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Towards the feasibility of using ultrasound to determine mechanical properties of tissues in a bioreactor

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Abstract

Introduction—Our ultimate goal is to non-destructively evaluate mechanical properties of tissue-engineered (TE) cartilage using ultrasound (US). We used agarose gels as surrogates for TE cartilage. Previously, we showed that mechanical properties measured using conventional methods were related to those measured using US, which suggested a way to non-destructively predict mechanical properties of samples with known volume fractions. In this study, we sought to determine whether the mechanical properties of samples, with unknown volume fractions could be predicted by US.

Methods—Aggregate moduli were calculated for hydrogels as a function of SOS, based on concentration and density using a poroelastic model. The data were used to train a statistical model, which we then used to predict volume fractions and mechanical properties of unknown samples. Young's and storage moduli were measured mechanically.

Results—The statistical model generally predicted the Young's moduli in compression to within < 10% of their mechanically measured value. We defined positive linear correlations between the aggregate modulus predicted from US and both the storage and Young's moduli determined from mechanical tests.

Conclusions—Mechanical properties of hydrogels with unknown volume fractions can be predicted successfully from US measurements. This method has the potential to predict mechanical properties of TE cartilage non-destructively in a bioreactor.

Keywords

Poroelastic wave propagation; hydrogel; aggregate modulus; tissue engineering; quality control

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Introduction

Multiple approaches are being employed to develop tissue engineered (TE) cartilage for the repair of diseased or damaged articular cartilage.¹⁸ It is likely that the mechanical, biochemical and biological properties and composition of TE cartilage will need to be similar to those of the native tissue. Considerable donor-dependent variability in the proliferation, and differentiation potentials, and in the biosynthetic activity of cells results in TE constructs with highly variable properties. Establishing that the properties and composition of TE cartilage are in acceptable ranges prior to implantation is an important component of the TE process. Currently, most mechanical or biochemical assessment protocols are destructive endpoint-tests and/or violate the sterile bioreactor environment.^{2,12,17,19,23,26,28} Such methods render a construct no longer suitable for implantation.^{16,26,28} While using multiple test samples, some for end-point evaluation and others for implantation is possible, biological variability makes it unlikely that the tested and implanted constructs will have identical properties.

This investigation focuses on one aspect of quality evaluation, namely compressive mechanical properties. While mechanical properties may not be the only determinants of TE success, we expect that they should be in the same range as native cartilage if the tissue is to survive. Engineered cartilage should have the mechanical integrity needed to carry the stresses that it will be subjected to in a joint, and, ideally, it should be able to function in this environment immediately after implantation. Although it might be possible to adapt commonly used mechanical testing methods to maturing TE constructs, avoiding contamination of the sample poses a considerable design challenge. Quality control of TE constructs should therefore use noninvasive and nondestructive evaluation. Non-invasive methods such as magnetic resonance imaging (MRI) can measure glycosaminoglycan (GAG) concentration and water distribution nondestructively in tissue, but these methods do not predict biomechanical properties.¹⁵

Ultrasound has been evaluated as a technique for noninvasive evaluation of TE cartilage, but also has not yet been fully utilized for biomechanical assessment.⁹ As transducers can be acoustically coupled to the outside of the reactor, and the construct is coupled to the reactor by the fluid culture medium, ultrasound is a potential means for evaluating mechanical properties without the risk of contamination of a maturing construct. Material properties such as elastic modulus and density are known to correlate with the speed of sound (SOS) within a test sample, but computation of these properties requires knowledge of the composition of the material.³

In a previous investigation using agarose hydrogels we showed a positive correlation between the aggregate modulus (H) derived from ultrasound measurements and Young's modulus (E) derived from a one-dimensional, mechanical, unconfined compression (UCC) test.²⁷ Agarose was used, since like cartilage, it is a fluid saturated porous-elastic material and it has been used as a scaffold material in TE. This result suggests that it may be feasible to use ultrasound to assess the mechanical properties of TE cartilage nondestructively as it is developing in a bioreactor. Our previous work showed that it was necessary to use a poroelastic model for wave propagation in agarose gels.²⁷ This model requires the volume

