Quasi-static elastography comparison of hyaline cartilage structures

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Abstract. Joint cartilage, a load bearing structure in mammals, has only limited ability for regeneration after damage. For tissue engineers to design functional constructs, better understanding of the properties of healthy tissue is required. Joint cartilage is a specialised structure of hyaline cartilage; a poroviscoelastic solid containing fibril matrix reinforcements. Healthy joint cartilage is layered, which is thought to be important for correct tissue function. However, the behaviour of each layer during loading is poorly understood. Ultrasound elastography provides access to depth-dependent information in real-time for a sample during loading. A 15 MHz focussed transducer provided details from scatterers within a small fixed region in each sample. Quasi-static loading was applied to cartilage samples while ultrasonic signals before and during compressions were recorded. Ultrasonic signals were processed to provide time-shift profiles using a sum-squared difference method and cross-correlation. Two structures of hyaline cartilage have been tested ultrasonically and mechanically to determine method suitability for monitoring internal deformation differences under load and the effect of the layers on the global mechanical material behaviour. Results show differences in both the global mechanical properties and the ultrasonically tested strain distributions between the two structures tested. It was concluded that these differences are caused primarily by the fibril orientations.

1. Introduction
Cartilage is a structural load bearing tissue which is found throughout the bodies of mammals. Articular joint cartilage is subject to rigorous cyclic loading patterns in everyday use, but is prone to damage under sudden shock loading conditions (Vertebramo and Seedhom 2007). Once damaged, articular cartilage has limited ability for self-repair (Klein et al. 2003; Yu et al. 1997) and consequently is a target for regenerative medicine (Yu et al. 1997; Klein et al. 2003; Ma et al. 1995; Grellmann et al. 2006). Articular cartilage has a fibril-reinforced layered poroviscoelastic structure (Fung 1993; Maroudas 1979; Mow et al. 1991) and as such has poorly characterised mechanical behaviour. In order to provide a prescriptive specification of the tissue properties required for engineered tissue, the contributions of the various structural features need to be determined.

Articular cartilage is a specialised structure of hyaline cartilage (Maroudas 1979). Hyaline cartilage consists of a porous, collagen fibril reinforced proteoglycan matrix containing small clusters of cells and an interstitial fluid (Fung 1993). The layered structure of cartilage tissue is shown in figure 1 (Mow et al. 1991). The direction of the collagen fibrils within the layers is a primary determinant of their mechanical behaviour. In the superficial zone the fibrils are in parallel alignment with the tissue surface, in the middle zone the fibrils are randomly aligned and in the deep zone the fibrils are perpendicular to the subchondral bone. In the superficial and deep zones the collagen fibrils exist in a higher density than in the middle zone Mow and Guo 2002. The global (depth-independent) mechanical behaviour of the tissue is determined by an interaction of the matrix porosity, the intrinsic matrix viscoelasticity and
the fibril reinforcement (Mak 1986; DiSilvestro and Suh 2002; Haider and Schugart 2006). Under loading conditions the fluid in the pores is pressurised and subsequently flows down the pressure gradient. This pressurisation and flow coupled with a dependency of permeability on strain (Mansour and Mow 1976) causes the properties to be strain and strain rate dependent (Park et al. 2008). The fibrils also exhibit tension-compression non-linearity (Soultat et al. 1999) and strain dependence (Fortin et al. 2000). The layered structure and properties of articular cartilage are essential for correct tissue function (Chen et al. 2001; Klein et al. 2003).

![Diagram of articular cartilage cross-section showing the fibril alignment and depth zones](image)

**Figure 1:** Illustration of the cross-section of articular cartilage. The fibril alignment is highlighted. Taken from Mow et al. (1991).

Previous investigations into cartilage behaviour have included both ultrasonic (Berry et al. 2006; DiSilvestro et al. 2001; Toyras et al. 1999; Zheng et al. 2002) and mechanical (Mak 1986; Fortin et al. 2000; Buschmann et al. 1998; Park et al. 2008) testing methods. The collagen fibrils have been seen to affect the backscattering characteristics of the ultrasonic signal (Laasanen et al. 2005). Measurements of attenuation and reflection characteristics have both been demonstrated to show potential for diagnosis of cartilage degeneration (Nieminen et al. 2004; Toyras et al. 1999), but neither of these parameters allows direct mechanical property measurements. Characterisation of the global mechanical behaviour of cartilage has been extensive (DiSilvestro et al. 2001; Toyras et al. 1999; Zheng et al. 2002; Mak 1986; Fortin et al. 2000; Buschmann et al. 1998; Park et al. 2008), but although the depth-dependent mechanical properties contribute to the global properties, the importance of the separate layers also needs to be considered for tissue engineering purposes.

