Changes in Acoustic Parameters at 30 MHz of Human and Bovine Articular Cartilage Following Experimentally-Induced Matrix Degradation to Simulate Early Osteoarthritis

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Matrix degradation and proteoglycan loss in articular cartilage is a feature of early osteoarthritis. To determine the effect of matrix degradation and proteoglycan loss on ultrasound propagation in cartilage, we used papain and interleukin-1α to degrade the matrix proteoglycans of human and bovine cartilage samples respectively. There is also minor collagen alteration associated with these chemical degradation methods. We compared the speed of sound and frequency dependent attenuation (20 to 40 MHz) of control and experimental paired samples. We found that a loss of matrix proteoglycans and collagen disruption resulted in a 20–30 % increase in the frequency dependent attenuation and a 2 % decrease in the speed of sound in both human and bovine cartilage. We conclude that the frequency dependent attenuation and speed of sound in articular cartilage are sensitive to experimental modification of the matrix proteoglycans and collagen. These findings suggest that ultrasound can potentially be used to detect morphologic changes in articular cartilage associated with early osteoarthritis.
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Chapter 1

Introduction

1.1 Osteoarthritis

Osteoarthritis is described as a group of degenerative diseases affecting the weight-bearing joints [1]. Osteoarthritis targets the articular cartilage found at the end of long bones, and is unlike rheumatoid arthritis, which is an auto-immune disease. Osteoarthritis is the second leading cause of morbidity in the population aged over 50, behind cardiovascular disease.

The principal treatment for advanced osteoarthritis is total joint replacement. Total hip and knee arthroplasty are currently the most common procedures performed.

1.1.1 Articular Cartilage

The hyaline cartilage which covers the articulating ends of the bones of diarthrodial joints is a highly specialized connective tissue. This articular cartilage has biochemical and biophysical characteristics which are well suited to its roles as a bearing surface and shock absorber in an articulating joint. The tissue is unique when compared with other types of cartilage in the body, and it has certain defining characteristics.
Articular cartilage is an alymphatic, aneural and avascular tissue [2]. It is located at the ends of long bones, and is relatively distant from a direct vascular supply. Consequently, articular cartilage relies upon diffusion of synovial fluid within the synovial capsule of the joint, rather than a vascular supply, for nutrition and lymphatic drainage. The gradient of diffusion requires that nutrients diffuse from the vasculature of the synovium, traverse the synovial membrane into the synovial fluid, and subsequently diffuse through the dense matrix of the cartilage in order to reach the chondrocytes, the cellular component of the cartilage [2].

The matrix of articular cartilage is a molecular framework which is permeable to nutrient molecules. The matrix is composed primarily of collagen fibres and highly negatively charged proteoglycan molecules, both of which are discussed in detail later in this section. The high concentrations of proteoglycans (up to 33 % dry weight) create an effective electronegative molecular sieve, which restricts the flow and limits the size of these nutrient molecules [1]. The ‘pore’ size in the cartilage matrix has been estimated to be 6.8 nm [3,4]. Thus, it is this limiting pore size which dictates the flow of molecules through the cartilage.

Chondrocytes, the cellular component of cartilage, are distributed in low concentration throughout the cartilage. The cells constitute a minority of the dry composition of cartilage, which is dominated by components of the extracellular matrix, collagen and proteoglycans [5]. The cell density of cartilage is considerably lower than that of the surrounding tissue, the synovial membrane. In conjunction with the avascularity, this hypocellularity explains the low turnover rate for macromolecules of the cartilage as well as the limited healing ability of articular cartilage. Photomicrographs of articular cartilage demonstrate a sparse organization of chondrocytes. In spite of this hypocellularity, cartilage has been shown to be a metabolically active tissue [6].

The extracellular matrix of the articular cartilage is responsible for the shock absorption as
well as the low friction articulation of the body's joints. Articular cartilage is a highly hydrated tissue, with water bound by hydrogen bonds to the matrix via collagen fibrils and the hydrophilic proteoglycans. It is estimated that the water content of articular cartilage is approximately 80% [7, 8]. The collagen fibres and proteoglycans are estimated to comprise about 60% and 25–35%, respectively, of the tissue's dry weight [8].

Collagen

Collagen is a fibrous molecule that plays an integral role in the structure and function of all animals. Collagen molecules constitute a major portion of tissues such as skin, muscle and bone. They are characterized by a high strength-to-weight ratio, while also being flexible and extensible. The collagen molecule has a characteristic tri-helical arrangement of monomer pro-collagen fibrils. The collagens of skin and bone, type I collagen, are composed of two $\alpha_1$ chains and one $\alpha_2$ chain. The type II collagen fibres of articular cartilage contain three identical $\alpha_1$ chains.