fractions and bulk densities of the fluid and solid components of a gel.²⁷ Because Walker *et al.* used carefully-fabricated hydrogels, the volume fractions and densities of the fluid and solid components were known to a high degree of certainty.²⁷ However, in TE cartilage these proportions are not known, and they change during maturation. A correlation between volume fraction and some nondestructively measurable property is needed to develop the correlation between ultrasound-derived properties and UCC test properties. A retrospective analysis of the data from our previous study suggested that gel concentration (volume fractions) might be correlated with SOS, but the results were inconclusive (unpublished). The likelihood of obtaining such a correlation is supported by other work that showed a positive linear correlation between hydration and SOS in animal muscle.²⁵

A relationship between water content and ultrasound velocity is needed to non-invasively quantify the mechanical properties of TE cartilage during maturation in a sterile bioreactor. The purpose of this investigation was 1) to determine prospectively if SOS is related to volume fraction in hydrogels, and 2) to determine in samples with unknown volume fractions, the accuracy of E estimated from predicted volume fraction and speed of sound. In this study, agarose hydrogels were used as phantoms for articular cartilage since, like cartilage, they are a fluid-saturated porous material. If the determinations above proved reliable in agarose, this would support using our approach for TE cartilage. This investigation followed two paths to achieve these goals. In the first path (Figure 1, **left-hand column**), we determine H and thence E in gels with known composition using the procedures we previously used in Walker *et al.*²⁷ In the second path (Figure 1, **right-hand column**), we estimated volume fractions of unknown samples based on the response of SOS to compressive deformation, then used these estimates to predict H , and thence E . Finally, we compare the estimated values of E to mechanically measured E .

Materials and Methods

Apparatus

An apparatus for ultrasonic evaluation of constructs in a bioreactor was designed and built (Figure 2). The apparatus consists of a rigid frame (Minitec (Victor, NY) struts and rigid aluminum plates) which aligns a sample stage between a load cell and ultrasound transducer at the bottom, and a polished reflector at the top. The load cell/transducer assembly and the reflector are maintained in coaxial alignment by the precision-machined jig. As we intend to use this system for biological samples, the stage is designed to hold (and position in X and Y) our standard bioreactors. In these experiments a bioreactor was used to hold the test samples, but the top membrane of the reactor (see ²⁹) was omitted for easy sample exchange and as sterility was not a concern here.

The 10 N load cell (Omegadyne LCMFD-10N) and associated controller (DP25B-S-A) were obtained from Omega Engineering (Stamford, CT). The ultrasound transducer is an Olympus V208-RM 20 MHz (Olympus NDT, Waltham, MA). A Panametrics 5072PR pulser-receiver (Olympus NDT) and an Agilent DSO-X 2012A 100 MHz oscilloscope (Agilent Technologies, Santa Clara, CA) were used for data acquisition. The load cell/transducer assembly is axially adjustable, so that the top of the transducer touches the membrane of the bioreactor; it is then fixed in place during the experiment. The bioreactor

stage can be positioned in X and Y using rack and pinion linear actuators (Edmund Optics, Barrington, NJ) to align the sample with the US beam. The reflector can be positioned in the Z-axis using a Parker-Hannafin (M4004-DM, Cleveland, OH) micrometer-driven linear stage.

The pulser-receiver settings of pulse repeat frequency (PRF), signal energy, damping, and gain were set at 1kHz, 13μJ, 100Ω, and 0dB, respectively, with no hardware filtering used. The oscilloscope sampling rate was 250 Ms/s. The oscilloscope was triggered by the pulser-receiver external trigger signal, and for each sample 1024 reflections were averaged to reduce random noise. Y-Axis resolution was 12 bits. To accommodate the signal averaging, ten seconds were allowed to elapse after any setting change before recording the data.

Test samples

Agarose gels were prepared as described previously and tested on the same day.²⁷ Briefly, for an N% gel, N/2 g of ultrapure agarose powder (Invitrogen, Carlsbad, CA) were dispersed in (50 - N/2) ml of deionized water (diH₂O) and then heated to boiling in a microwave oven. The beaker and mixture were weighed before and after heating and evaporative losses were replaced by adding diH₂O to restore the original weight. The hot agarose solution was allowed to solidify in acrylic molds, (5.000 ± 0.010 mm by 12.7 mm in diameter) sandwiched between two heavy glass bars to ensure smooth and parallel gel surfaces. Six different concentrations were tested: 2%, 4%, 5%, 6%, 8% and 10%. After the US-tests, the samples were stored in diH₂O at 4 °C for later mechanical testing.