Elastography is an ultrasonic technique initially developed for the detection of cancerous tumours (Ophir et al. 1991). The elastography procedure involves acquiring ultrasonic backscattered signals from a material before and after the application of a strain. The shifts observed in the arrival times of signals from internal scatterers are used to determine localised tissue strains under the assumption of a constant sound speed in the material. The strains in the tissue are inversely proportional to the elastic moduli and hence this provides a method for the characterisation of depth-dependent moduli, as long as sufficient information regarding the material stresses is known. The feasibility of material characterisation of poroviscoelastic materials (such as cartilage) via elastography has been investigated (Cohn et al. 1997a; Cohn et al. 1997b; Righetti et al. 2005; Zheng et al. 2002; Zheng et al. 2005). The main limitations of these previous cartilage investigations were the boundary conditions from the method of strain application and the sample orientations. In the studies by Zheng et al. the ultrasonic
data were obtained using a 50 MHz transducer, the beam from which was significantly attenuated before reaching the furthest layers of the material (Zheng et al.). The work reported here is an improvement of the methodology first reported by Zheng et al. The nasal cartilage samples used in the present study were approximately double (3.93±0.55mm) the thickness of the articular samples (1.57±0.30mm), which would cause an unacceptable degree of attenuation, so a lower frequency transducer was selected. The other key difference is that in this present study the deflection of the load cell was quantified using a linear variable differential transformer (LVDT); the level of load cell deformation (approximately 0.0016mm/N) is only one order of magnitude smaller than the displacement applied to the cartilage, so are therefore relevant to the results. The experiments performed by Zheng et al. included only a linear approximation for the load cell deflection.

This current work is a preliminary study for future work involving cyclic loading protocols to link the layered structure of cartilage to the behaviour in more physiologically relevant conditions.

2. Materials and Methods

In this study a quasi-static elastography method was used to compare the depth-dependent mechanical behaviour of two types of hyaline cartilage. These were bovine articular cartilage and porcine nasal cartilage. Nasal cartilage is a hyaline cartilage with a non-layered structure (Fung 1993) whereas articular cartilage has three layers. The global mechanical properties were measured simultaneously with the acquisition of the ultrasound A-scans for elastography. Under constant strain the tissues undergo stress-relaxation, causing a reduction in the stress required to maintain the global strain (Mak 1986). The localised shift behaviour within the two types of cartilage provides information regarding the contribution of the various zones of articular cartilage to the overall tissue deformation.

Samples: Cartilage samples were obtained from a local abattoir (Blixies Farm, Ranks Green, Essex, UK) within 4 hours of slaughter, excised from surrounding tissue and prepared into 8mm diameter discs using biopsy punches. Once prepared the samples were stored in phosphate buffered saline solution (PBS) prior to testing. Ten samples for each test of each cartilage type were prepared. As shown in figure 1, the axial orientation of the articular cartilage samples was important in the tests. Nasal cartilage sample discs were axial orientation independent due to the homogeneity of the structure.

Mechanical testing: A schematic of the apparatus is shown in figure 2. The mechanical test equipment consisted of an actuator (Physik Instrumente, M-227) in series with a load cell (Interface, SM-50N) within a rigid frame. To quantify the load cell deformations a LVDT (Omega, GP911-0.5-S) was placed in parallel with the load cell. The data from the LVDT was used to determine the actual strain applied to the samples at any given point.
Strains of 2, 4, 6 and 8% were applied to the top surface of the samples via the actuator and held constant for 20 minutes. The bottom surface of the samples (cartilage-bone interface of articular samples) rested on the rigid fixed surface of the Perspex delay-line. The load cell continuously recorded data of the force required to maintain the strain applied to the sample. The LVDT continuously recorded the small deformations across the load cell.