The pro-collagen fibrils are composed almost exclusively of hydroxylysine, lysine and hydroxyproline amino acids. There is a strong hydrogen bonding interaction among the three pre-collagen fibrils which twists them into their characteristic tri-helical structure. There are interactions between the adjacent individual collagen fibrils, which allow multiple collagen fibrils to link together to form long, strong collagen fibres.

Collagen type II fibres are organized into distinct layers in articular cartilage. Cartilage has a characteristic tri-laminar appearance when viewed by polarized light microscopy. The layers are named according to the position and orientation of the collagen fibres in the cartilage. Figure 1.1 is a schematic representation of the distribution of collagen in mature cartilage. The tangential layer is the thinnest layer and makes up the surface of the articular cartilage. The collagen fibrils here are oriented parallel to the articular cartilage surface, and exhibit a preferential alignment parallel
to the direction of joint articulation. The transitional zone is characterized by the appearance of collagen fibres organized in a Gothic arcade structure. The radial layer, or deep layer comprises the lower two thirds of the articular cartilage thickness, and collagen fibres are oriented perpendicular to the articular cartilage surface. Finally, below the radial layer exists the calcified cartilage, a dense zone which serves to anchor the cartilage to the underlying bone, and also anchors the collagen fibres of the cartilage, in order to reinforce the overall structural integrity.

Figure 1.1: Schematic of the collagen fibre distribution in mature articular cartilage.

Proteoglycan Aggregate

The proteoglycan aggregate is a long, hydrophilic molecule. The core of the aggregate is a long molecular chain of hyaluronic acid. It comprises 25–35% of the dry weight of the articular cartilage [8]. The proteoglycan molecule consists of a hyaluronic acid core, a molecule which tends to form hydrated gels in solution. Branching out from the hyaluronic acid core are several polysaccharide chains. These chains bind to the hyaluronic acid core by means of globular proteins. Two
specific glycosaminoglycan molecules, chondroitin sulphate and keratan sulphate, branch out from
the oligosaccharide chains, as illustrated in Figure 1.2.

Figure 1.2: Schematic representation of the structure of the proteoglycan aggregate.

The function of the proteoglycan aggregate is to provide rigidity to the cartilage tissue. This
is achieved by the high concentration of water, bound by the hydrophilic proteoglycan molecules,
present in the cartilage.

As cartilage is compressed by an external joint loading force, some of the tightly bound
water will be squeezed out of the cartilage, into the space between the articular surfaces. This thin
film of water provides articular surface lubrication, which facilitates virtually friction-free motion
of the opposing cartilage pieces on one another.

Shock absorption by the articular cartilage occurs as a direct result of the proteoglycans.
When cartilage surfaces are compressed, they deform slightly, and absorb the force. Internally, the proteoglycans exert a restoring force on the collagen framework surrounding them. This restoring force is caused by the highly negative charge of the proteoglycan aggregate. At a molecular level, there exists a Coulomb repulsion between the individual molecules as they near one another. Thus, this introduces a limitation on the deformation and compressibility of the articular cartilage. Further restoration of the collagen framework is caused by an osmotic potential between the synovial fluid outside the cartilage and the cartilage's internal conditions. This osmotic potential causes water to permeate the cartilage and helps to restore its original shape. The cartilage can thus be deformed to the hydraulic limit of the compressibility of the medium, which provides the cushioning to the bones of the body.

1.1.2 Changes in Articular Cartilage with Osteoarthritis

The morphologic changes in joints affected with osteoarthritis were originally identified in writings of the Hunter brothers over 200 years ago [9]. A monograph in 1942 by Bennett, Waine and Bauer described the progression of changes seen in the developments of osteoarthritis of the knee [10]. In 1949, Collins established a classification system for the joint changes which is a precursor to much of the current work [11]. This work identified that the earliest changes in cartilage were visible at the surface layer of the tissue. These changes involved an increase in cellularity, as well as a decrease in metachromatic staining. This loss of metachromatic staining is characteristic of proteoglycan depletion in the cartilage. This depletion of proteoglycans has also been described using an orthochromatic stain, Safranin O, by Rosenberg [12]. At a biochemical level, the earliest changes to appear reflect this loss of staining by chromatic dyes, indicating a depletion of proteoglycans. This is confirmed by Mankin and Lippiello's results, which elaborate that the amount of proteoglycan depletion of osteoarthritic cartilage is directly
proportional to the severity and advancement of the disease [13]. This focal loss of proteoglycans is thought to be a key characteristic of early osteoarthritis in articular cartilage. Progression of the disease is often marked by the appearance of surface inclusions, or clefts in the articular surface. With further disease progression, these inclusions can develop into fibrillations, which can extend from the articular surface of the cartilage to the radial layer with time [11]. These fibrillations can be exacerbated by the repetitive use of the joint over time, and deepen quickly.