Procedure

Calibration—We established the accuracy of the ultrasonic measurements described below by measuring the SOS in water; we chose water as the sample since its SOS has been accurately measured and documented as a function of temperature.^{1,4} Therefore, it can serve as a standard for our US measurements.

Hydrogel ultrasonic evaluation—For ultrasonic evaluation, the sample was placed in diH₂O in an open bioreactor in the test apparatus (Figure 3). The acoustic reflector is immersed in the water above the sample using the micrometer-driven linear stage, while the transducer is acoustically coupled to the underside of the bioreactor (Aquasonic 100 gel, Parker Labs, Fairfield, NJ).

The height of the sample and time of flight of US were measured using a technique that we developed and validated. This approach is based on the position of echoes from the bioreactor membrane-sample interface, and the sample-reflector interface; the SOS in the bioreactor membranes is thus irrelevant.

Zeroes were established for time and sample height using the contact between the lower membrane and reflector in a fluid-filled open bioreactor as follows. Typical reflections from the lower bioreactor membrane, and the reflector a few millimeters above are shown in Figure 4A. As the reflector approaches the lower membrane (no sample is present during this operation) the membrane and reflector echoes begin to merge (Figure 4B). If the reflector was lowered beyond the point of contact with the lower membrane, echoes shifted

to the left on the oscilloscope. Zeroes for time and sample thickness were determined as the point of incipient left shift of the echoes from the lower membrane. At this point, the micrometer was zeroed and a cursor (t_0) was placed at the lowermost point of the trough of the echo from the reflector (arrow, Figure 4B).

Gel samples were placed in the bioreactor, and the height of each sample was measured by lowering the reflector until it contacted the top surface of the gel. As previously, contact with the top of the sample was identified as the point of incipient left shift of the echoes from the top of the sample. Contact was verified by a small change, ≈ 0.020 N, in the load cell output. A second cursor (t_f) was placed on the lowermost point of the trough of the echo from the reflector. The time difference between the two cursors was the time of flight for the uncompressed sample. The thickness of the uncompressed sample was the micrometer reading (h). This approach was used instead of the peak of the signal envelope determined using low-pass finite impulse response (FIR) filtering as in Walker *et al.*²⁷ In Walker *et al.*, distinct reflections were obtained from both the top and bottom of the hydrogel as both those surfaces were in contact with water.²⁷

Hydrogel mechanical tests—Gel samples were tested in uniaxial unconfined compression using a Rheometrics RSA-II Solids Analyzer (Rheometrics Inc., Piscataway, NJ) to determine Young's modulus. Compression was applied at constant strain rate of 0.01 s^{-1} for 15 seconds. The slope of a least-square fit of the stress-strain data between 0.015 and 0.02 strain was used as a measure of Young's modulus. In addition, frequency dependence of the storage modulus was evaluated at 1, 5, 10, 20, 30, 50, 70, 100 rad/sec, with an intended amplitude of 0.02 strain after a 0.04 pre-strain.

Material Modeling and Wave Propagation

The SOS (c) was calculated from the expression

$$c = \frac{h}{\frac{(t_1 - t_0)}{2}} \quad (1)$$

The time-of-flight is halved to allow for the round trip to the reflector and back to the transducer. SOS was found for each sample at its full height and after four sequentially applied $50 \text{ }\mu\text{m}$ compressive displacements. Gels were allowed to relax for several minutes after each displacement before measurements were made.