A pre-load of 3N was applied to each of the samples prior to the strain application to ensure effective contact of the samples and the compression surfaces. The application of pre-loads is standard practice in the mechanical testing of cartilage (Fortin et al. 2003; Julkunen et al. 2008). The pre-loads were maintained for approximately 5 minutes until the load cell reading reduced to zero (i.e. when no additional force was required to deform the samples). All tests were conducted with the samples fully submerged in a tank containing PBS at room temperature (22.7 ± 1.4ºC).

Ultrasonic testing: The 15 MHz focused ultrasound transducer (Panametrics, V319) was inserted through the base of the tank. The lower plate of the rigid frame had a hole cut in the centre as an acoustic window and a Perspex delay-line was used for the sample to rest on. The delay-line thickness was chosen so that the ultrasound focussed into a column in the samples. Perspex was selected due to the absence of grain boundaries that would be found in a metal. The signal was transmitted and received by the same transducer using a pulser-receiver (Panametrics 5055PR). A-scans were automatically acquired at 3 second intervals throughout the test using the oscilloscope (LeCroy 9310M) controlled by an in house LabVIEW (National Instruments Corporation 2008) program.

Speed of sound values were obtained using the time of flight method demonstrated in equation 1, where \( a \) is the sample thickness, \( v \) is the sound speed and \( t_1 \) and \( t_2 \) are the arrival times of echoes from the two sample surfaces.

\[
v = \frac{2a}{t_2 - t_1}
\]

The pre-strain A-scans were compared to post-strain A-scans at three different time intervals: immediately post-strain application and 10 and 20 minutes after the strain was applied.

Analysis: Calculations of global elastic properties were made by defining the stress (\( \sigma \)) from initial sample dimensions and the load cell data. Global applied strain values were corrected.
with the LVDT data to obtain the actual strain ($\varepsilon$). Young’s modulus, $E$, was calculated from these values as shown in equation (2):

$$E = \frac{\sigma}{\varepsilon}$$ (2)

Ultrasonic A-scans pre- and post-straining were processed in Matlab (The MathWorks 2008) by splitting the signals into windows and applying the sum squared difference (SSD) algorithm. The equation describing the SSD algorithm is as follows:

$$R_{SSD}(\tau) = \int_{-T/2}^{T/2} (s_1(t) - s_2(t + \tau))^2 \, dt$$ (3)

Over a window of $\pm T/2$, signal $s_2$ is moved across signal $s_1$ in increments of $\tau$. When the value of $R_{SSD}$ is found to be a minimum, the corresponding value of $\tau$ is the shift between the two signals.

The windows which were one tenth of the signal length (corresponding to approximately one tenth of the sample depth) were applied to both the pre- and post-compression signals. The post-strain signal is moved step-wise through the pre-strain signal applying the SSD algorithm at each step. The SSD integral values for each step are calculated. The minimum value of the integral indicates the best match between pre- and post-strain signal shape and the corresponding step value is recorded. This step value is then converted into a time shift using the known sampling interval. The time shifts are then plotted in terms of their representative depth within the tissue. With accurate measurements of tissue thickness before and after the compression, these time shift values could be converted into displacement measurements in terms of actual tissue depth. Each window overlapped the preceding window by one third.

To ensure no erroneous data was plotted on the shift traces from portions of the signal with very little correlation, the correlation coefficient of the two signals at the step-position corresponding to the minimum SSD was also calculated. The correlation coefficient ($C$) is a measure of the degree of similarity between two signals. Whereas the SSD detects the shift at which the minimum difference occurs, the correlation coefficient provides a normalised value of the sum of the window contents for comparison. The individual points in the pre- or post-compressional signal, for example $s_{1i}$ where $i = 1,2\ldots$ (number of points in the window, $n$) is compared to the mean of the window ($\bar{s}_1$), combined with the same values in the comparison window and divided by the corresponding standard deviations ($\sigma_{s_1}$) and normalised to $n$:

$$C_{s_{1i}s_{2i}} = \frac{\sum ((s_{1i} - \bar{s}_1)(s_{2i} - \bar{s}_2))}{(n-1)\sigma_{s_1}\sigma_{s_2}}$$ (4)

The value returned is between 0 and 1, where 0 indicates no correlation between the signals and 1 indicates identical signals. A threshold value is set for the minimum acceptable correlation coefficient. Shifts corresponding with correlation values below the threshold are not included in the graphical output of the shift profile.

