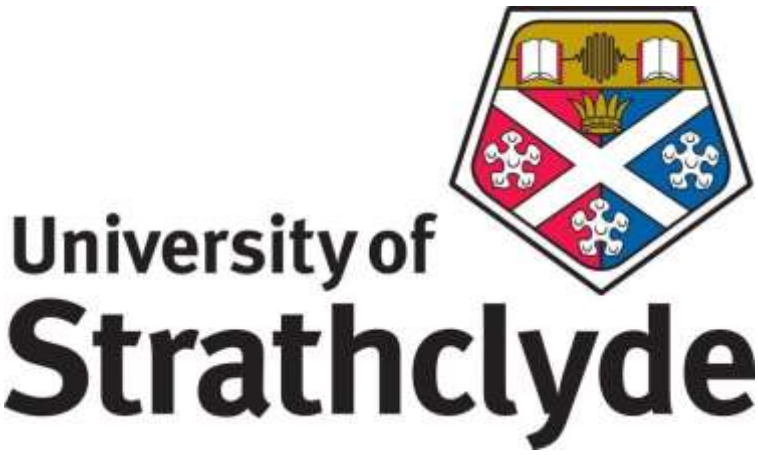

Department of Biomedical Engineering

Masters of Science Dissertation

Dynamic Mechanical Analysis of Articular Cartilage Using Nanoindentation

Written By David Bryant Moore Jr.

Supervised by Dr. Philip Riches

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Declaration

‘This thesis is the result of the author’s original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.’

Signed: Date:

Acknowledgments

I would not have been able to complete this project without a host of people helping me along the way. My supervisor Phil Riches was a fountain of knowledge throughout the experience, lending insightful help when I hit a wall or directiom when I became unsure. He was also a calming force; whenever an issue arouse with technology or the ever dwindling time frame he sagely supplied what needed to happen next in order to fix or fulfil that hurdle I was currently struggling with. I'd also like to specifically thank Milovan Cardona, who provided equipment training and was a sounding board for all of my questions and ideas. Not only did he go out of his way to help me on multiple occasions, he also treated me as a friend from the very start. Finally I'd like to acknowledge and thank the rest of my support group, from my university class mates to my family sending support from overseas.

Abstract

The aim of this study was to use nanoindention in a novel attempt to characterize the behaviour of articular cartilage. Using bovine femoral heads, viscoelastic properties of the tissue were determined for five frequency loading magnitudes; 1 Hz, 2 Hz, 5 Hz, 10 Hz, and 25 Hz. Dynamic mechanical analysis was used to measure the δ, storage and loss modulus of the samples. The testing showed that the storage modulus displays frequency dependence up until higher frequencies where the response plateaus and becomes insensitive to frequency. The loss modulus was found to have no frequency dependent qualities. Throughout all frequencies the storage modulus has far greater values than loss modulus, with the disparity between the two increasing with frequency. . The excess energy being stored as opposed to lost can cause tissue damage, which potentially is a mechanism of the onset of Osteoarthritis.

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1. Introduction

Osteoarthritis is the most prevalent joint condition in the world, affecting hundreds of millions of people every year. There are no actual treatments for this condition, just symptom mitigation or joint replacement. It is hoped that with an early detection of this condition preventative measures may be taken to avoid later stage osteoarthritis, along with the symptoms and risks that go along with it. Currently there are no techniques or technology capable of detecting the onset of the condition, with only late stage degradation being visible via standard imaging techniques. Early stages of this condition are characterized by biochemical and morphological changes throughout the tissue. An endoscopic tool that can detect changes in tissue surface and behavior could be an enormous asset in earlier diagnosis of this disease. But for such a diagnostic tool to be created, an intimate knowledge of the cartilages surface mechanical characteristics needs to be achieved. This project hopes to support or even add to what is known about articular cartilage's mechanical properties, using a novel application of a proven method.

1.1. Tissue Structure

Articular cartilage is a highly specialized connective tissue of diarthrodial joints that provides a smooth, lubricated surface for articulation of motion and promotes transmission of loads with a low frictional coefficient. The mechanical behavior depends on the interaction between its fluid and solid components (Mansour et al 2014). The complex structure of articular cartilage makes its treatment and repair a problem. With this in mind many scientists over the decades have tried to better understand and define this specialized tissue so that with a greater understanding can lead to improved medical diagnostics and potentially treatments.

Cartilage is composed of chondrocytes cells. These chondrocytes are characterized by their production large amounts of extracellular matrix. The extracellular matrix is in turn composed of collagen fibres, proteoglycan, and elastin

fibres. Even though cartilage has a vast amount of fluid in the tissue, there are no blood vessels in cartilage to supply the chondrocytes with a constant blow support, meaning all oxygenation and nutrients must be brought into the cells through a much slower concentration gradient system or through convection. This has a large effect on the tissues inherent growth and more importantly for this studies interests, the tissues regeneration, or lack thereof. Cartilage is notorious for its lack of any advanced tissue replacement, which is why conditions such as osteoarthritis are so prevalent in mid to late stage patients, but that will be discussed a little later on. Back in the components of cartilage, the collagen fibrils play a huge role in both structure and function, especially in the higher specialized subset of cartilage that we are interested in characterizing. Articular cartilage is widely characterized by having different zones. These zones include the superficial tangential, the transitional, the deep, and the calcified zone. Each zone is separated due to differences in cell orientation, shape, and concentration of the main components of the ECM (extracellular matrix) as shown in Figure 1. The superficial tangential zone is characterized by densely packed thin collagen fibrils that are aligned parallel to the tissue's surface, while the chondrocytes in this zone have an elongated shape. In the transitional zone collagen fibrils are more loosely aligned and no longer in an organized parallel to the surface but more in a splattering of different orientations. The fibrils themselves appear to be thicker, while the chondrocytes take on a less elongated and more rounded. Both these occurrences may be a result of the looser packing. In the deep zone, collagen fibrils and chondrocytes are arranged in a columnar fashion, parallel to each other, aligned perpendicular to the tissues surface. The fibrils are also the largest in diameter in this zone, This layer has the highest proportion of proteoglycan and lowest concentration of water. Finally there is the small calcified zone of cartilage that connects the tissue to the cancellous bone underneath. This is all quite a radical change through a single tissue structure, that scientist have attributed to the cartilage's function as a compressive loading pad, with the superficial and transitional zones allowing for shock absorption and immediate deformation, while the deep zone provides for the true loading platform with minimal deformation. Deeper layers are relied on more in regards to supporting applied loads and dissipating energy within articular cartilage

(Setton et al 1993). The orientation and packaging of the fibrils also is attributed to many of the mechanic properties of the tissue. Collagen fibril orientation has been attributed as the main reason for a lower tensile stiffness and higher lateral expansion possessed by articular cartilage than seen in other superficial tissue (Mow et al 1995). Chondrocyte behaviour also alters depending on zone, which in turn affects the behaviour of the tissue as a whole, even though all of this is at a micro scale. Chondrocytes provide a huge difference in the mechanical environment of articular cartilage, significantly altering the principal stress and strain magnitudes in modelling as well as mechanical testing (Kovach 1996).

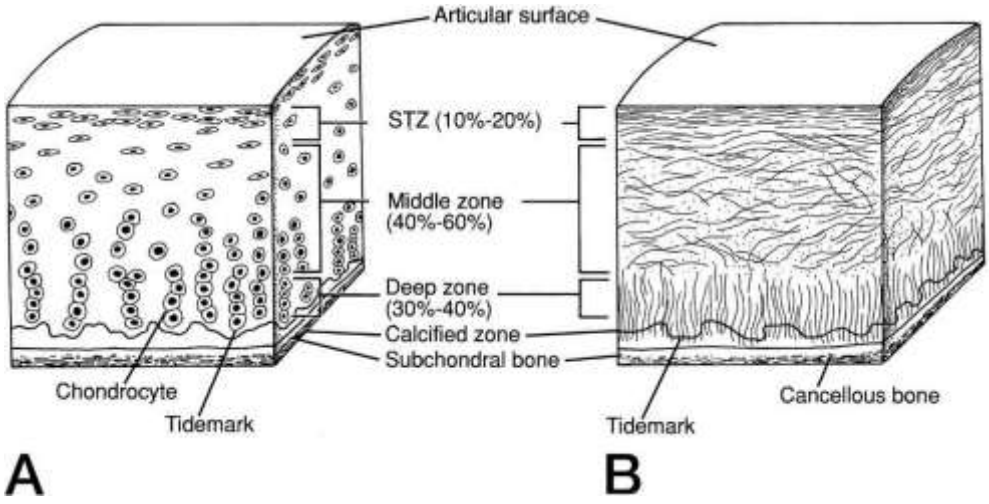


Figure 1: Zonal structure of articular cartilage: A. cellular organization B.collagen fiber architecture. (J Am Acad Orthop Surg 2(4): 193., 1994).