The collagen content of osteoarthritic articular cartilage, assayed biochemically, is thought not to change over the progression of the disease [13]. It is, however, thought that the thickness of the collagen fibres might increase in size with disease progression [14,15]. In addition, the collagen fibres produced by the chondrocytes during disease progression, collagen type I and type II, are similar to those found in human skin and bone [16]. This resembles the type I collagen found in scar tissue during wound healing. Thus, the reaction of the body to collagen loss is to treat it as a normal lesion in the body and produce collagenous scar tissue to repair the damaged area. Results by Rosenberg support this theory [12].

The advanced stages of osteoarthritis include remodeling of the joint's bones. Osteophytes, small bony outgrowths, form around the margins of the joint. In the knee, the tibial head and the distal femur are involved. These features may impinge the motion of the osteoarthritic joint and represent a source of pain.

1.1.3 Models of Experimentally Induced Osteoarthritis

Although the genetic predisposition of individuals to osteoarthritis is not well understood, the intermediate and end results are well documented [1]. Osteoarthritis can be induced either experimentally, or occur in a spontaneous biological model, as in rhesus macaques or mice [17]. These spontaneous osteoarthritis models, however, do not allow for the study of the disease in human
CHAPTER 1. INTRODUCTION

tissue. An experimental disease model of osteoarthritis makes use of the prior knowledge of these intermediate disease stages and outcomes, and usually employs either a mechanical or chemical means to produce them from disease-free tissues [17]. These disease models can be used to induce osteoarthritis-like changes in vivo or in vitro.

Osteoarthritis is characterized by articular cartilage edema, fibrillation, and erosion accompanied by chondrocyte proliferation and decreased staining of matrix proteoglycans, thickening of the subchondral bone, deformation of the articular surface and osteophyte formation [17]. A disease with such a wide range of associated tissue changes is best modeled in vivo, where all facets of the disease are involved, and physiological changes can take their course over time. However, these models are inherently more difficult to keep under controlled conditions, when compared to an in vitro model [18]. Excellent in vivo models of osteoarthritis have been developed and established in a variety of animals, including macaques, dogs and guinea pigs [19–21]. These models induce joint instability, which, over time can lead to progressive degenerative arthritis in the joint [17].

A number of biochemical models of induced osteoarthritis exist in addition to the mechanically induced models. These in vivo models involve the intra-articular injection of a disruptive enzyme or substance, such as saline or papain [22–24]. These models rely upon the chemical behaviour of a substance upon introduction to the cartilage, either by injection, or digestion. The extracellular matrix and its constituents, such as proteoglycans, are primarily targeted by these models. Examples of such enzymes, are collagenase, an enzyme that degrades collagen, and papain, an enzyme that degrades matrix proteoglycans and collagen. At low doses, papain has been shown to deplete the proteoglycan distribution in cartilage while sparing the collagen framework [25]. Papain offers a good experimentally-induced osteoarthritis model for in-vitro studies. In addition, certain experimentally-induced osteoarthritis models are well suited to the induction of disease-like
conditions in cultured tissue. These biochemical models culture cartilage in vitro in a medium containing degrading enzymes of interest, which are taken up by the chondrocytes and incorporated into the cellular cycle. The result is that these enzymes can be used to alter the normal metabolism of the cartilage and affect the normal production of its molecular constituents.

Interleukin-1 is a catabolic cytokine found in most cells of the body. It affects the production of cartilage constituent molecules by the chondrocytes [26]. Interleukin-1 is involved in matrix degradation by promoting the secretion of proteinases from the chondrocytes, which in turn degrade the matrix proteoglycans and cause minor collagen degradation. In addition, it also suppresses the further synthesis of new proteoglycans and collagen type II [26]. Interleukin-1 has been documented to stimulate proteoglycan release and minor collagen degradation, and can be incorporated into cartilage cultured in vitro. Hence, interleukin-1 can provide a good model of experimentally-induced osteoarthritis in cartilage.