An expression for the one-dimensional aggregate modulus (H) was computed using speed of sound and assuming that the agarose gels could be modeled as poroelastic materials with incompressible components.³ Under these conditions

$$H = \frac{c^2 (\eta_f^2 \rho_{b,s} + \eta_s^2 \rho_{b,f})}{\eta_f^2} \quad (2)$$

where the aggregate modulus is in Pa, c is the speed of sound in m/s, the unitless η_i 's are the volume fractions of the fluid (f) and solid (s) per total volume, and the $\rho_{b,i}$ are the bulk densities of the fluid and solid components (the mass of each constituent over the total volume of the hydrogel) in kg/m³. For the agarose hydrogels the compositions were known as these were fabricated gels, and the bulk densities were calculated by:

$$\rho_{b,s} = \frac{m_{\text{agarose}}}{v_s + v_f} = \frac{N \times 10^{-3}}{\frac{N \times 10^{-3}}{\rho_{\text{agarose}}} + \frac{(100-N) \times 10^{-3}}{1000}} \quad (3)$$

$$\rho_{b,f} = \frac{m_{\text{fluid}}}{v_s + v_f} = \frac{100 \times 10^{-3}}{\frac{N \times 10^{-3}}{\rho_{\text{agarose}}} + \frac{(100-N) \times 10^{-3}}{1000}} \quad (4)$$

where N is the number of grams of dry agarose powder in 100 g of gel, $\rho_{\text{agarose}} = 1.64 \times 10^3$ kg/m³ (see Johnson *et al.*¹¹), and v_i are the volumes of the solid and fluid, respectively.

The volume fraction of solid in the gels was calculated with the assumption that water is bound to the helical agarose fibrils at a ratio of 0.625 solid agarose to water:¹¹

$$\eta_s = \frac{N/100}{\rho_{\text{agarose}} \times 0.625} \quad (5)$$

and the volume fraction of the fluid component is thus

$$\eta_f = 1 - \eta_s \quad (6)$$

Data Analysis

Data modeling—The ultimate goal of our data modeling was to first fit a model relating SOS to displacement and concentration using “training” data with known concentrations, and to use results of this model to estimate the unknown concentration of a future sample, which can then be used to obtain estimates of other mechanical properties such as Young's modulus. When a regression of Y on a single X is fit for the purpose of estimating X given Y for a future sample, this is termed the “calibration” problem (*e.g.*, Graybill⁷), and here we extend this approach to the case where the underlying model is a mixed model accommodating repeated measures from the same gel sample.

SOS measurements obtained on the same gel at each volume fraction and displacement were averaged arithmetically prior to analysis. Within gels of each volume fraction, the relationship between average SOS and displacement was well-described by a linear

regression (median $R^2=0.98$). Interestingly, the least-squares slopes of the straight-line fits appeared to be linearly related to the gel percent ($R^2 = 0.983$, Figure 5A), whereas the relationship between the least-squares intercepts and gel percent was slightly curvilinear and was better approximated by a quadratic regression (*i.e.*, where the independent variables are concentration and concentration-squared). Based on these observations, a linear mixed model was developed to describe the relationship between SOS, gel concentration and displacement, accounting for the within-gel correlation of measurements obtained at different displacements on the same gel. If SOS_{ijk} is the measured SOS of the i^{th} gel at the known concentration C_j measured at the known displacement D_k , the model is:

$$SOS_{ijk} = (\alpha_0 + \alpha_1 C_j + \alpha_2 C_j^2 + a_{ij}) + (\beta_0 + \beta_1 C_j + b_{ij}) D_k + e_{ijk} \quad (7)$$

where $\alpha_0, \alpha_1, \alpha_2, \beta_0, \beta_1$ are the unknown parameters, a_{ij} and b_{ij} are random effects representing the deviation of the i^{th} gel percent at concentration C_j from the population average intercept and slope, respectively, and the e_{ijk} are independent normally distributed errors with mean 0 and variance σ^2 , assumed independent of (a_{ij}, b_{ij}) . The random effects (a_{ij}, b_{ij}) are assumed to be from a bivariate normal distribution with zero mean vector and 2×2 covariance matrix Φ . Maximum likelihood estimates of parameters were obtained using the SAS Proc MIXED software (SAS Institute, Cary, NC).

Confirming the findings of the preliminary analyses, we found that there was a significant nonlinear quadratic effect of concentration on SOS (measured by α_2 in Equation (7)), but the quadratic effect of concentration on slope was not significant. Therefore, Equation (7) includes a term for $C_j D_k$ with coefficient β_1 measuring the linear relationship between concentration and slope, but not a term $C_j^2 D_k$ which would represent the quadratic effect of concentration on slope.

