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Ultrasonic wave propagation assessment of native cartilage explants and hydrogel scaffolds for tissue engineering

Sean S. Kohles^{*}, Shelley S. Mason, Anya P. Adams, Robert J. Berg, Jessica Blank, Fay Gibson, and Johnathan Righetti

Regenerative Bioengineering Laboratory, Departments of Mechanical & Materials Engineering and Biology, Portland State University, Portland, Oregon, USA

Iesha S. Washington and Asit K. Saha

Center for Allaying Health Disparities through Research and Education (CADRE), Department of Mathematics & Computer Science, Central State University, Wilberforce, Ohio, USA

Iesha S. Washington: wiesha87@yahoo.com; Asit K. Saha: ASaha@centralstate.edu

Abstract

Non-destructive techniques characterising the mechanical properties of cells, tissues, and biomaterials provide baseline metrics for tissue engineering design. Ultrasonic wave propagation and attenuation has previously demonstrated the dynamics of extracellular matrix synthesis in chondrocyte-seeded hydrogel constructs. In this paper, we describe an ultrasonic method to analyse two of the construct elements used to engineer articular cartilage in real-time, native cartilage explants and an agarose biomaterial. Results indicated a similarity in wave propagation velocity ranges for both longitudinal (1500–1745 m/s) and transverse (350–950 m/s) waveforms. Future work will apply an acoustoelastic analysis to distinguish between the fluid and solid properties including the cell and matrix biokinetics as a validation of previous mathematical models.

Keywords

transmission wave elasticity; ultrasonic elasticity; acoustoelasticity; cartilage engineering; hydrogel biomaterials; cartilage biokinetics; biomedical engineering; bioengineering

1 Introduction

Cartilage tissue engineering (TE) is a promising solution for cellular and tissue replacement therapies as well as an alternative for animal models used in clinical research; however, many of the current TE approaches have not been fully validated. Most mechanical and biochemical assessment of TE constructs require destructive endpoint-testing and/or compromise the sterility of the bioreactor environment used during construct formation (Walker et al., 2011). Ultrasonic imaging is a standard clinical diagnostic tool that is based on propagating sound waves and offers a technology for tissue characterisation and stimulation in a non-invasive and non-destructive manner. The response to mechanical stimulation induced in passive tissues by external sources such as low to high frequency vibration or compression can be used to analyse tissue elasticity as a metric for tissue health (Hein and O'Brien, 1993). Recently, it has been shown that ultrasonic assessment can

predict the process of native cartilage regeneration and engineered cartilage histology (Hattori et al., 2005).

Native articular cartilage is divided into four distinct histological zones (superficial tangential or resting zone; the proliferative or middle zone; the hypertrophic or deep zone; and the calcified zone) based on the preferential orientation of matrix molecules, cellular morphology, and biochemistry (Buckwalter and Mankin, 1997). Ultrastructural studies of adult cartilage have shown that the preferential orientation of collagen fibrils varies from the articular surface to the intermediate and deep zones (Agemura et al., 1990). The depth-dependent structural arrangement of chondrocytes and extracellular matrix (ECM) macromolecules, such as proteoglycans (PG) and fibrillar collagens (mainly collagen type II), results in a structurally heterogeneous and mechanically anisotropic material. For example, at any given depth from the articular surface, PG concentration may change as a function of the distance from the chondrocytes (Jurvelin et al., 1997). The zonal distribution of ECM molecules gives rise to the tissue's unique poroelastic properties and ability to act as a load-bearing cushion in which mechanical forces transmitted through the joint to the underlying subchondral bone can be properly distributed (Waldman et al., 2004).