**Cartilage Cultured with Interleukin-1**

Chondrocytes are the cellular component of cartilage, responsible for the synthesis of essential matrix components. This is accomplished by the constant remodeling of the cartilage constituent molecules by the chondrocytes. An ideal disease model for osteoarthritis would attempt to maintain conditions as close to those in vivo as possible. Resection of bovine cartilage from freshly slaughtered animals, followed by culture in a maintained environment, has been demonstrated to preserve chondrocyte function and viability [27]. By using cartilage with viable chondrocytes, a disease model of osteoarthritis can be applied which introduces an enzyme into the cellular cycle. Interleukin-1 is a cytokine secreted by chondrocytes which promotes the degradation of collagen and proteoglycans by mediating the secretion of proteinases and other degradative molecules into the cartilage and inhibiting the further synthesis of proteoglycans.
An investigation by Smith et al. demonstrated a significant release of matrix proteoglycans following the treatment of bovine nasal cartilage explants with recombinant human interleukin-1α [28]. This study also demonstrated that recombinant human interleukin-1α also inhibited the further uptake of radio-labeled sulphate into cartilage, indicating the inhibition of further sulphated glycosaminoglycan synthesis in the cartilage. Hence, the use of interleukin-1α on cultured cartilage explants offers a method of simulating the morphological changes associated with early osteoarthritis in cartilage tissue. When the viability of the cellular chondrocytes is maintained, and matrix proteoglycan degradation is induced by interleukin-1α, the early stages of osteoarthritis can be partially simulated in an in vitro environment.

Chemical Digestion of Cartilage with Papain

Papain is a protease enzyme originating from the bark of the papaya tree. It is a strong protease which has the ability to degrade many biochemical molecules. It has been used previously, via intra-articular injection in rabbits and guinea pigs, to stimulate osteoarthritis-like changes in articular cartilage in vivo [18,22,24]. This previous work has focused primarily on inducing osteoarthritis-like changes in animals, such as pigs and rabbits, and is an established in vivo model of the disease.

The mechanism of proteoglycan degradation by papain appears to act on the areas of the proteoglycan associated with binding to collagen fibrils [15]. Work by Junqueira demonstrated an increase in Picro-Sirius Red staining following papain digestion of cartilage sections for histology. Papain most likely interacts with the link proteins of the hyaluronic acid core of the proteoglycan aggregate, thus promoting degradation of the entire proteoglycan molecule [1]. As previously mentioned, an in-vitro papain digestion model of osteoarthritis in cartilage provides a valuable alternative model, as obtaining cartilage post-mortem would offer questionable and inconsistent chondrocyte viability.
Papain is a powerful degradative enzyme, and in strong concentrations can digest articular cartilage in its entirety [29]. At low concentrations, however, papain primarily degrades the proteoglycans of the articular cartilage and only degrades the collagen network to a minor extent [22]. By using lower concentration of the enzyme, there should be little disruption of the collagen network, with proteoglycan digestion. These changes are similar to those encountered in articular cartilage during early stages of osteoarthritis. This is the basis for the use of papain digested cartilage samples in vitro as a model of the disease process for human articular cartilage.

1.1.4 Current Diagnostic Imaging Modalities for Imaging Cartilage

Articular cartilage presents a challenge in medical imaging. Cartilage is an avascular tissue located at the ends of bones involved in articulating joints, is primarily composed of water in a gel-like matrix, and thus is relatively transparent to some modalities. Current modalities used for imaging cartilage in vivo include arthroscopy, plain film radiography (X-ray), magnetic resonance imaging (MRI) and ultrasound.

Arthroscopy

Arthroscopy of articular cartilage is an invasive modality for visual examination of the joint area. It offers a direct, extensive and magnified view of the articular surfaces of the knee, as well as the synovium. Unlike other modalities, arthroscopy requires an invasive procedure to provide images. Previous literature has stated that direct joint visualization through the arthroscope is more sensitive than magnetic resonance or plain radiograph in detecting cartilage lesions [30]. Although arthroscopy can only provide information of the cartilage surface, it is the 'gold standard' for the assessment of articular cartilage, against which all other methods are judged [31].

Arthroscopy has been used to monitor the course of knee osteoarthritis using a baseline and
follow-up approach, and has demonstrated itself to be of value in diagnosis of several disorders of the knee joint [1]. It is principally used for diagnostic purposes, such as the evaluation of the cartilage surface, where it is referred to as chondroscopy [32]. There currently exist several classification systems of articular cartilage for arthroscopy [33–35]. These classification systems are generally based upon probing of the cartilage surface with a hook-like probe, while visually inspecting the cartilage with the arthroscope. Cartilage surface integrity is typically classified on a qualitative scale of I to IV, with IV representing extreme cartilage loss [33].