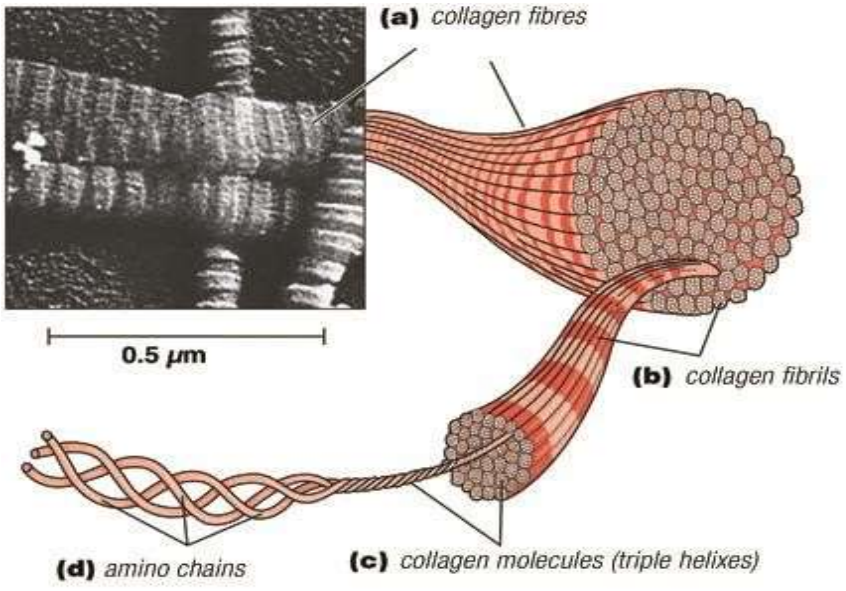


Figure 2: Collagen Fiber microstructural formations. (Image from Scitec Nutrition®. Copyright © 2015).

Nanostructure of the individual cell structures must also be considered when considering complex biological behaviour. Inside each collagen fibres many different fibrils, which in turn are composed of many collagen molecules. Finally those molecules are triple helix formations of coiled polysaccharide chains. These minute scale structural configurations have been shown to have definite mechanical implications. For instance the volume dependence of the polysaccharide configuration can be used to describe the charge independent portion of cartilage elasticity (Mow et al 1995). Other characteristics of cartilages mechanical behaviour, such as its timedependent creep can be explained by its free draining coil structure (Mow et al 1995). Similar inferences would be expected from chondrocytes but the details of how the mechanical loading of joints influences the function of said chondrocytes remains unknown, but the deformation of the matrix produces mechanical, electrical, and physiochemical signals that can have major roles in stimulating chondrocytes. On an even smaller scale there has been research into the effects of individual pieces of a polysaccharide chain in the bundle fibres of a helix, dealing with drag on small segments of the GAG chains, the frictional drag associated causing fluid flux (Mow et al 1995). What's more is that even non cell types effect how the tissue behaves. The interstitial fluid inside the ECM is just as important to the compressive loading response of the tissue as the fibres and chondrocytes themselves. A non-linear

permeability function has shown drastic effects on the compressive creep and stressrelaxation behaviour of articular cartilage tissue, due to the phenomenological viscoelastic effects, which is mostly determined by the rate of interstitial fluid flow. It was shown that compaction of the tissue will increase its fixed-charge density and hence decrease permeability (Fox et al 2009). This permeability would also change due to tissue damage and wear associated with conditions such as osteoarthritis. It is plausible that understanding any of these structural indicators and mechanisms can give scientists an indicator as to what to look for in early diagnosis and treatment of OA. But knowing how the miniature structures function to help the tissue preform its task is only half the picture, since the tissues behaviour as a whole is just as crucial. The tissue is not just a sum of its parts, there are many characteristics and behaviours that cannot be described by molecular and individual microstructural analysis. To try and predict these complex interactions modelling techniques are used.

1.2. Viscoelastic Theory

Viscoelastic theory is the idea that there are materials that exhibit both solid and liquid, or elastic and viscous, behaviours. In essence a material in between a solid or a liquid, at least in the context of responses external stresses or strains. All biological tissues fall into this category to various degrees. This theory characterizes the initial response of a material to a stress or strain and then again as it changes as the material goes towards equilibrium. In contrast a single elastic or viscous material such as a pure metal or water will have a single and constant response that will perform as either purely elastic or viscous, with no change in overall time dependent behaviour.. The different segments are described mathematically as the storage and loss moduli. The storage modulus being the elastic characterization of the energy stored in the material when a stress or strain is applied, while the loss modulus is the viscous portion that describes the energy that is lost from the material when a stress or strain is applied. The pair can then be shown as a ratio that can mathematically represent the tissue, which is described as the δ. This δ is the final description mathematically of how viscoelastic a material is, and is what is used to compare various tissues to one another. So since a tissue such as articular cartilage displays both fluid and solid reactions to stress, it is characterized as a viscoelastic material. More precisely at mechanical

equilibrium, articular cartilage is usually characterized as an isotropic elastic material with no interstitial fluid flow (Korhonen et al 2002). But before it reaches equilibrium cartilage exhibits viscous behaviour, as would seem likely since there is a great deal of fluid in the tissue such as the ECM. Because of these facts articular cartilage as a whole is usually characterized as an elastic solid. This type of characterization has be determined by a varied array of tests over the decades, mainly due to the classic confined and unconfined compression tests, with more specialized and smaller scale testing following after. Using compression testing the Young's modulus of articular cartilage was determined to be in the range of 0.45 to 0.90 MPa, the Possion's ratio was found to be between 0.0 to 0.28, and the permeability was found typically in the range of 10E-15 to 10E-16 m4/Ns (Royce 2001), (Korhonen et al 2002). There is variability between individual tissue samples as well as between species, as would be expected in a biological tissue. Interestingly there is even variability in the tissue due to location in a joint (Bayliss et al 1999). Differences in stiffness may be attributed to the permeability of the interstitial fluid as opposed to differences in the collagen bundles. In softer cartilage the chondrocyte is subject to large elastic matrix strains coupled with little fluid flow, whereas chondrocytes in stiffer cartilage samples are subject to smaller elastic strains of the surrounding matrix coupled with greater local fluid flow (Barker et al 2000). This puts great emphasis on the fluid flux in the tissue as determinate of behaviour. Interstitial fluid flow may be considered a rate-limiting process in governing tissue behaviour under conditions of low hydraulic permeability, which is the case for healthy articular cartilage. For small loading times and a few other specific loading situations, the flow of interstitial fluid through the ECM can be considered as the dominant transient response, controlling both the solid stress and strain fields, as well as how the forces dissipate. The intrinsic viscoelasticity of the solid matrix of the ECM has been shown to significantly alter the load-carrying and energy-dissipating behaviours of the tissue (Setton et al 1993). This means a characterization which incorporates both solid matrix viscoelasticity and biphasic behaviour would be crucial to fully characterize such a complex tissue response.