The localised biomechanics (stresses, strains, and elastic properties) of articular cartilage ECM is thought to be actively modulated by chondrocyte-matrix interaction driving biosynthesis regulation (Korhonen et al., 2008; Saha and Kohles, 2012). Characterising the mechanical properties of cartilage constituents including cells and ECM as a means to define constitutive relationships between stress and strain may elucidate their physical interactions and contribute to the understanding of cartilage development, adaptation, and degeneration (Kohles et al., 2007). In addition, the distinct histologic zones within articular cartilage have exhibited distinct material properties at both the cell and tissue level (Ginat et al., 2006; Shieh and Athanasiou, 2006).

Selection of cells and replication of matrix organisation based on zonal mimicry may provide strong design specifications when constructing regenerative or tissue replacement therapies. In this work, ultrasonic wave transmission was investigated as one of many baseline bioengineering design metrics with the objective of characterising and comparing key engineered cartilage constituents.

2 Methods

2.1 Articular cartilage harvest and ultrasonic testing

Full-thickness articular cartilage was harvested from a two-year old steer (Mark's Meat, Canby, OR) within 4 hours of slaughter. The cartilage was extracted from the metacarpophalangeal joint using a scalpel (Figure 2). Full-thickness cartilage parallelepipeds (typically $x = 4$ mm, $y = 4$ mm, and z or $t = 1.5$ mm) were created from the tissue samples. Ultrasonic longitudinal (v_{ij}) and transverse (v_{ij}) wave propagation velocities were measured in three orthogonal orientations (planar and thickness directions indicated by i, j subscripts) for 6 measurements per sample (Figure 1). The experimental test set-up included a pulser-receiver (Model 5058PR, Panametrics, Billerica, MA, USA), a multichannel oscilloscope (Model TDS460A, Tektronix, Beaverton, OR, USA), and an array of sending-receiving transducers: 5 MHz (Model V156), 10 MHz (Model V112), 50 kHz (Model X1021), and 100 kHz (Models X1020 and V1548) transducers (Panametrics) facilitating a total of 1050 measurements. Aggregate tissue density (ρ_l) of the samples was determined and system time delays were accounted for during each transducer arrangement.

2.2 Hydrogel scaffold fabrication and ultrasonic testing

An agarose hydrogel formulation has been widely accepted as a host-biomaterial for cartilage TE culture (Nicodemus and Bryant, 2008), providing an appropriate technology for the bioengineering of modern scaffolds (Mata, 2011; Morsi et al., 2011). Here, a 2% agarose solution was made by slowly adding low-melting agarose (Type VII, Sigma-Aldrich, St. Louis, MO, USA) to a beaker containing Dulbecco's phosphate buffered solution (DPBS, Sigma-Aldrich) while being stirred on a hot plate. The concentration change due to excessive heating was taken into consideration in order to maintain a 2% concentration by weighing the beaker and solution prior to heating. The solution was brought to a boil for 10 minutes stirring continuously until the agarose was completely dissolved. Hot sterile water was added to return the contents to the original weight and mixed continuously. The mixture was allowed to cool to 45°C and casted in a 5 mm × 5 mm × 5 mm mould, immediately being placed in a refrigerator at 4°C for 10 minutes. For an equal-weighted statistical comparison with the cartilage explants, 35 agarose samples were prepared (Figure 2). Due to the homogeneity of the 125 mm³ agarose cubes, ultrasonic longitudinal (v_{ij}) and transverse (v_{ij}) wave propagation velocities were only measured in the z-direction. Three measurements per sample from each of the five sending-receiving transducers, described above, were used facilitating a total of 525 measurements per sample. Aggregate biomaterial density (ρ_b) of all of the samples was determined and system time delays were accounted for during each transducer arrangement.

2.3 Elastic and statistical analysis

The measured propagation velocities were examined as a distinguishing elastic feature between the cartilage/hydrogel structure (when wavelength, $\lambda > t$, typically at kHz) or the constituent matrix/polymer material (when $\lambda < t$, typically at MHz) (Kohles and Roberts, 2002). Transverse isotropy was tested and confirmed in the cartilage samples, while full-isotropy was affirmed in the agarose samples (Kohles et al., 1997). Generalised stiffness or moduli (as a Young's and Shear Moduli variants) were calculated (ρv_{ij}^2 and ρv_{ij}^2) for both the cartilage explants and agarose samples. An analysis of variance was applied for all comparisons (JMP v5.0.1, SAS Institute, Inc., Cary, NC, USA). Means (+/- standard deviation, SD) are shown for graphical comparisons.