Prediction of properties of unknown samples—Once a mixed model (Equation (7)) has been developed and found to fit adequately to the training data, results can be used to estimate the unknown gel concentration C_0 of a new gel based on measurements of its SOS: $SOS_k^0, k = 1, \dots, K$ measured at displacements $D_1, \dots, D_K$. The maximum likelihood estimate of C_0 using the initial sample as a training set is obtained by fitting a non-linear mixed model in SAS Proc NLMIXED to the original data set, with the measurements on the new gel: $SOS_k^0, k = 1, \dots, K$ added. This simultaneous estimation approach, treating C_0 as an additional unknown parameter to be estimated using the maximum likelihood method, extends a similar approach outlined by Graybill for the calibration problem based on a simple linear regression of Y on X.⁷ This is done in the NLMIXED program by specifying the Gaussian likelihood of the original data SOS_{ijk} based on the linear mixed model (Equation (7)), as well as the Gaussian likelihood of $SOS_k^0, k = 1, \dots, K$, obtained from the Gaussian non-linear mixed model:

$$\text{SOS}_k^0 = (\alpha_0 + \alpha_1 C_0 + \alpha_2 C_0^2 + a_0) + (\beta_0 + \beta_1 C_0 + b_0) D_k + e_k^0 \quad (8)$$

where $\alpha_0, \alpha_1, \alpha_2, \beta_0, \beta_1$ are the same as in (Equation (7)), (a_0, b_0) are from the same distribution as are (a_{ij}, b_{ij}) , and e_k^0 are from the $N(0, \sigma^2)$ distribution. This is a non-linear model because the expected value of SOS_k^0 is not a linear function of the parameters $\alpha_0, \alpha_1, \alpha_2, \beta_0, \beta_1$ and C_0 . Maximum likelihood estimates of all parameters including C_0 are obtained using the default dual quasi-Newton algorithm in SAS Proc NLMIXED, using a Laplace approximation to the integrated log-likelihood (qpoin = 1). An example showing the data setup and the SAS code used for this analysis is included in the Supplementary Material. The feasibility of this approach was tested using the original data, using a leave-one-out approach, where each of the 40 gels was treated as a new sample at an unknown concentration, while using the other 39 gels as the “training” data set.

To evaluate the feasibility and precision of our approach, we used the regression relationships for aggregate modulus predicted based on known volume fractions and mechanically measured moduli, but replaced the aggregate modulus by that predicted from the model (Equation 8) described above. Thus, using the concentration predicted by the model, aggregate modulus was recomputed for each sample using the leave-one-out approach. The corresponding Young's and storage moduli were then separately obtained from correlations at zero displacement between predicted aggregate modulus and measured Young's and storage moduli. For validation, these were then compared to the actual Young's and storage moduli determined from the SOS and known concentrations.

Results

Using the newer apparatus and the improved method of finding the time differences between reflections, accuracy and precision were improved, and previously published results were confirmed.

For accuracy calibration purposes, five measurements in water at a known temperature resulted in an average SOS of 1490.3 ± 0.60 m/s, which is within 0.086% of the published value of 1488.9 m/s.⁴ In addition, we reduced the standard deviation in the overall computed SOS in gels from ≈ 31 m/s (using the apparatus described in Walker *et al.*²⁷) to ≈ 2.4 m/s using the new setup.

We also confirmed that there was a positive linear relationship between Young's modulus (E) and the aggregate modulus (H) determined from ultrasound, $E = 0.01259H - 546127$. Using the new apparatus, the linear regression had an $R^2 = 0.976$ (Figure 6), up from 0.94 in Walker *et al.*²⁷ We also found a linear relationship between storage modulus (E'), measured using mechanical tests, and the ultrasound-derived aggregate modulus. Regression coefficients and R^2 values are shown in Table 1.

SOS under compression

US SOS measurements performed uncompressed and at four equal 50- μm compressive displacement increments showed that for each gel concentration the SOS decreased linearly as the deformation increased (Figure 7).