Arthroscopy is an established method of examining the articular cartilage surface and staging osteoarthritis. Arthroscopy is limited, however, by an inability to identify tissue variations common in early stages of osteoarthritis, such as loss of proteoglycans in the matrix and cloning of the chondrocytes. For early stages of osteoarthritis, when changes are occurring below the surface, it is important that a diagnostic tool provide this information.

**Plain Film Radiography**

Plain film radiography is a frequently performed diagnostic imaging procedure. Images are produced by exposing a subject to a collimated beam of x-rays and placing a photographic film beneath the subject. The intensity of the resulting image is proportional to the density of the tissue through which the x-rays have passed. The resulting image is termed a radiograph. Cartilage is not a dense enough tissue, relative to surrounding bone, to appear on most radiographs; hence, the joint space appears dark on film.

Plain film radiographs have been used for decades to investigate osteoarthritis of joints [36, 37]. The grading of osteoarthritis progression is typically inferred by evaluation of joint space narrowing, and appearance of several features associated with the disease [31]. These features include osteophytes, bony outgrowths appearing around condyles in the advanced stages of osteoarthritis,
cysts and sclerosis of the subchondral bone.

The staging of osteoarthritis in plain film radiography is based upon the indirect evaluation of joint integrity by means of the previously listed features on plain film radiographs [37]. Although widely used, plain film radiography provides only indirect information about the cartilage in the joint. It can only provide information to assist in the later stages of osteoarthritis, because early changes in cartilage are extremely difficult to quantify indirectly.

**Magnetic Resonance Imaging**

Magnetic resonance imaging (MRI) has become an important modality for the evaluation of internal derangements of the knee, as well as other joints. MRI is an imaging modality based on a natural magnetic behaviour of atomic nuclei, the magnetic signal from hydrogen atoms in the water found in the body. The physics of MR imaging of cartilage have been described in detail [1]. Recently, there has been growing interest in applying MRI to the study of human arthritis. MRI has the advantage of being able to provide a high resolution, non-invasive imaging method, with excellent soft-tissue contrast as well as 3D imaging capabilities.

The heterogeneous composition and complex biochemical composition of articular cartilage are reflected by its complex appearance when imaged with MRI [1,38]. Several factors affect the appearance of cartilage in MRI. The content and distribution of the proteoglycan aggregates, along with the anisotropic organization of collagen both influence the amount of water in cartilage, as well as the water’s relaxation properties, both of which tend to give cartilage a characteristic zonal appearance in MRI images [39,40]. This zonal appearance, described as tri-laminar, is thought to be a result of collagen orientation and water distribution in the cartilage [41]. At present, there has been limited success in correlating the MR images with the morphologic and histologic changes observed in cartilage with osteoarthritis, such as proteoglycan loss [42].
MR imaging has shown success in detecting larger defects in articular cartilage. However, the spatial resolution has yet to reach the level where MR can be used to detect morphological changes in the cartilage associated with the early stages of osteoarthritis. As MR technology improves, its ability to play a role in the early stages of diagnosing degenerative joint diseases such as osteoarthritis will grow.

Ultrasound Imaging

The use of ultrasound to investigate cartilage was first reported in the 1950s, by Dussik and Fritch [43]. Their work investigated the change in properties of ultrasound, primarily velocity of sound and acoustic attenuation, as the ultrasound propagated through articular cartilage tissue. This initial characterization was essential to the understanding of the behaviour of ultrasound in cartilage. However, the ultrasonic properties of cartilage remain inadequately characterized.

The development of portable pulse-echo scanners in the 1970s and 1980s promoted interest in scanning articular cartilage. Initial studies of cartilage with ultrasound, such as Cooperberg et al., investigated the effects of arthritis on ultrasound B-scan (brightness) images of human and bovine cartilage [44]. These early studies were carried out at ultrasound frequencies of 7.5 MHz or less [44]. At these frequencies, the spatial resolution of the ultrasound in the cartilage is 220 μm or more, assuming a velocity of sound in cartilage of 1665 m s⁻¹ from Dussik and Fritch [43]. This relatively low spatial resolution, compared to the thickness of cartilage (≈ 2.0 mm) is a significant limitation. With the available resolution, imaging studies of cartilage at the time focused primarily on examining the echoes from both the surface and the calcified cartilage layer [44]. Consequently, several studies tried to score osteoarthritis based upon the quality and sharpness of the echoes received from the cartilage [44,45].