3 Results

In this work, ultrasonic measurements analysed an agarose hydrogel formulation as a scaffold substrate for cartilage TE in comparison with native cartilage samples. The analysed cartilage samples confirmed an aggregate density (here $\rho_t = 1330 \text{ kg/m}^3$) and both longitudinal and transverse propagation velocities similar to that reported in the literature ($v_{ij} = 1500\text{--}1720 \text{ m/s}$) (Agemura et al., 1990) (Figure 3). The statistical influence of propagation orientation on longitudinal ($p = 0.8763$) and transverse ($p = 0.0006$) wave velocities in the native cartilage samples was statistically inconsistent. However, the wavelength of propagating waves relative to the propagating distance as indicated by MHz ($\lambda < t$) versus kHz ($\lambda > t$) frequencies was statistically significant for both longitudinal ($p = 0.0001$) and transverse ($p = 0.0001$) waves within the cartilage samples.

In comparison, the fabricated agarose cubes had a mean density of $\rho_b = 1110 \text{ kg/m}^3$. A longitudinal propagation velocity range of 1595–1745 m/s was also determined for the biomaterial indicating a statistical similarity ($p > 0.05$) with native cartilage (Figure 4). Overall, the basic calculation addressing the ultrasonic elastic and shear moduli for both the cartilage and hydrogel samples was dominated by similar density, propagation velocity measurements, and water content (nearly 80%) (D'Arrigo and Paparelli, 1988). These influences produced statistically similar ($p > 0.05$) hydrated stiffness values having large

magnitudes dominated by the incompressible nature of water when assessed with both longitudinal and transverse wave propagation modes (Figure 5).

4 Discussion

The application of ultrasonic wave propagation was explored as a means to compare an agarose biomaterial as a scaffold for cartilage TE design. Here, the results provided baseline measurements for analysing future neotissue growth in a non-destructive manner. It was found that 2% agarose constructs had an ultrasonic propagation velocity similar to that of native cartilage, and that this approach may be used to assess the integrity of agarose constructs seeded with chondrocytes while ECM macromolecules are being synthesised without destroying the engineered tissue.

Due to the aqueous nature of both the articular cartilage explants and the agarose hydrogels, the reported propagation values are highly dominated by the mechanical wave transfer through the water constituent. As a limitation in this approach, the ultrasonic propagation velocities themselves may not provide critical information in assessing native or TE cartilage. However, the signal resolution may be tuned in a manner sufficient to identify cellular or molecular influences on wave propagation during chondrocyte and ECM biokinetics. The difference in ultrasonic signals, when comparing isolated agarose with cell-seeded agarose, may be used to assess the elastic properties of chondrocytes and newly developing ECM. Mechanical loading of hydrated materials such as cartilage tissue has also been shown to influence ultrasonic propagation velocity measurements (Niemenen et al., 2007). Tissue-equivalent phantoms may eventually aid in standardising the accuracy of developing cell-biomaterial construct measurements (Singh et al., 2008).

Articular cartilage is a highly hydrated tissue due to the negatively charged PG that binds fluid within its matrix. The mechanical performance of cartilage is dominated by these solid/fluid interactions. When the tissue degenerates through injury or disease, the PG and collagen network is disturbed, altering the load transfer to the subchondral bone. This cascade of degeneration ultimately compromises the tissue and joint mechanical properties, as recently assessed with ultrasound (Brown et al., 2007). Ultrasonic wave transmission offers a highly resolved technique for characterising these subtle changes in the tissue properties. However, distinguishing the ultrasonic propagation through its water content (~ 1482 m/s) from the composite solid components (ECM and cells) will be very challenging. Ongoing efforts will apply forward and reverse acoustoelastic analysis to decipher bulk ($K = \rho_f v_{if}^2$) and aggregate ($H_a = \rho_s v_{if}^2$) moduli, which can be measured from separated fluid (f) and solid (s) ultrasound signals, respectively (Shull, 2002; Kobayashi and Vanderby, 2007).