All regression parameters in the linear mixed model (Equation 7) were significantly different from zero (Table 2), and means of SOS estimated under the model were close to the observed means of SOS across concentrations and displacements. A possible exception were the means of SOS at higher deflections for the 5% gel samples (Figure 7).

When using the leave-one-out method to predict concentration for each of the 40 gels using the other 39 samples as the training sample, the 95% confidence intervals covered the true gel percent in 38/40 (95%) of the samples, as would be expected on average. The two confidence intervals that did not cover the true concentration were (2.51, 3.93) at gel concentration 4% and (4.46, 5.99) at gel concentration 6%. The average width of the 95% confidence intervals for the unknown concentration was ± 0.85 .

Prediction of mechanical properties of unknown samples

The average error in the predicted value of Young's modulus decreased with increasing gel concentration. Nineteen of the twenty-one samples with a concentration of 6% or more had a predicted Young's modulus that was within 10% of the measured value (Figure 8).

Discussion

In a previous study (Walker *et al.*²⁷) we showed that mechanical properties of a poroelastic material, measured by traditional methods, could be predicted from US measurements of SOS. This method works for known materials, but could not be applied to an arbitrary material, as we needed to know the volume fractions and bulk densities of the solid and fluid phases *a priori*. The current work demonstrates the feasibility of using this approach to determine mechanical properties of an arbitrary poroelastic material. To the best of our knowledge, this is the first study to predict mechanical properties based solely on SOS measurements and wave propagation in poroelastic materials. Others (*e.g.*, Saarakkala *et al.*) have taken an analogous approach, but modeled cartilage as an elastic material.²⁴ In our previous study, we found that, in highly hydrated materials, assuming a 1D elastic model yielded modulus values approximating the bulk modulus rather than the Young's modulus.²⁷ In addition, the current approach allows us to make predictions regarding the composition (fluid *vs.* solid phase) of the material. This may be valuable as an assessment tool for TE cartilage, as the composition is known to change as a function of tissue maturity. The eventual goal of the methods tested in this paper is to non-invasively quantify the mechanical properties of the bioreactor-grown tissue as a predictor of the maturity. A crucial step is to model a relationship between SOS and level of hydration in a poroelastic, fluid-saturated medium. We used defined agarose hydrogels as phantoms to show the feasibility of this method. Agarose gels exhibit a fluid-filled solid matrix, analogous to cartilage, and agarose is commonly used as a scaffold material in cartilage TE, further justifying its use.

Since quasi-static mechanical properties are well-established for tissues, we sought to relate the modulus computed from ultrasound measurements to those measured mechanically. We have now shown that it is possible to predict mechanical properties from those measured entirely from SOS measurements. The error in this prediction was lower for high concentration gels than for low concentration gels. This trend suggests that when applied to tissue-engineered cartilage, which has a higher solid content than even our most concentrated gels, the error in predicting Young's modulus should generally be below 10%. In principle, this could be applied to other tissues in a bioreactor. However, other uncertainties such as inhomogeneity may complicate measurements in TE cartilage.

The mixed model (Equation (7)) can be best envisioned as a two-stage model. It assumes that, for each sample, the SOS measurements are related to deflection by a linear regression with a sample-specific intercept and slope. This assumption is well-supported by the high individual R^2 values of the regressions of SOS vs. deflection observed for the separate gel samples. The second stage allows the average intercepts and slopes of these regressions, which are assumed to be random, to vary, depending on concentration. Through exploratory analyses including the plot in Figure 7, we found that the slopes of SOS vs. deflection were linearly related to concentration. However, the relationship between the intercept of the SOS vs. deflection regressions and concentration was somewhat nonlinear (Figure 5B), and we found that a quadratic regression fit significantly better than a linear relationship in the mixed model. Hence, using Equation (7), for a gel at concentration C , the regression of SOS vs. deflection is predicted to have an intercept of $\alpha_0 + \alpha_1 C + \alpha_2 C^2$ (m/s) and a slope of $\beta_0 + \beta_1 C$ (m/s/units of deflection). This implies that for a gel sample with concentration C measured at deflection D , the predicted SOS is $\alpha_0 + \alpha_1 C + \alpha_2 C^2 + (\beta_0 + \beta_1 C)D$ m/s. We use a mixed model here rather than multiple regression because there are two sources of variability – between sample and within-sample – where multiple measurements of SOS at different deflections measured on the same gel sample are correlated. Multiple regression is not valid to use in this case because it assumes that all measurements are independent.