These early methods demonstrated the potential of ultrasound to be a valuable real-time
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tool for cartilage imaging. Cooperberg et al. investigated the difference between in vivo B-scan images of normal and osteoarthritic human cartilage [44]. The femoral condyles were imaged, and the properties of the surface and deep layer echoes were investigated. Cooperberg et al. were able to demonstrate the ability to detect loss of cartilage thickness in osteoarthritic patients accurately with ultrasound in vivo, as well as describe qualitatively the surface of the cartilage according to a grading scale. Results were dependent upon accurate positioning of the transducer based upon external landmarks, and only a limited area of the knee could be studied [44]. This study demonstrated both the impressive potential of ultrasound and its weaknesses in imaging articular cartilage.

High frequency ultrasound, at frequencies greater than 20 MHz, offers higher resolution than conventional scanners, as well as decreased penetration depths. Recent work has begun to investigate the imaging of articular cartilage at frequencies of 25, 50 and 100 MHz [46–49]. This higher frequency ultrasound, offering resolutions of 50 μm and better, can provide more information about the underlying morphology of the cartilage tissue. Many of these studies focused on correlating in-vitro ultrasound images of cartilage with corresponding histology for both normal and osteoarthritic tissues [46,48,50,51]. Investigators have primarily attempted to correlate histological changes associated with osteoarthritis, such as matrix proteoglycan loss, with changes observed in ultrasound images. To date, limited success has been achieved in correlating backscatter signal from ultrasound images of cartilage with corresponding histology [48,51].

There has also been work in examining the ultrasonic parameters of both normal and osteoarthritic cartilage tissue [43,49,52]. Parameters of interest, which describe the behaviour of ultrasound in tissue, include acoustic backscatter, acoustic attenuation and velocity of sound. These values can often change dramatically between normal and diseased tissue, where morphology has
been altered. Agemura et al. studied the velocity of sound and acoustic attenuation in bovine cartilage which had been subjected to several chemical procedures aimed at producing morphological changes similar to that of osteoarthritis [49]. Biochemical digestion to deplete matrix proteoglycans and to degrade collagen fibrils was used to induce experimental osteoarthritis in vitro. Their results confirmed that ultrasound was sensitive to these changes in the morphology of the cartilage and this was observed in the corresponding acoustic parameter changes. The change observed was a decrease in the speed of sound when bovine cartilage was digested in vitro with chondroitinase ABC, a chemical that degrades the matrix proteoglycans [49]. Similar results were also reported in a study on the acoustic attenuation by Senzig et al. [52].

Previous work has demonstrated the ability of ultrasound to detect morphological changes in cartilage, via imaging or basic ultrasound parameters. B-mode imaging has been able to exhibit limited correlation with histological findings and may improve with the use of higher frequencies with improved spatial resolution. Tissue characterizations of basic ultrasound parameters have demonstrated a sensitivity to the morphological changes in cartilage occurring with early osteoarthritis [46,49,52]. These morphological changes are consistent with those observed histologically in early osteoarthritis, and can be induced in vitro by simple biochemical digestion models [22]. Hence, ultrasound tissue characterization may offer, in the future, a useful method of diagnosing the changes in cartilage composition associated with early osteoarthritis and provide a tool for the early detection of osteoarthritis.
1.2 Ultrasound Imaging of Articular Cartilage

1.2.1 Introduction

Ultrasound is defined as sound waves at frequencies greater than the audible limit, that is greater than 20 kHz. The objective of diagnostic ultrasound imaging is to obtain information about living tissue by probing it with these sound waves.

1.2.2 B-Mode Imaging of Cartilage

External Ultrasound Imaging

B-mode imaging is a two dimensional imaging method available on commercial ultrasound scanners. Ultrasound imaging of the musculoskeletal system is currently performed as an external diagnostic technique. The ultrasound transducer is placed on the skin surface, on the joint of interest, and the image is formed by the ultrasound penetrating the various tissues overlying the joint. These tissues include skin, fat, tendon and muscle. It was found that optimal B-mode imaging of the articular cartilage of the femoral condyles was obtained by maintaining the knee joint in maximal flexion [53].

By maintaining the joint in the greatest degree of flexion permitted by range of motion, imaging of the weight bearing region of the condyles is allowed without interference from the patella. Further reports by McCune et al. support this approach to the imaging of cartilage [54]. These initial studies described images of normal articular cartilage as a smooth, uniformly hypoechoic band found beneath the echoes of the overlying skin and muscle [53]. The interface between the synovial fluid and the articular surface produced a thin echoic line at the surface of the cartilage [53]. This echoic line is most likely caused by the difference in acoustic impedance between the synovial fluid and the articular cartilage. Consequently, there was also a hyperechoic layer corresponding to
CHAPTER 1. INTRODUCTION

the signal from the echogenic interface between the cartilage and the subchondral bone layer. From these early external sonographic images, the thickness of the cartilage was able to be determined by measure of the thickness of the hypoechoic band [53,54].