There have also been quite a few studies involving cartilage in microindetation style testing combined with dynamic material analysis. These indention tests give information on how the tissue reacts on not only on a more isolated scale but also gives

a picture of how a tissue reacts at different frequencies of loading stress. Response to frequency loadings of varied degrees simulates different real world conditions, such as slow flexion of a knee to a sharp sudden force application while during running. Differences in force application effect the tissues response, as shown by theoretical studies that suggest that flow-dependent viscoelasticity is less significant than flowindependent viscoelasticity at higher frequencies of loading (Hayes et al 1978). Not only that but it has been shown with increasing loading frequency the tissue increasingly stores the energy input in the form of elastic strain (Lia et al 2005). Studies in frequency dependence loading in regard to the elastic and storage moduli of the tissue and the phase angle all characterize the viscoelasticy of the tissue in that certain instance, which can be compared and extrapolated to the tissue as a whole. The phase angle of articular cartilage has been shown to reduce to zero at a 40 Hz loading in microindetation, which suggests that above this frequency cartilage behaves effectively as an elastic solid and that the dynamic modulus should not be expected to increase past said point, remaining constant at higher frequencies (Guilak et al 1999). This is a characterization of the microstructures of the tissue which inherently has issues to be considered, chief among them the fact that the tissue is not acting as a whole. Other issues that are to be aware of with such testing of individual parts is tissue damage. In micro indentation, extraction of the cylindrical samples from the tissue damages its fibrous structure, which leads to reduced compressive stiffness. A higher Young's modulus was found from the indentation test than in both confined and unconfined compression tests, indicating a difference between the mechanical status of an intact and isolated cartilage samples (Park et al 2004) (Jurvelin et al 1990). This damage inherently changes the mechanical function. By using a nano indention test it is hoped that this isolation damage effect will be minimized due to the minute nature of the testing zone and mechanical implications. There are other considerations beside the tissues condition that have been looked into as well. Strain rate related effects can contribute to the overall indentation behaviour of tissue. By sampling at a varied range of frequencies it is hoped that these differences will normalize and not become biased by a specific sampling strain rate. It has also been shown that more accurate analysis of indentation readings could be gathered by taking into account the intrinsic matrix viscoelasticity (Lia et al 2004). These are some of the considerations and properties

that have been concluded about articular cartilage within the bounds of the viscoelastic theory's thinking.

1.3. Biphasic Theory

An alternative approach to tissue mechanical anaylsis is Biphasic Theory. Biphasic or Poroviscoelastic theory is similar to the viscoelastic theory in the sense that both theories describe materials as being of both a viscous and elastic nature. The two theories differences lie in how the tissue is shown to react in comparison to its components. In viscoelastic theory the material is treated as a whole, reacting to the stresses or strains applied in a homogenous and uniform way. The entire material reacts immediately as one way and then shifts to another as it reaches equilibrium. In biphasic theory the material is considered as having two separate phases, one elastic and one viscous. These two heterogeneous phases both work together in one space, but are not completely dependent on each other. Both phases react to the same stress or strain at the same time, just at different rates. Both phases move towards equilibrium at separate rates as well. For a material described using biphasic theory, the stress relaxation or creep comes from the reaction of the fluid flowing relative to the solid, as opposed to the entire tissue acting as one to relax. This method of thinking is a better, more thorough approach to dealing with tissues with such large fluid flux like that of articular cartilage, but it has many shortcomings in the experimental side. It is difficult to observe and record data of internal fluid flux while preforming most standard stress testing. To this effect, most studies into biphasic theory are currently theoretical, using models to predict and characterize tissue behaviour. That being said there have been a few recent studies that have produced Biphasic poroviscoelastic models that have been shown to accurately predict experimental data response of articular cartilage in unconfined, confined, and indentation testing (DiSilvestro et al 2004). The data was compared to separate experimental tests in a specific loading situations, which implies the model may be correct in a certain well defined situation, but that does not show evidence of a full working model. These models tend to become quite complex in their efforts to effectively map the correct tissues responses, though there have been claims

of simplification using a Conewise Linear Elasticity model to describe the solid phase of the tissue. This was shown to reduce the number of elastic constants by a factor of 3 when modelling, therefore making the model less arduous to apply and compare with experimental data (Soltz et al 2000). This is not uniformly recognized however as this methods robustness has been questioned. Many state that the complexity of the tissue with its myriad microstructural components and zonal changes cannot be simplified as such, even if in the simplification it remains immensely complex. It is argued that the structures from collagen fibre to fibril, as well as the chondrocytes, must be taken into account as a primary concern in relation to the biphasic's ever present fluid flux. In this vein a fibril-reinforced cartilage model that accounts for the complex arcade structure of the collagen network in the tissue was developed, though at the time was not fully vetted (Wilsona et al 2004). This accountants of the microstructural components in the effect on the tissue is moving the field in a promising direction, but seems to still be in the early stages. There have been many other models developed over the years as well, all in the hope to characterize articular cartilage behaviour (Guilaka et al 2000) (Ehlers et al 2002). The issue becomes that there have been a vast number of models produced, but very few in the similar fashion, and none of which are truly universally recognized and considered as a gold standard to work with and to compare against. This is not a unique problem for this particular tissue by any means, but the underlying problem remains; there is not enough known about how biological tissue reacts, especially not from the early diagnostic and tissue replacement/engineering standpoint.

1.4. Osteoarthritis

Understanding the tissue, its mechanical function, its micro and nano structural composition is not just a purely academic pursuit that scientists have pursued over the decades for curiosity. This research is important in many different medical regards, chiefly among them in the diagnosis and treatment osteoarthritis. Osteoarthritis is the

most prevelant joint condition in the world, affecting an estimated 27 million people in the United States alone, with an untold millions of people worldwide (CDC). The condition is described as a wearing down and damaging of the cartilage covering the ends of the bones. The cartilage gradually roughens and thins as the condition progresses. Once the tissue covering the bone becomes thin enough the bone itself is affected and starts to thicken underneath. As the tissue tries to repair the damage, cells become more active, including the synovium which may thicken and produce extra synovial fluid. This in turn causes joint swelling and increased irritation of the site, causing pain to the patient. In late stages when the cartilage is nearly depleted the bone at the edge of the joint grows outwards, forming bony spurs called osteophytes. Capsules and ligaments slowly thicken as well, contracting in an effort to stabilize the no longer smooth joint motion, which is seen in patients as loss of movement range and joint stiffness. Current diagnostics relies heavily on symptom occurrence and severity, relying on the condition to progress to a point where significant pain or irritation brings the patient in for a check-up. The medical practitioner then makes a diagnosis based on the physical evidence, the patient's testimony. Possibly an X-ray may be taken of the joint in question, looking for osteophytes or a significant reduction in distance between bones, an indication of cartilage loss. In some cases MRI are used to closer inspect the soft tissue, usually in regards to surgical possibility. Current treatment options for osteoarthritis rely heavily on treating the symptoms as opposed to the condition itself. A myriad of pain killers are commonly prescribed, from organically derived capsaicin creams to drugs such as tramadol, nefopam or meptazinol. There have been trials using stronger painkillers such as oxycodone, but the risk of side effects makes it not a widely used course (Cadwell 1999). Other options such as steroidal and non-steroidal anti-inflammatory drugs are often prescribed in concurrence. There are other physiotherapist regimes that have shown some improvement in patients, if in an arguably small manner (van Barr et al 1998) (Garfinkel et al 1994). The final non-surgical option is known as viscosupplumentation. In this procedure a hyaluronic acid is injected into the knee joint through a series of injections over aperiod of weeks. This hyaluronic acid is a chemical found in the synovial fluid surrounding the joints that acts as a lubricant for the smooth articulation and load transmission. Patients with osteoarthritis have been shown to have

a lower concentration of this hyaluronic acid in their joints. By adding hyaluronic acid to the arthritic joint over the course of the treatment the procedure hopes to refacilitate smooth articulation and reduce the patient's pain. This procedure while interesting in its use of biological changes in the joint to return joint function has not been proven to be very effective as a treatment. There have been a few studies done on its effectiveness, none coming back with promising results (Balasz et al 1994) (Lussier et al 1996). Only in severe situations is treatment of the underlying condition considered, via surgical methods. The most common and effective is total knee arthroplasty or replacement. This procedure removes the entire joint and replaces it with an artificial one, usually of both metal and plastic components. The metal parts of the implant are commonly of titanium or cobalt-chromium based alloys while plastic parts are normally of an extremely high molecular weight polyethylene, made for both durability and biocompatibility. The NHS states that nearly 70,000 total knee replacements occur in the UK every year, making it by far the most commonly practiced surgical procedure for OA treatment. There are of course complications with this type of procedure, such as implant compatibility, the lifespan of the implant (20 years average), and surgical risks. Due to these type of considerations total knee replacement is only considered for patients in "severe" pain.