Recent efforts have correlated matrix content and mechanics in developing engineered cartilage constructs with positive success by applying reflective ultrasound (Rice et al., 2009). In that work, the speed of sound (SoS) and slope of attenuation (SoA) were compared between developing cell-biomaterial constructs and non-degrading hydrogel controls from 50 MHz to 100 MHz ultrasound data. SoA was shown to be a better indicator of the density of accumulated matrix molecules than SoS, while SoS correlated better with mechanical modulus than SoA. These promising data have encouraged the incorporation of ultrasound sensors as described within two recent reports characterising a novel bioreactor design for cell and tissue engineering (Mason et al., 2011; Popp et al., 2012). This non-destructive approach reduces the bench-top assessment modality to a practical tool for online, real-time evaluation of the developing analogous living system created within the bioreactor environment.

As neotissue forms within the cell-seeded agarose constructs, it will be important to detect any critical changes to the mechanical properties in a non-destructive manner in order to optimise TE development. Overall, ultrasonic wave propagation offers a potential means to gather constituent content and mechanical metrics for characterising the biokinetics of native and engineered cartilage.

5 Conclusion

In this work, we explored the application of ultrasonic propagation velocity as a metric for comparing native cartilage explants and a hydrogel biomaterial as design specifications for ongoing cartilage tissue engineering. This approach has identified both the benefits of interfacing ultrasonic sensors with a novel bioreactor as well as the limitations of assaying hydrated materials, where the resulting wave propagation is dominated by the water content. A more refined acoustoelastic analysis will facilitate distinguishing the cell and matrix biokinetics as modelled previously.

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Biographies

Sean S. Kohles is an Adjunct Professor and Director of the Regenerative Bioengineering Laboratory at Portland State University. He completed a doctoral degree in Bioengineering and bachelors/masters degrees in Engineering Mechanics at the University of Wisconsin-Madison. He has published over 200 journal and conference papers in the areas of cell, tissue, joint, biomaterials, and forensic biomechanical engineering and serves on the Editorial Boards for the *Journal of Biomechanics*, the *Annals of Biomedical Engineering*, and the *Journal of Biomechanical Engineering*. He has maintained funding for his research from the National Science Foundation, the National Institutes of Health, and private foundations, while training over 1200 students.

Shelley S. Mason is a Biology doctoral student at Portland State University specialising in bioengineering applications. She received her bachelors of science in Biology from Portland State University after serving in the US Navy. She currently works as a Research Assistant in the Regenerative Bio-engineering Laboratory examining the treatment of vitamin D metabolites on the growth of engineered bone and cartilage tissue grown in a novel bioreactor. She is also investigating the phenomenon of mechanotransduction during engineered tissue development. Her research interests include both regenerative and preventative medicine. After graduating, she is interested in continuing her research career as a Postdoctoral Associate.

Anya P. Adams is currently a graduate student at Stanford University, pursuing a masters degree in Mechanical Engineering with a concentration in Mechatronic Design. She previously received a bachelors degree in Biomechanical Engineering with a focus on Medical Device Design, also from Stanford University. During her undergraduate experience, she served as an intern within the Regenerative Bioengineering Laboratory at Portland State University. After her graduate training, she intends to seek an engineering design position within the medical device industry.