In this study, we showed that SOS is a nonlinear function of concentration at zero compression. This is in contrast to our previous work that had suggested a linear relationship between SOS and concentration.²⁷ We believe that the non-linear relationship was previously obscured by the higher variability and lower accuracy in our prior measurements.

SOS in agarose gels was also found to decrease under compression. Current modeling, based on linear poroelasticity cannot explain this behavior. In fact, just the opposite might be predicted. As the material is compressed it is reasonable to expect that fluid would be squeezed out of the matrix, which results in locally higher concentration. However, as we have shown, higher concentrations result in higher, not lower SOS. This is an inherent limitation of linear theories of material behavior. Nonlinear continuum models that are based on higher-order material constants, and which may include chemical as well as mechanical effects, are some approaches that can be used to predict changes in SOS in a stressed material.^{6,10,31} In cartilage, SOS decreases under ramp compression but then returns to near initial levels as stress relaxes.^{13,20} Nieminen and Lötjönen have modeled these changes as a linear function of changes in void ratio and collagen orientation, determined using a sophisticated finite element model.^{13,20}

In summary, our earlier work showed that there was a relationship between modulus measured using ultrasound, and that measured mechanically. Application of this approach to an arbitrary material was, however, limited by lack of knowledge of the volume fractions (or concentration) of the material. We have now shown that it is possible to estimate volume fractions and quasi-static mechanical properties of agarose hydrogels entirely from measurement of SOS. Thus, the traditional mechanical properties of a porous-elastic material can potentially be derived entirely from US measurements.

These results support the feasibility of extending this method to TE cartilage while it is contained in a sterile bioreactor. While in this study, we used an open bioreactor for the convenience of rapidly switching samples, from a measurement perspective, it makes no difference whether the upper membrane is present or not; this portion of the US signal is simply ignored.

Limitations

In this paper, we used agarose hydrogels as a model system to characterize the system and to show the feasibility of the approach, as these are well-characterized samples with uniform properties. Extension to another material, *e.g.*, alginate, would require the development of a training set for that material. At this point, we do not see any practical way to bypass this requirement. In real tissue, uncertainties such as regional inhomogeneities may complicate measurements. However, as in most mechanical tests, our approach yields average properties of the specimen, so that these measurements will likely be as predictive of the behavior of the sample in aggregate as properties determined by “conventional” methods. Heterogeneity is captured by other approaches which we are currently exploring (*e.g.*, elastography), but which exceed the scope of this study.³²

It is important to note that the current approach uses one-dimensional modeling of wave propagation, which is often assumed in studies using ultrasound. Importantly we have shown that it is feasible to correlate mechanically measured modulus with modulus measured from ultrasound. However, if we were interested in determining parameters such as Poisson's ratio, 1D propagation would not be the correct assumption to use.

There is emerging research on the effects of ultrasound irradiation on the development of engineered tissue. Where an effect of ultrasound was noted, it appeared to be neutral to beneficial.^{5,8,14,21,22,30} By comparison to these studies, the exposure time and energy is quite small and unlikely to have much effect.

In this study, bulk densities were computed as we knew what they were, having fabricated the gels. These would not be known for developing cartilage, and may vary over time. However, an analysis of error propagation suggests a lack of sensitivity to this variable (see supporting supplementary data for an analysis of error propagation).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Flow diagram of the procedure used to validate the predictions of modulus of a gel with unknown volume fraction. The column of boxes on the left side represents computing the modulus as was done in Walker *et al.*²⁷, where volume fractions are known. The column of boxes on the right side represents the method used to estimate the modulus as described in this paper, where the volume fraction is not known *a priori*.

Figure 2.