So there are many different treatment options available for the treatment of this condition, but few of them really even consider treating or fixing the tissue. Either the symptoms are treated while the problem persists, or the entire joint is removed and replaced. From a medical stand point there seems to be a lack of middle ground. This is why research into the exact nature of how OA begins and progresses is so important. The possibility of stalling the onset of such a condition, even preventing it, is a huge driving force behind current research in the field. But for this to occur our knowledge of the degradation process of articular cartilage must be as comprehensive as possible, while also having a way of detecting the onset of the deterioration. It is thought that damage to the collagen fibril network is likely to be one of the earliest signs of osteoarthritic cartilage degeneration (Radin et al 1970). Another of the earliest biochemical and morphological evidence of cartilage degradation is proteoglycan depletion and collagen disruption of the articular surface (Stoop 2001) (Stockwell 1983). These early surface zone changes in the tissue are difficult to detect and

recognize with standard mechanical testing because the mechanical behaviour of the tissue is averaged over the full depth of the cartilage, averaging all five zones with the superficial being where the changes would first occur. Since the test cannot flag such localized changes, the spatial and temporal relationships among the changes and the loss of mechanical function of the tissue are not well defined (Desrochers 2012). It remains unclear what occurs first, the functional or structural changes, as well as exactly how said functional changes at tissue level relate to microscale structural and functional changes. As the deterioration process progresses however changes in overall mechanical function become increasingly apparent. The tissue is highly sensitive to biochemical alteration of its matrix. Increased cross-linking causes significant increases in the matrix storage modulus while proteoglycan depletion and collagenase digestion cause significant decreases, which also causes a significant difference in complex shear moduli (Alexopoulosa et al 2005). Once the condition progresses into diagnosable osteoarthritis the tissue has changed in such a way that the tissue rapidly begins to deteriorate. Osteoarthritic cartilage has a lower modulus and increased permeability as compared to healthy tissue, which results in greater and more-rapid deformation of the tissue than normal under stress. This change may influence the activity of the chondrocytes, due to their connection to the mechanical environment (Radin et al 1970). Meaning that as with many diseases the further OA progresses, the faster the tissue deformation rare becomes.

In the look through previous research and papers some issues with testing and analysis became apparent that should be considered while implementing our own experimental design. There was research done showing that any derogative process which compromises the tensile properties, such as articular surface fibrillation or in our case simulated micro indentation, will have a detrimental effect on the dynamic compressive properties of the tissue (Vanwanseelef et al 2002). The scale of this experiments compressive testing is on the minute scale, in an attempt to not only characterize a smaller response but also to minimize the documented tissue damage shown by repeated tissue testing. It was also shown that the tissues attachment to bone may affect results, because a bony substrate would stiffen the structural response (Trickey et al 2000). As this is the tissues natural state, and this experiment is not an individual fibril response study, it was determined that leaving the tissue attached to

its boney substrate was a more logical and endemic approach. Finally there was research done showing that indention and retraction rates of a nanoidentor affect the tissues response (Austin et al 2013). This experiment was using a ramp indention test, and as our own will be doing a sustained ramp the hold with oscillations, the indention and retraction rates were not judged to be a factor as long as the matched the sampling rate so as no reading spikes occurred. Just for certainty only the second half of the oscillations were analysed, giving tissue time to normalize.

2. Aims and Objectives

In this experiment it is hoped that using nanoindention and dynamic material analysis will result in a characterisation of the surface mechanics of articular cartilage. The objectives of the project are determining if there is any frequency dependency of the δ, storage modulus, and loss modulus, using nanoindentation. This information will be useful as a baseline measure for future work into the effect of tissue degeneration on these parameters. Any insights from that can then hopefully be applied to the development of an endoscopic tool capable of detecting biochemical and morphological changes through changes in surface dynamics, which will facilitate an earlier diagnosis of Osteoarthritis.

3. Methods

The samples used for this experiment were obtained from a local abattoir. All samples were freshly butchered and then immediately frozen, being continually stored in a -18 C freezer except for sampling harvesting and the experiment itself. Samples were taken from four bovine thigh bones, with the femoral heads being the point of interest. During sample preparation the femoral head was removed from the rest of the thigh using a hack saw. Once separated, a 3mm tissue plug extractor was used to extract 25 samples from the 4 different femoral heads. This was done as quickly as possible to avoid the tissue from thawing out in an attempt to limit as much recrystallization in the tissue as possible, therefore minimalizing tissue damage caused by the freezing and preservation process. The extracted plugs had a diameter of 3mm, with a full surface depth as well as subchondral bone attachment of a few millimetres used for attachment. The subchondral bone was sanded down to be as flat as possible, creating a level cartilage tissue surface, for the purpose of making measurements and later setup more accurate. The samples were then attached to 10mm petri dishes using a pressure-sensitive adhesive medium. The samples were kept hydrated through this process as well as the entirety of the experiment with a standard phosphate buffer solution. This was achieved by filling the petri dishes with the buffer solution up to the cartilage itself, making sure that the liquid did not cover the surface layer of the tissue where the indenter would be interacting with it. All 25 samples were subsequently labelled and then arranged for testing. Sample prep was adapted from previous university work done by Megan Austin (Austin et al 2013).

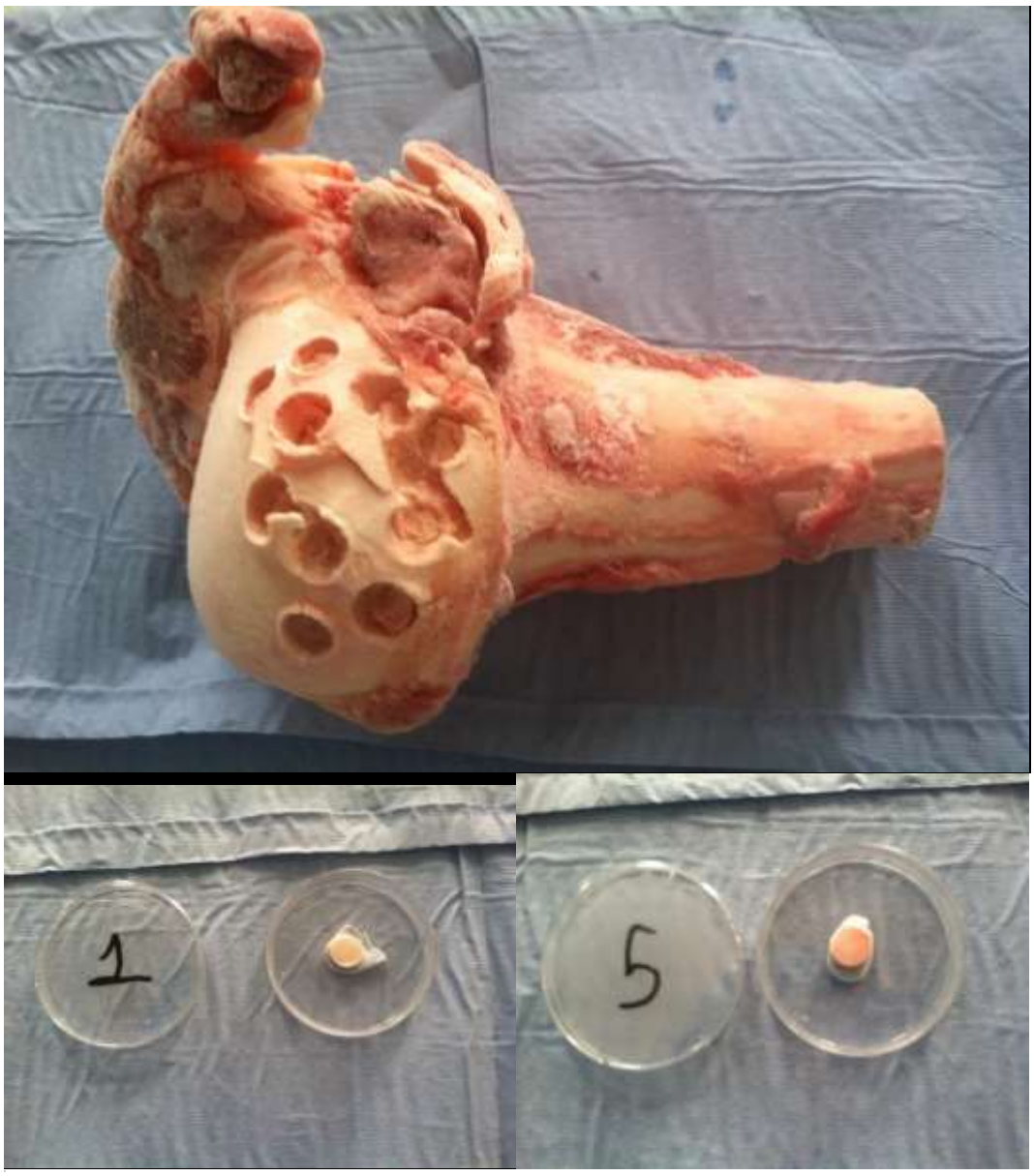


Figure 3: Above: Femoral head with tissue plugs extracted. Below: individual samples adhered to trays and labelled.