The anatomy of the knee imposed limits on the chondral surfaces of the joint which could be imaged by external sonography. Several studies demonstrated transverse scans of the trochlea, the depression between femoral condyles, at frequencies of 7.5 MHz obtained with the knee positioned in flexion [53]. Images of the anterior femoral condyles have also been obtained using the same imaging technique [54].

External ultrasound imaging of articular cartilage provides non-invasive information about the underlying structures of the knee. However, there are difficulties in employing this technique to image articular cartilage. The resolution of the ultrasound image is directly related to the ultrasound imaging frequency used; similarly, penetration is inversely proportional to frequency. Thus, by improving the penetration of the ultrasound, the imaging resolution is sacrificed. For images of articular cartilage produced at 7.5 MHz, the image resolution is approximately 0.2 mm [55]. However, the average thickness of articular cartilage in the knee is approximately 2.2 mm [53,54]. The lower frequencies of ultrasound which are required to perform external sonography of the knee provide lower resolution images of the articular cartilage. Good correlation was obtained, however, between pre-operatively measured cartilage thickness using external ultrasound imaging and that measured histologically following knee arthroplasty [56].

Initial examination of articular cartilage by external ultrasound B-mode imaging were intended to describe the imaging of normal cartilage tissue [53,54]. It is also of interest, however, to describe the observations between normal and osteoarthritic cartilage. Cartilage thickness measurements have been made of normal and osteoarthritic cartilage; however, due to flexion restriction
in the degenerated joint, it is often difficult to obtain optimal imaging conditions [53]. It was found that the normally smooth and well demarcated interface between the articular surface and the surrounding soft tissue appeared blurred [54]. Some ultrasound investigators have concluded that qualitative estimates of the degree of osteoarthritis, based on degree of blurring and cartilage thinning, may be more useful than the efforts to measure the cartilage thickness directly from these images [54].

External B-mode imaging can provide a measure of articular cartilage thickness in the knee joint. However, the lower frequencies used to image cartilage externally have difficulty in showing detail of the internal cartilage structure [53]. The earliest observations associated with the progression of osteoarthritis involve changes in the internal morphology of the cartilage, including proteoglycan loss, collagen alteration and edema [1]. External ultrasound B-mode imaging can determine cartilage thickness, and has demonstrated a difference in measurable thickness of normal and osteoarthritic cartilage [56]. While the clinical role of external ultrasound imaging of the osteoarthritic knee joint has not been completely evaluated, it appears the technique has limitations in monitoring disease progression in articular cartilage.

**High Frequency Ultrasound Imaging**

Currently, of the methods available for the evaluation of articular cartilage in osteoarthritis, only direct arthroscopic inspection of the joint can quantify roughening of the articular surface, detect swelling of the cartilage, or map the extent of chondral ulceration [57]. Arthroscopy reveals little information, however, about the cartilage thickness, or the morphological structure beneath the surface. Higher frequency ultrasound imaging may be incorporated into a minimally-invasive imaging tool, used in conjunction with arthroscopy, for diagnosis of the knee joint cartilage. A minimally-invasive tool would be one that caused minimal damage to the surrounding structures,
in the area being diagnosed. Arthroscopic imaging, where the joint capsule is compromised only by a small incision, is an example of a minimally-invasive modality.

Preliminary investigations established the feasibility of high frequency ultrasound (20 MHz) in cartilage thickness measurements of the human acetabulum, *ex vivo* [58]. These early studies showed that the echoes from the articular surface could be distinguished from those of the hypoechoic cartilage matrix, as well as from echoes received from the subchondral bone interface. Initial studies at 20 MHz demonstrated an accuracy of thickness measurement of 0.08 mm, when compared to histology. Sanghvi *et al.* (1990) produced initial images of canine articular cartilage at 25 MHz [47]. These initial B-mode images displayed cartilage with a three layered appearance. Their study also examined correlation between ultrasound images and Safranin-O stained histology, with findings of limited correlation between echoes and histology [47]. Further investigation indicated feasibility for the determination of cartilage thickness from ultrasound measurements. These measurements indicated good correlation between thickness determined using ultrasound and photomicrographs. This work demonstrated that higher frequency ultrasound images of cartilage showed a more heterogeneous appearance than observations at lower frequencies, where the cartilage appeared as a homogeneous hypoechoic signal [47,53].