Above the bioreactor, a micrometer (Parker Hannifin Daedal M4004DM) was attached to a metal rod acting as an acoustic reflector. Using this assembly the height of the sample, (*i.e.*, the distance the US signal travels) is always known. A load cell (Omegadyne LCMFD-10N), placed coaxially with a 0.125" diameter, 20-MHz center-frequency ultrasonic transducer (Olympus V208-RM), was used to measure the compressive load on the sample. Water-soluble ultrasound transmission gel (Parker Labs Aquasonic® 100) was used to couple the bioreactor to the apparatus. A pulser-receiver (Olympus Panametrics 5072PR) was used to generate the ultrasound signal and to sense and amplify its reflection. The reflected signal was digitized and displayed on an oscilloscope (Agilent Infiniivision DSO-X 2012A, 100 MHz bandwidth). Finally, a National Instruments NI USB-TC01 with a type-T thermocouple was used to measure the temperature of the water in the bioreactor. All devices were calibrated before use.

Figure 3.

Schematic of a hydrogel sample in an open bioreactor. The bottom of the sample is constrained in the vertical direction but not in the radial direction, and displacement is applied to its upper surface by the micrometer in 50 μ m increments. We then waited several minutes to allow for stress relaxation before taking SOS measurements.

Figure 4.

Zeroes for sample height, and US time of flight are determined by the merging of echoes from the reflector and lower bioreactor membrane. In a sealed bioreactor, there would be an additional 200 μm membrane thickness, which would be gated out. For reference, each division on the X-axis is 200 ns.

Figure 5.

A - Plot of the mean slopes of the SOS vs. deflection lines from Figure 7, for each gel concentration. $R^2 = 0.983$. Left Y-axis shows the SOS vs. absolute deflection in mm, right Y-axis shows SOS vs. strain (mm/mm). **B** - Plot of the mean intercepts of the SOS vs. deflection lines from Figure 7 (at zero deflection). Quadratic fit, $R^2 = 0.996$.

Figure 6.

Positive linear correlation between Young's modulus (E) measured mechanically and the aggregate modulus (H) determined from ultrasonic SOS measurements processed using Equations (2) to (6), using the known gel concentrations. The regression is computed using all the data. The equation (*see text*) is also used to predict Young's modulus from SOS for an unknown sample.

Figure 7.

Fit of the model $SOS = a_0 + a_1 (C) + a_2 (C)^2 + \beta_0 (D) + \beta_1 (C \cdot D)$ for each gel concentration to the mean measured speeds. C is the concentration and D is the deflection. The coefficients for the model are in Table 1. The slopes of these lines are the data used in Figure 5A, the intercepts are used in Figure 5B. Bottom X-axis is absolute applied compressive displacement in mm. The top axis shows applied compressive strain (mm/mm)

Figure 8.

Percent error calculated from the mechanically measured Young's modulus vs. the predicted Young's modulus from the statistical model. The diamond symbol is the prediction computed from the measured SOS for each sample, and the vertical bars represent the upper and lower limits of the 95% confidence intervals of the predicted Young's modulus based on a statistical model of 40 gel samples and six gel concentrations.

Table 1

Estimates from mixed model relating SOS on concentration and deflection. Concentration is expressed in percent, deflection in mm; the estimated $SOS = \alpha_0 + \alpha_1 * C + \alpha_2 * C^2 + \beta_0 * D + \beta_1 * D * C$ (Figure 7).

Effect	Estimate	95% Confidence Interval	p-value
Intercept (α_0)	1483.88	(1481.72, 1486.03)	<.0001
Concentration (α_1)	4.00	(3.27, 4.73)	<.0001
Concentration² (α_3)	-0.08	(-0.13, -0.02)	0.005
Deflection ($\alpha\beta_0$)	9.64	(6.26, 13.02)	<.0001
Concentration* Deflection (β_1)	-6.96	(-7.45, -6.48)	<.0001

Table 2
Coefficients for the regression of mechanically measured storage modulus on US-derived aggregate modulus

Frequency	Slope	Intercept	R ²
1	0.0122	-157580	0.973
5	0.0140	-154698	0.960
10	0.0143	-105841	0.960
20	0.0151	-113621	0.960
30	0.0155	-109884	0.964
50	0.0163	-117553	0.965
70	0.0169	-138270	0.966
100	0.0176	-142223	0.968