The actual testing involved using a MFP-3D SA Nanoindenter system to run a series of ramp hold functions with different osculating frequencies in the hold phase. This system was fully isolated inside a noise dampening enclosure; with the entire system sitting atop a vibration cancelling table. The sample was illuminated using a fibrotic cable set that had an external power source outside of the enclosure. All of these measures helped keep any interference to a minimum. Before any testing began the machine was calibrated, maxing out the sum and correcting for any virtual deflection. A known spring constant and previously calculated InVols number was

were used. For this test a spherical sapphire tip with a radius of 300 microns was used as opposed to the more commonly used Berkovich tip. The reasoning for this is due to the viscoelastic nature of the tissue, a smoother more spread surface being used for the indention reduces the tissues own viscoelastic effect, in an effort to decrease any biphasic or lag stages in the tissues reaction to the oscillations (VanLandingham 2003). The nanoindentor was set up in contact scanning mode to have a contact force trigger of 2.5 µN with an indention of 900 nm and a scan rate of 1 Hz. The surface was found using the indenter probes Z voltage sensor while visually confirming surface contact with the attached diffraction-limited optics.

Figure 4: Asylum Research Nanoindentor system with vibration isolation table and external light source.

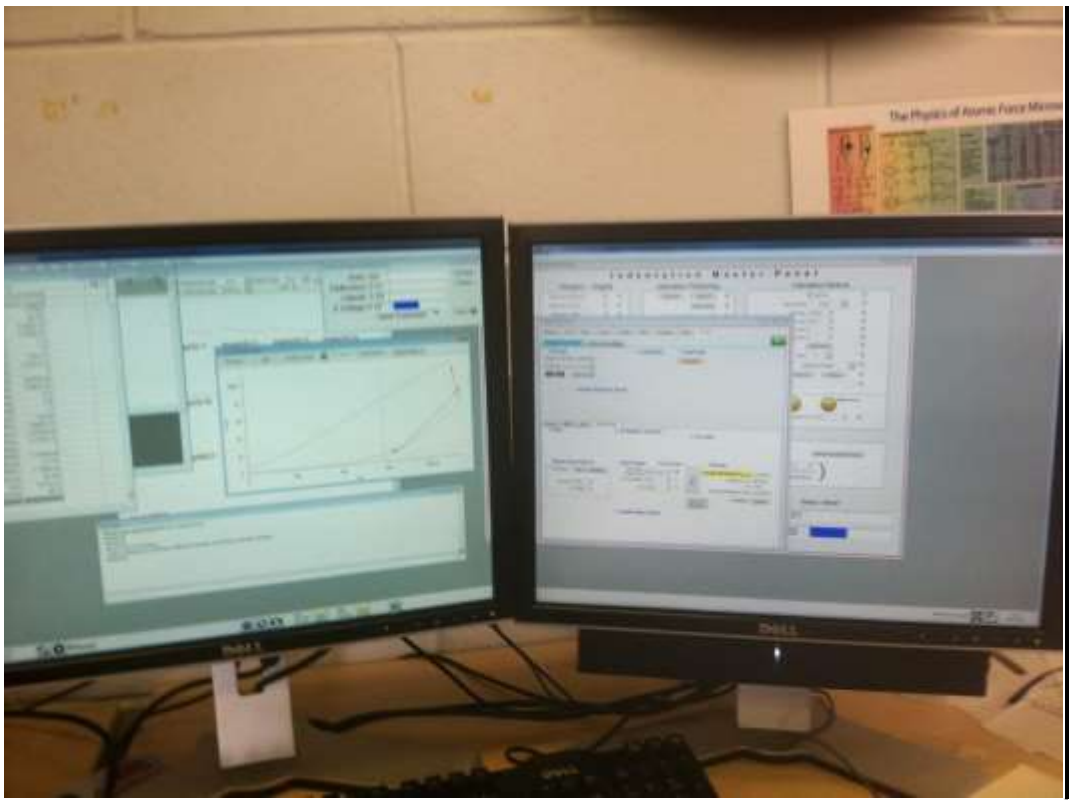


Figure 5: Nanoindentation control panel.

The loading and unloading velocities remained constant and similar to the scan rate throughout all tests so as to reduce any searching to contact velocity changes that might affect readings. The actual ramp hold consisted of 5s loading, a varied hold dependent of frequency, and a 5s unloading period. The hold time was proportional to the frequency of the oscillation being put into the tissue during the hold phase, allowing the sinusoidal wave to oscillate ten complete cycles. For example a frequency of 0.1 Hz has a hold of 100 seconds, while a frequency of 5 Hz has a hold of 2 seconds. To make sure that sufficient data resolution was achieved the sampling rate is also proportional to the frequency, twenty times the frequency. This will give twenty points on each complete wave form. This was the case for all but the last two (25 Hz and 50 Hz), which due to machine restrictions the program limited the sampling rate to their max at those frequencies Each sample was tested at 8 frequencies; 0.1 Hz, 0.5 Hz, 1 Hz, 2 Hz, 5 Hz, 10 Hz, 25 Hz, and 50 Hz. The table below gives a complete spread of the tests done on each sample.

Table 1: Hold Sample Oscillatory frequency Setups.

Oscillating Frequency	Sampling Rate	Hold Time
0.1 Hz	2 Hz	100 s
0.5 Hz	10 Hz	50 s
1 Hz	20 Hz	10 s
2 Hz	40 Hz	5 s
5 Hz	100 Hz	2 s
10 Hz	200 Hz	1 s
25 Hz	337.84 Hz	0.4 s
50 Hz	171.82 Hz	0.2 s

Each test was monitored and real time data observed to make sure no machine errors occurred such as a false or late trigger, using sensor data, graph readings, as well as visual confirmation with the optical feed. All data was gathered and stored on both the sampling computer and a secondary hard drive for later review and analysis.

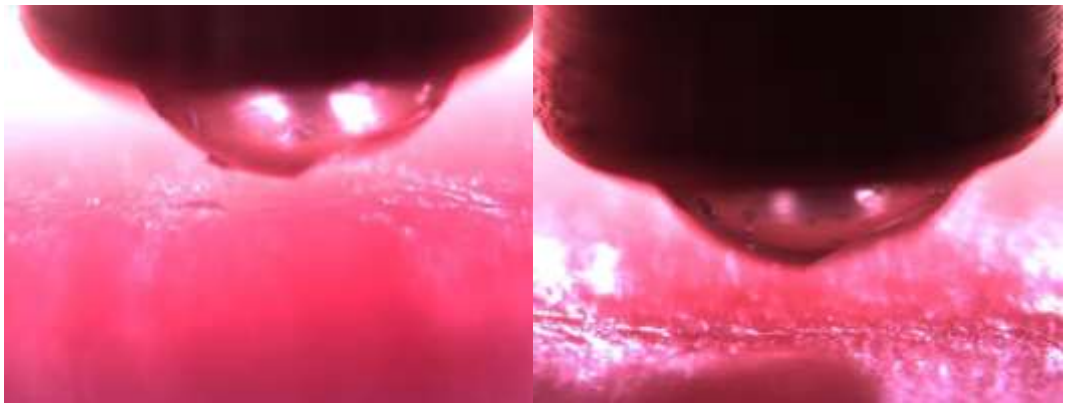


Figure 6: Nanoindentation tip above surface of the tissue.

