus collagen concentration for SHG and texture simulation images (A), and for TPF signal components (B). Signal image area fraction versus collagen concentration for SHG and texture simulation images (C) and for TPF signal components (D). Mean image skewness versus collagen concentration for SHG and texture simulation images (E) and for TPF signal components (F). Mean speckle contrast versus collagen concentration for SHG and texture simulation images (G) and for TPF signal components (H). SHG values are filled circles; simulation values are open circles; cell-derived TPF (uncrosslinked gels) are open circles; matrix-derived TPF (uncrosslinked gels) are open diamonds); and matrix-derived TPF (crosslinked gels) are filled diamonds. Data points from SHG and TPF data represent an average of five images per gel. R^2 coefficients for the linear best fits (A,B,D), logarithmic fits (C,D) and power-law fits (E-H) are given in the text.

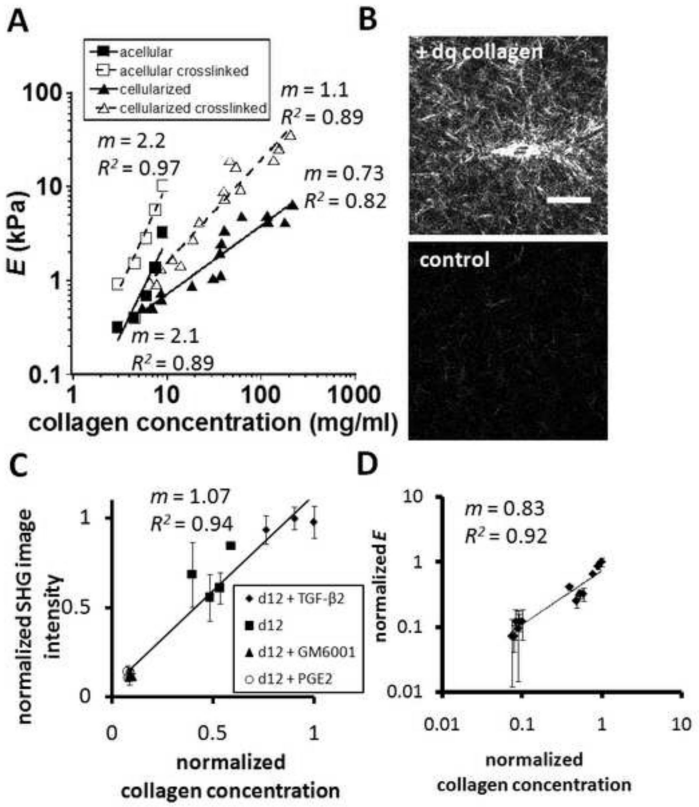

Figure 4.

(A) E measured from uncrosslinked cellularized gels (□, $n = 16$), glutaraldehyde crosslinked cellularized gels (□, $n = 16$), uncrosslinked acellular gels (□, $n = 5$), and crosslinked acellular gels (□, $n = 5$) as functions of collagen concentration. E data points represent five averaged measurements per gel. Error bars are omitted for clarity. Lines represent linear best-fits to the log-transformed data (averaged over coarse and fine-structured gels). Best-fit slopes and R^2 values are indicated next to best-fit lines. (B) Representative confocal fluorescence images of cellularized gels polymerized with DQ collagen (upper panel) and without DQ collagen (lower panel) and incubated for 7 days. Scale bar is 50 μm. (C) Normalized mean SHG image intensity versus normalized collagen concentration for cellularized gels incubated for 12 days (d12) in DMEM+10% FBS supplemented with 2 ng/ml TGF-β2, 35 pg/ml PGE2, or 10 μM GM6001. (D) Normalized E versus normalized collagen concentration for the same gels, determined by indentation testing as in (A). Slopes and best-fit coefficients are indicated in the figure.

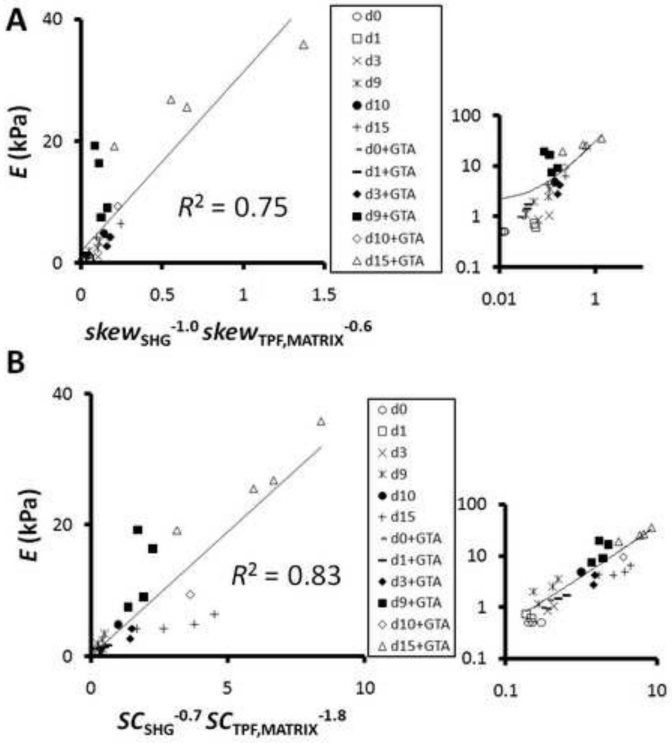

Figure 5.

(A) Nonlinear best-fit model for E on SHG signal skewness and matrix-derived TPF signal skewness. Markers indicate days in culture of the collagen gels and presence of GTA crosslinking. The inset on the right is a loglog plot of the data. (B) Nonlinear best-fit model for E on SHG signal speckle contrast and matrix-derived TPF signal speckle contrast. Markers indicate days (notation "d1" for day 1) in culture of the collagen gels and presence of GTA crosslinking. The inset on the right is a log-log plot of the data. Power-law best-fit exponents and R^2 values are indicated in the figure. E was averaged from five measurements per gel for $n = 16$ gels.