3. Results

Mechanical results: Figures 3 (a) and (b) show the mechanical results from the study. The results were averaged over the 10 samples for each strain level in each tissue type; the error bars show the standard deviation at each point. Values are plotted at 15 second intervals. Figures 3 (a) and (b) compare the apparent elastic moduli throughout the full 20 minute test periods of the nasal (a) and the articular (b) samples at each of the strain levels. The apparent
elastic moduli for articular cartilage appeared to be dependent on the strain applied. This was not the case for nasal cartilage samples.

Ultrasonic results: The sound speed of the cartilage was calculated using the time-of-flight method (equation 1) and the strains known to be applied to the material. The sound speeds were found to be $1920 \pm 95$ m/s and $1690 \pm 109$ m/s prior to strain application for the articular and nasal samples respectively. The sound speeds were also calculated immediately post-strain application and at 10 and 20 minutes post-strain. It was found that for both the nasal and the articular cartilage the speed of sound propagation increased under strain, however not as a function of the strain directly but rather as a function of the time the strain had been applied. Nasal cartilage sound speed increased by $0.6\% \pm 0.05\%$, $1.3\% \pm 0.05\%$ and $1.9\% \pm 0.05\%$ compared to unstrained cartilage at the instant of strain application and at 10 and 20 minutes respectively. Articular cartilage sound speed increased by $1.2\% \pm 0.05\%$, $3.0\% \pm 0.05\%$ and $4.0\% \pm 0.05\%$ compared to unstrained cartilage at the instant of strain application and at 10 and 20 minutes respectively.

Figures 4 (a)-(d) show the nasal strain maps at (a) 2\%, (b) 4\%, (c) 6\% and (d) 8\% global strains. Internal strains at the instant of load application and 10 minutes and 20 minutes later are shown with lines of best fit plotted through the data. A reference line is plotted in each figure for the strains expected in a homogeneous linear elastic material. ‘Initial tissue depth’, as displayed on the x-axis of figures 4 and 5, refers to the linear distance in the unstrained tissue from the bottom of the sample. The strain maps for the nasal cartilage samples show approximately linear trends at gradients lower than the reference lines. The coefficient of determination ($R^2$) values were recorded as a measure of how closely the trend lines fitted the data. All the $R^2$ values were above 0.8, which denotes a reasonably close fit, with the exception of the value for 6\% strain at 10 minutes which was 0.72. Figures 5 (a)-(d) show the corresponding articular cartilage strain maps at each of the applied global strains. Lines of best fit have not been fitted to this data; only the reference lines are shown as above. The strain maps from the articular cartilage samples show regions of different gradients which deviate from the reference lines.
Figure 4: Elastographic strain maps from nasal samples following instantaneous strain application and after the initial strain has been held constant for 10 and 20 minutes (a) 2% applied strain; (b) 4% applied strain; (c) 6% applied strain; (d) 8% applied strain.

Figure 5: Elastographic strain maps from articular samples following instantaneous strain application and after the initial strain has been held constant for 10 and 20 minutes (a) 2% applied strain; (b) 4% applied strain; (c) 6% applied strain; (d) 8% applied strain.
4. Discussion

The aim of this study was to investigate the depth-dependent deformation of two different cartilage types using ultrasound elastography. Ultrasound elastography was selected as a non-destructive technique to simultaneously collect global and depth-dependent elastic properties of the tissues. The two cartilage types were compared to demonstrate which behaviours of articular cartilage are likely to be directly linked to the three-layer structure of the material.