4. Analysis

After the testing was completed, the gathered data was first compiled into each samples specific eight tests. The force, deflection, and time data were then isolated, with the offsets being removed, normalizing the data so that any differences in sample

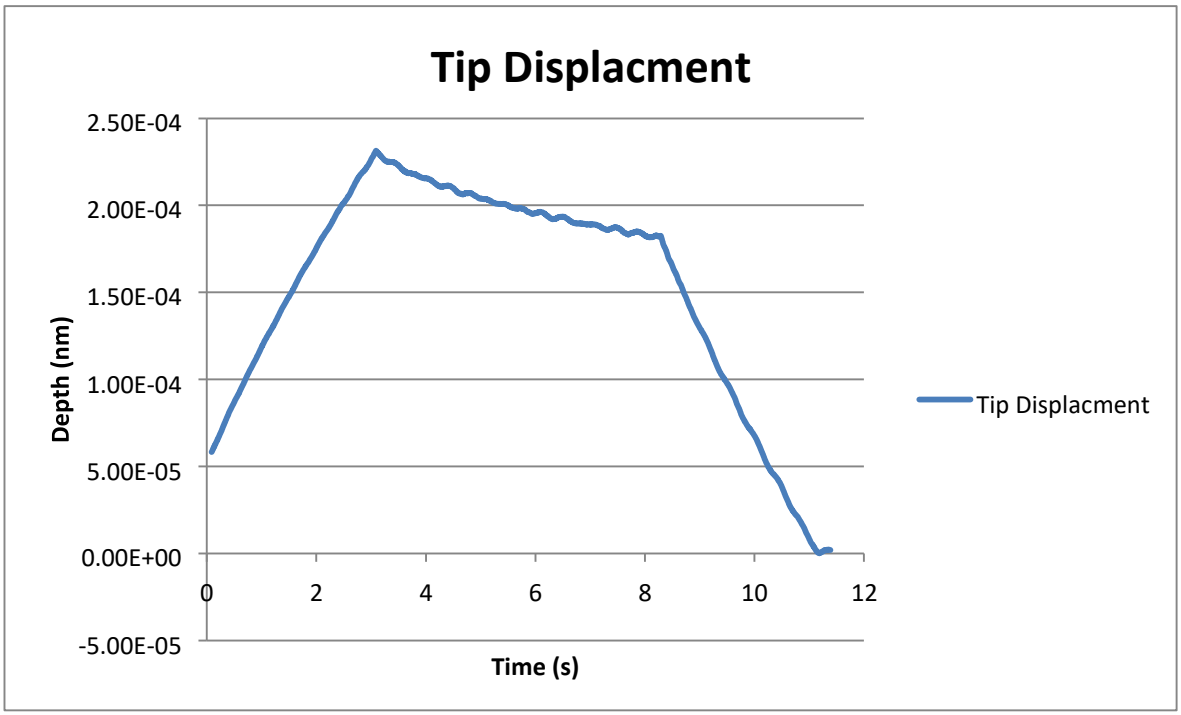
height or deflection offset would be accounted for. The data was then transferred over to Microsoft Excel where the oscillation peaks were manually identified for both force and deflection. For each data the last 5 oscillations were used for the calculations, due to variability of the viscoelastic material making the first half slightly more variable. Due to difficulty discerning the apex of certain wave forms, the 0.1 Hz, 0.5 Hz and 50 Hz testing runs mechanical behaviour were omitted from later analysis. This is not particularly troubling since measuring properties at low loading frequencies predicts properties that do not occur during gait activities or other normal mechanical behaviour. The 50 Hz is also not recorded during normal gait behaviours, though it is recorded in sudden fall situations. For the remaining 5 loading frequency ranges the average time difference between peak stress $\sigma 0$ and peak strain 0 was then determined in each cycle over the last 5 cycles. This difference was the multiplied by 2(pi)(Fq), where Fq is the oscillating frequency. This gives the δ in radian form, which can be used to define samples viscoelasticity, as well as to calculate their storage and loss moduli. To convert from delta to the different moduli the below equations were used.

$$E' = (\sigma 0/\ 0)\cos\delta \quad (1)$$

$$E'' = (\sigma 0/\ 0)\sin\delta \quad (2)$$

Also to be noted is that the analysis was only able to be carried out on 10 of the previously stated 25 samples due to time constraints surrounding this project. Further analysis will likely be performed at a later date to expand the data set, but for now 10 samples from 4 different animals will be discussed, using 5 frequency ranges.

5. Results



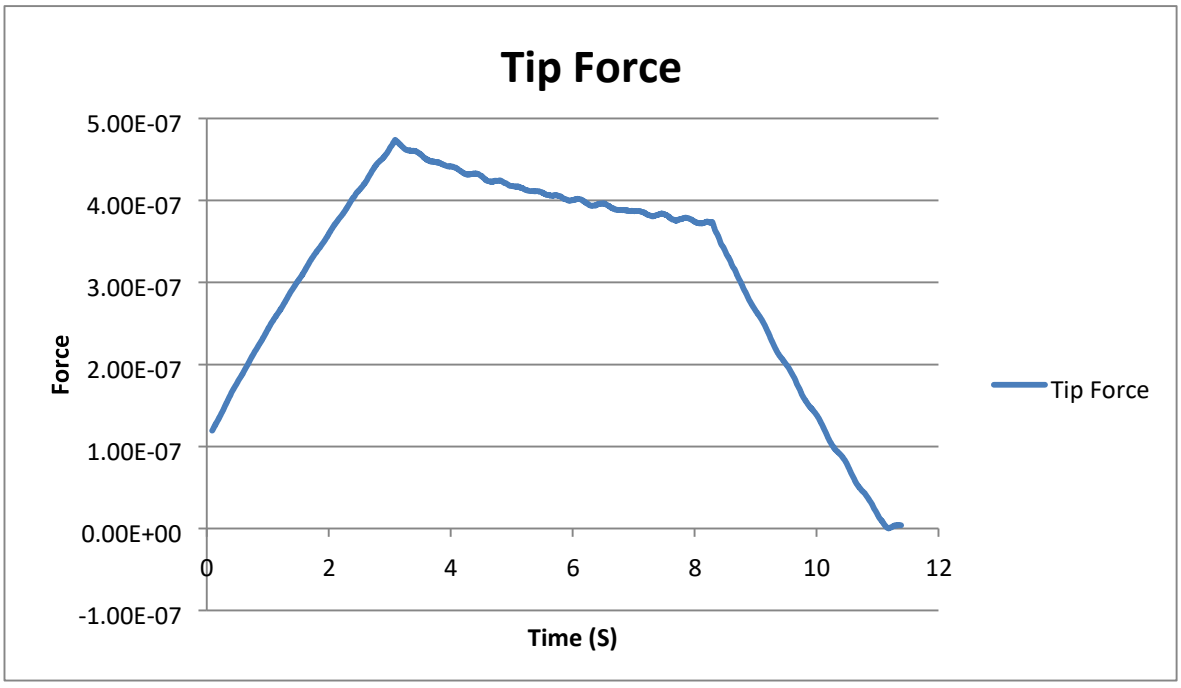


Figure 7: Examples raw traces of tip displacement and force. The phase lag was calculated from the time between the peaks of these elements.

The frequency dependence of the mechanical response and viscoelasticity of the tissue is observed through the changes of the δ, the storage modulus, and the loss modulus. Below are graphs detailing the response of each of these three indicators over the 5 loading frequencies. The storage modulus was far greater in magnitude than the

loss modulus in all frequencies. In fact it was nearly ten times the magnitude over the entire range.

The δ is shown below in Figure 9. At lower frequencies there is minute variation amid the different samples. As the frequency increases however the variation between different tissue reactions also increases. Between 1and 5 Hz frequencies in phase angle, but after 5 Hz a steady upwards trend occurs. It should be noted that not all tissues respond to frequency changes the same rate.