Robert J. Berg is a National Science Foundation Graduate Research Fellow in the Electrical and Computer Engineering Department at the University of Texas at Austin where he is working towards his masters and doctoral degrees. He is currently studying software engineering with a strong emphasis on machine learning algorithms for distributed systems. His work is particularly aimed at knowledge discovery and efficient mining of Big Data. During his internship within the Regenerative Bioengineering Laboratory, he was an undergraduate in Mechanical Engineering at Tufts University. While earning his bachelors degree, he focused on control systems and the dynamic mechanical performance of biomaterials in *vivo*.

Jessica Blank is currently a graduating senior from Tualatin High School in Oregon. She will be completing a challenging curriculum of college level courses via the International Baccalaureate Diploma Program. Upon graduation, she will be entering the Oregon State University Bioengineering and Pre-Medical Studies Programs as a Presidential Scholar in the Honours College. She served as a Saturday Academy Science and Engineering Intern during her apprenticeship in the Regenerative Bioengineering Laboratory. Her future interests include pursuing a bioengineering graduate degree or medical school with a specialisation in Oncology.

Fay Gibson is currently a senior in the Mechanical & Materials Engineering Department at Portland State University. She is Lab Assistant within the Regenerative Bioengineering Laboratory, having been supported by an Undergraduate Leadership Award and currently as a member of the MCECS Research and Mentor Program. Her projects have included work on biomaterial scaffold design and validation of a mechanical bioreactor. After completing her bachelors degree in Mechanical Engineering, she intends to pursue graduate training in Biomedical Engineering. Her career goals are to design and develop products that improve the quality of life for those who are limited in movement and function, whether as a result of trauma, genetic defects, or degeneration.

Johnathan Righetti is an undergraduate Biology and Chemistry student at Portland State University, pursuing a specialty in Organic Chemistry. He received a bachelors degree in Economics from the University of Montana, with an emphasis in health systems and student debt. He is currently a Lab Assistant and member of the MCECS Research and Mentor Program in the Regenerative Bioengineering Laboratory, specifically aiding in the harvest and culture of bovine chondrocytes, and their reintroduction into three-dimensional scaffolds. He intends to enrol in medical school in the fall of 2014. His areas of interest in medicine include Orthopaedics/Sports Medicine, Emergency Medicine, Cardiology, or Anaesthesia.

Iesha S. Washington is currently an undergraduate biology major at Central State University pursuing an interest in musculoskeletal physiology with a minor in mathematics. She previously served as a CADRE Bioengineering Intern in the Regenerative Bioengineering Laboratory. Upon graduation, she hopes to matriculate into medical school and pursue her interest in Oncology. She is currently a member of Beta Beta Beta, the Lambda Iota Chapter of the National Honor Society for biology majors.

Asit K. Saha is an Associate Professor of Mathematics at Central State University. He completed his second doctoral degree in Knowledge Based Tissue Engineering from the University of South Australia in 2007. His research areas include system dynamics modeling approaches to cell-matrix interactions, human immune systems, stress related disorders in the brain, and embryonic pattern formation/morphogenesis. He has over 30 journal publications describing his research in biomathematics and serves on the editorial board for

the *International Journal of Computers in Healthcare*. His research is funded by the National Institutes of Health and addresses health disparities.

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Figure 1.
Schematics of explant anatomic orientation and propagating ultrasonic waveforms

(a)

(b)

Figure 2.
Images of (a) harvested bovine cartilage tissue and (b) fabricated agarose hydrogel scaffold cubes

Figure 3.

Mean ($\pm$ SD) (a) longitudinal and (b) transverse propagation wave velocities as gathered from through-thickness (anterior-posterior) and within-plane (superior-inferior plus medial-lateral) orientations of harvested cartilage explants

Figure 4.
Comparison of mean ($\pm$ SD) ultrasonic propagation velocities of $n = 35$ agarose biomaterial samples as driven by longitudinal wave impulses at varying frequencies

Figure 5.

Fluid-dominated stiffnesses of the agarose biomaterial scaffolds and cartilage explants as measured using the 100 kHz longitudinal and transverse wave transducers