The mechanical results demonstrated a difference between nasal and articular cartilage in terms of stress relaxation and instantaneous modulus characteristics. The articular cartilage was found to be nonlinear with the instantaneous moduli being a function of the applied strain. The instantaneous modulus was greatest at 2% compression (1.19±0.3MPa), and then decreased with applied strain (0.82±0.09MPa at 4%, 0.62±0.08MPa at 6% and 0.53±0.07MPa at 8% strain). The instantaneous moduli of the nasal cartilage samples appeared independent of the applied strain (average 0.56±0.06MPa, maximum 0.59±0.06MPa and minimum 0.52±0.05MPa). The equilibrium moduli of all the cartilage types at all applied strains were similar (approximately 0.15±0.08MPa for the nasal cartilage and 0.13±0.09MPa for the articular cartilage). Elastographically, the strain maps from the nasal cartilage samples were roughly linear and the articular cartilage strain maps showed evidence of separate behaviour in different regions. The stress-relaxation behaviour of articular cartilage was not obviously depth-dependent as small increases were seen in the strains at 10 and 20 minutes, in all measured depths. These increases were found at each level of global applied strain. The nasal cartilage data decorrelated between pre- and post-strain far more readily than the articular data. Consequently there are some places in the nasal strain maps where either points have been omitted or where strains anomalous to the trend lines have been plotted as only one or two values were available to plot from the ten averaged samples. The pre- and post-strain correlation for the articular data was better with at least 5 data points to average at any tissue depth.

The speeds of sounds measured in the two cartilage types differed. The bovine articular cartilage sound speed also varied significantly from the values found in literature for the same material (1636 ± 25m/s (Patil et al. 2004)). There was a high degree of scatter in both the mechanical and the ultrasonic data, as demonstrated by the error bars in figures 3, 4 and 5. This scatter is likely to have been caused primarily by the biological variation between individual samples, but variation in the ambient temperature would have also affected the material behaviour particularly with respect to the speed of ultrasound propagation.

The ultrasonic results from the nasal tissue showed a scattered linear strain map, with no clear individual regions. These linear strains were seen to increase with the level of applied strain. At all global strains the nasal cartilage strain maps showed a slight increase in measured strain at all tissue depths at both 15 and 20 minutes. The strain maps from the articular cartilage demonstrated regions of similar elastic moduli. The approximate thicknesses of these regions are 0.5mm for the deeper zone (31%), 0.7mm for the transitional zone (44%) and 0.4mm for the superficial zone (25%). The steeper gradient from the top regions of the articular cartilage strain maps and high peak strain values reflect the lower elastic modulus of the tissue in this region (Zheng et al. 2002). The regions corresponding to the transitional and deeper zones have less steep gradients as a result of higher elastic moduli in these regions. The region of the strain maps roughly corresponding to the transitional zone of the articular cartilage closely resembles the appearance of the whole of the nasal strain maps. Articular cartilage and nasal cartilage have similar compositions with the main difference being the collagen fibril orientation. It is likely therefore that the differences seen in the strain maps, between the two cartilage types, can mainly be attributed to the fibril arrangements.
The instantaneous mechanical behaviour of the cartilage tissues demonstrates a clear difference between the two cartilage structures investigated. The global elastic properties of articular cartilage suggest a link between the layered structure and the nonlinearity of the response to an instantaneous load. The equilibrium elastic properties of both cartilage types were approximately equal, suggesting this is not dependent on the collagen fibril orientation of the cartilage. The variation in elastic properties in the articular cartilage appears to be physiologically significant, with greatest levels of deformation permitted far away from the bone.

The diameter of the samples with respect to the measurement area covered by the beam was considered to be sufficiently large for edge-effects to be disregarded in the ultrasonic data. However, it is likely that the time taken for all the cartilage samples to reach equilibrium was reduced compared to the period in vivo as the fluid could move freely out of the sample edges. The speed of sound in the articular and the nasal cartilage were respectively assumed to be constant through the thickness of the tissues and independent of strain. The assumption of depth-independence for sound speed in articular cartilage is likely to be a source of error, as Agemura et al. (Agemura et al. 1990) reported a variation of approximately 5% from the superficial to the deep zones. Greater accuracy could be obtained for the ultrasonic data by adopting methods for tighter control of the ambient temperatures.

5. Conclusion
Ultrasound elastography has been used for the first time to compare the depth-dependent mechanical behaviour of two different hyaline cartilage structures. Articular cartilage demonstrated strain dependence in instantaneous elastic moduli, which was absent in the behaviour of nasal cartilage. The elastography strain maps revealed evidence of depth-dependent elastic moduli for articular cartilage while indicating a homogeneous elastic modulus for nasal. As both materials are poroviscoelastic hyaline cartilages, this result implies a direct link between the layered collagen fibril alignment and the non-linear elastic response of articular cartilage tissue. This link between the layered structure and function is not trivial and is a challenge to be met in the future for tissue engineering of articular cartilage.

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