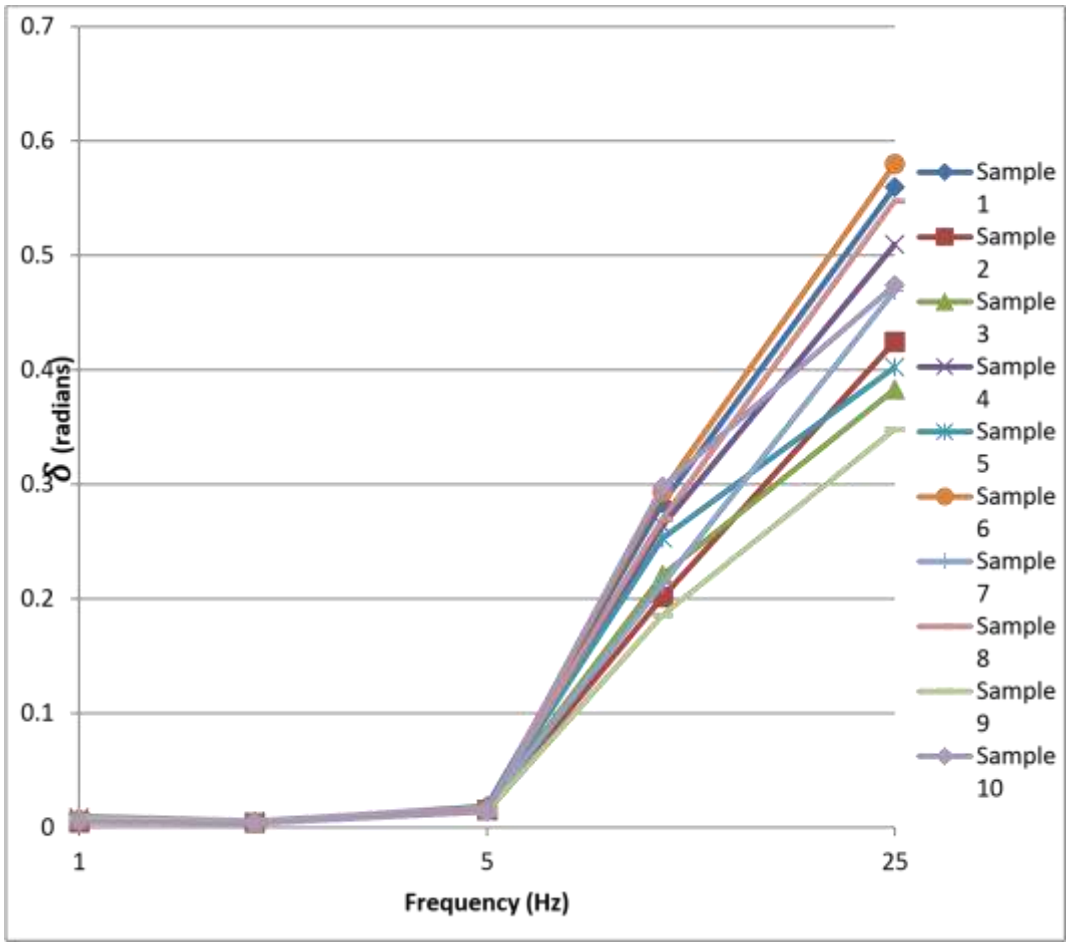


Figure 8: Variation in δ over increasing oscillating frequencies.

The Storage modulus or E' is shown below in Figure 10. The modulus is shown to have a similar trend through the variation of the frequencies, showing a steady climb from the lower frequencies until it peaks after 10 Hz, tapering off and holding steady or even slightly decreasing at 25 Hz. Interestingly many of the samples have a slightly reduced modulus between 1 and 2 Hz.

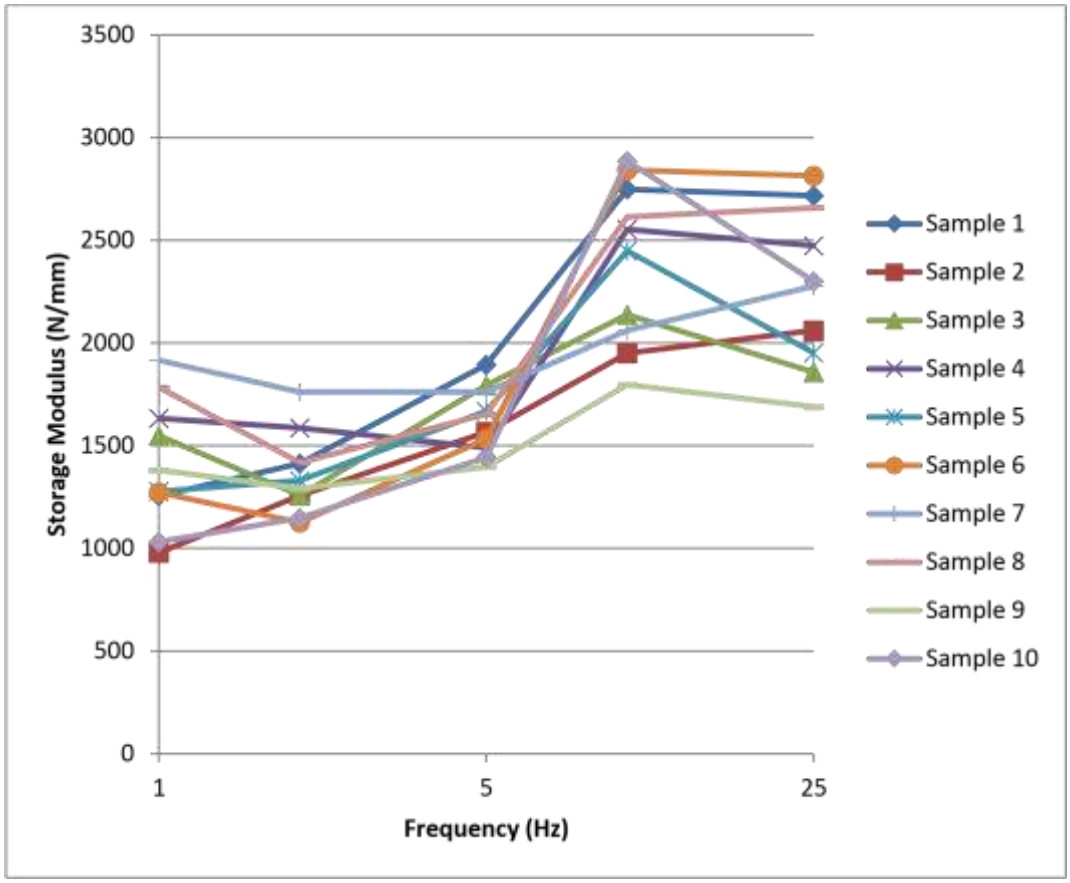


Figure 9: Variation in Storage Modulus over increasing oscillating frequencies.

The Loss modulus or E" is shown below in Figure 11. The loss modulus of articular cartilage was much less than the storage modulus over all frequencies tested and showed little to no frequency dependence. The loss modulus had a fairly constant value across all tested frequencies, ranging between 125 and 275 N/mm. There is considerably less similarities between samples for this modulus, with many samples behaving in direct contradiction to each other. Their reactions at the lower frequencies included some samples plummeting while others rapidly rose, such as sample 2 and sample 8. Similar inconsistencies occurred at high frequencies, where some samples continuously rise while others stayed nearly undisturbed, such as between sample 6 and sample 3. There seems to be a small upwards trend to the data, but as many of the samples start out higher than they end up it is not a storage enough correlation to truly define.

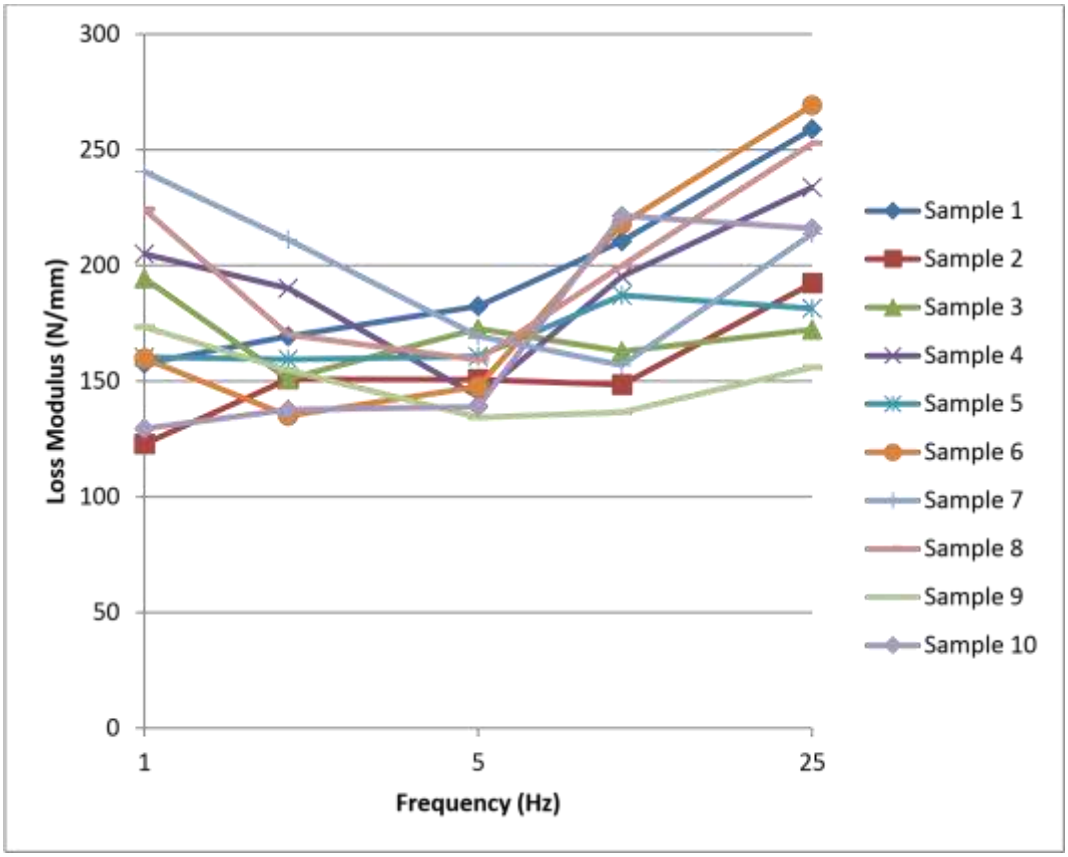


Figure 10: Variation in storage modulus over increasing oscillating frequencies.

Finally besides the δ there is no increase in modulus variation between samples due to any frequency dependency. The phase lag however does show an increase in variation between samples at higher frequencies.

Table 2: Table detailing average tissue response for the Delta, Storage, and Loss Moduli (N/mm).

Loading Frequency	Delta	Storage Modulus	Loss Modulus
1 Hz	0.007244 ± 0.001209	1408.02 ± 468.97	176.82 ± 58.89
2 Hz	0.004452 ± 0.001185	1359.24 ± 306.74	163.00 ± 38.09
5 Hz	0.015978 ± 0.002461	1619.84 ± 249.55	156.00 ± 24.04
10 Hz	0.248215 ± 0.0562	2404.14 ± 544.49	183.77 ± 36.47
25 Hz	0.469857 ± 0.1159	2280.03 ± 562.54	214.73 ± 51.49

6. Discussion

These results show that there do seem to be aspects of articular cartilage are frequency dependent, such as increasing δ and storage modulus with increasing

frequency. However the storage modulus itself seems to be only dependent to a certain point where the increase levels out into a plateau as the frequencies get higher. This is similar to other findings regarding frequency dependence using other testing larger scale testing methods (Fulcher et al 2009) (Guilak et al 1999). In these tests it was shown that after a certain point the storage modulus will become independent of any increasing frequency, as it has reached a maximum point. This data seems to suggest that the tissue behaves similarly at a nano scale as it does on the micro level, with a possibly quicker maxing out. This might be due to the scale the scale however, because the forces applied in the micro level tests were of a larger magnitude as well. A decrease in overall force loading may have a correlative decrease in the range of frequency dependence and how high a frequency it takes to level out the moduli. It seems apparent that to a point the higher the frequency the higher the storage modulus, but the rate of change of modulus is greatest at lower frequencies. At higher frequencies the rate of change tended towards a constant value, as indicative of a plateau. This result is consistent with the observation that articular cartilage is nearly insensitive to increasing rates of loading at high strain rates (Oloyede et al 1992). Previous studies have also shown that articular cartilage's loss modulus is far smaller than its storage modulus and that the moduli was non-frequency dependent, but again these were all on a larger micro scale.

Due to the fact that articular cartilage is a composite material made up of collagen, chondrocytes, proteoglycans, and fluid filled ECM, there are a few different mechanisms that may be the cause of this frequency dependent plateauing of the storage modulus. It could be caused the collagen fibrils that provide the bulk tensile strength of the tissue, specifically how in the different zones of the cartilage, the orientations of the fibrils are shifted to correspond to different necessities and intensities of stress withstanding. With greater loading frequencies, deeper layers of tissue would therefore be affected, stiffening its response. This seems less likely with the concept of the nanoindentation loading and depth. Such a small surface area was contacted and an indention under a single micrometre it seems a stretch to attribute deeper layers of tissue causing the plateauing effect. Another more probable reason for plateauing and stiffening of the tissue is interstitial fluid flow. High-frequency sinusoidal loading has a relatively lengthy amount of time for the fluid to move about.

With this increased time the fluid could re reach equilibrium before the loading is finished, becoming an incompressible substance, therefore stiffening the material. The shorter the frequency, the less fluid flow and distribution is allowed to occur, giving a more fluid response. As promising of an explanation as that is, it is never so simple with biological components. More likely the plateauing storage modulus is a response to the myriad of components that make up articular cartilage, but it would be safe to say that fluid flow plays a major part.

These differing behaviours of articular cartilage, in regard to the semi-frequency dependency of the storage modulus and non-frequency dependent loss modulus, have implications for mechanical degradation of the tissue. The storage modulus increases with frequency, until it plateaus off, and is significantly larger than the loss modulus. Since the loss modulus is not frequency dependent there is no increase of energy loss as the loading frequency increases. This relationship shows that more energy is stored in the system than dissipated and that the disparity between the moduli increases at higher frequencies. This excess energy then either needs to be reused or dispersed via a single or series of mechanisms or actions. It is believed that one of the main hazardous mechanisms for this excess energy dissipation is through the formation of cracks in the tissue itself (Verteramo et al 2007). So at higher frequency loadings such as sudden jolts or falls the disparity between the tissues moduli becomes too great for the normal energy relief, which is when permanent tissue damage occurs, slowly degrading the tissue at first, but becoming more rapid as the protective layer and therefore the energy absorbent potential decreases.

This apparent semi-frequency dependency of the tissue's δ and storage modulus is an insightful trend that is supported by literature, albeit on a larger scale. The variation between samples seems to imply that these characterizations are repeatable in the tissue, not limited to a single animal or topographical location. This is promising for further research into development of an endoscopic diagnostic tool, as anything of that ilk would require a fairly consistent response from tissue to be able to differentiate between healthy and deteriorating tissue. Further research should be focused on refining and confirming the nanoindentaion data, then simulating different degrees of tissue degradation using both chemical and physical methods. If there is a noticeable and significant change in the tissues frequency dependent responses between health

and moderately damaged, an endoscopic device may be a distinct possibility for the future.

7.Limitations

There are a few limiting issues and concerns that should be noted in this study. First of all the spherical sapphire head has a small portion missing out of its side. This is not thought to have caused and reading issues, as the contact point of the sphere is fully intact and no part of the tissue came close to the fault. There was also an issue of tissue dehydration during testing. This was mitigated as thoroughly as possible by keeping the tissue as fully covered with buffer solution throughout the testing process. The shrinkage of the tissue was noted as testing progressed, and whenever need be the equipment was readjusted to the new surface height in reach of the micro arm. There is of course the issue of the reduced size of the analysis, but the data gives a clear trend that is consistent with previous research, and completing the other sample's analysis should only stand to strengthen the trend. This data may be used at a later date. Another major issue is the accuracy of actually finding the surface of the tissue. Even with the sensitive equipment, fine-tuned trigger force settings, and the visual confirmation, re adjustments had to be made often. Finally there is an issue of overall tissue relaxation during the oscillatory loading sequence. Once the indention occurs and holds, even though the frequency loading is happening, the tissue begins to relax from the overall loading. This can cause difficulty in observing peak stresses and strains, and therefore the phase difference as the entire tissue is relaxes in response to the original indentation.

8.Conclusion

Articular cartilage displays viscoelastic behaviour through all the tested loading frequencies (1 Hz, 2 Hz, 5 Hz, 10 Hz, 25 Hz). This behaviour has health implications, as mechanical damage to articular cartilage and the onset of osteoarthritis may be partially attributed to the disparity of its moduli. The storage modulus is frequency dependent, increasing with frequency until a plateau region occurs. The storage modulus also displays far greater values than the loss modulus. Coupled with the fact that loss modulus is non-frequency dependent and does not increase with oscillatory frequency, a large rift forms between the storage and loss moduli, which translates into

more energy being stored by the tissue than dissipated. This effect is only exasperated at higher frequencies. The lack of built in dispersion mechanisms that can handle the higher frequency excess energy is a major cause of tissue damage, which potentially leads to Osteoarthritis. The data gathered in this experiment suggests a repeatable frequency dependent tissue response, which with further research may lead the way to an endoscopic diagnostic device that can distinguish tissue degradation early on from alterations of mechanical response behaviour.

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