Harasiewicz *et al.* (1993) presented images of normal porcine articular cartilage obtained at 50 MHz, thereby offering improved image resolution [59]. These images of femoral condyle provided near microscopic (≈ 40μm) resolution of tissue structure. Images of immature femoral cartilage as well as mature cartilage were presented, and possessed a two layered appearance of echogenic signals [59]. Cartilage thickness was determined only by electronic measurement from the images. This study clearly produced impressive high resolution B-mode images of articular cartilage *in vitro* and demonstrated potential for further experimentation [59].
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More recent work has employed high frequency ultrasound imaging techniques, at 50 MHz, to produce cross-sectional B-mode images of cartilage in vitro [45,46,48]. For in-vitro imaging, specimens in these studies were immersed in either degassed saline or water. The typical examination procedure involved scanning the high frequency transducer (20-50 MHz) over the articular surface of the specimen, in order to image the cartilage and subchondral bone [46,48]. Kim et al. (1995) investigated the imaging of immature porcine cartilage with high frequency ultrasound backscatter microscopy [48]. This study endeavoured to establish possible correlation between backscatter signal from high frequency B-mode images of cartilage and corresponding histology specific for the cartilage matrix. Although these images had superior resolution to previous techniques, investigators were unable to determine the source of the backscatter signal in the images. This was most likely due to the complex internal organization of articular cartilage [60]. Figures 1.3 and 1.4 display typical high frequency B-mode images, obtained in this laboratory, of human articular cartilage at 50 MHz, using techniques of Kim et al. [48].

Figure 1.3: High frequency ultrasound image of normal human cartilage imaged at 50 MHz. The small scale bars represent 0.2 mm.

These B-mode images, produced at 50 MHz, display cartilage with a two layer appearance. Figure 1.3 shows a superficial hyperechoic layer, demarcating the interface between saline and the articular surface of the cartilage. Below this is a hypoechoic layer deeper in the cartilage, and
CHAPTER 1. INTRODUCTION

Figure 1.4: High frequency ultrasound image of severely osteoarthritic human cartilage imaged at 50 MHz.

finally, a hyperechoic signal band corresponding to the boundary between the cartilage and the dense subchondral bone layer. Figure 1.4 is an ultrasound image of cartilage in a severely advanced stage of osteoarthritis. The two layer appearance has given way to an irregular and roughened surface echo, along with that from the subchondral bone. This severely diseased cartilage has a different morphology than that of normal human cartilage, which may explain the difference in the image clarity.

Higher resolution ultrasound imaging techniques offer improved resolution of internal cartilage structures [46–48]. Cartilage thickness and articular surface quality were parameters which could be established by the imaging techniques. However, current imaging has investigated primarily bovine and porcine articular cartilage. As a result, there is little information on the appearance of human cartilage at high frequencies in the literature.

1.2.3 Ultrasound Tissue Characterization of Articular Cartilage

Ultrasonic propagation properties, primarily attenuation and speed of sound, can be used to characterize tissues according to their composition and structural organization [61]. Ultrasound tissue characterization is a quantitative measure of the behaviour of the ultrasound beam as it propagates
through a tissue of interest [55]. As the ultrasound beam propagates, it undergoes many interactions in the tissue of interest – including scattering, reflection, absorption and others. Tissue characterization quantifies this behaviour, by means of acoustic parameters such as attenuation, speed of sound and backscatter [55]. These coefficients are specific and distinct for tissues of differing structure or composition, and can be used to distinguish between them [61]. Acoustic parameters are based on the composition of the structure of interest, and thus, morphological or physical changes associated with disease can cause a detectable change in these parameters [62].

Acoustic parameters such as attenuation and speed of sound can be measured using a variety of techniques [63]. Pulse echo reflection and transmission echo methods offer good precision on measurements in vitro [55]. Acoustic parameters are measured by comparing the properties of ultrasound pulses that have propagated through the tissue of interest with pulses that have propagated through water or saline alone. Further discussion of measurement methods will be provided in detail in the following sections on the attenuation and speed of sound parameters.

Attenuation

The ultrasound attenuation coefficient is used to describe the loss of energy of an ultrasound wave as it propagates through tissue. Pulse energy is lost through a variety of interactions. Attenuation in a tissue is a strong function of ultrasound frequency [64], with higher frequencies more highly attenuated than lower frequencies [55].

In tissues, particularly connective tissues, the collagen fibrils play an important role in determining the ultrasonic attenuation [49, 52, 62]. In many tissues, such as cartilage, muscle and tendon, collagen has an ordered arrangement, while others, such as scar tissue have random arrangements [1]. The acoustic properties of the healthy myocardium differ from myocardium where scar tissue has been induced [62]. The alteration in arrangement of collagen fibrils from
organized (normal) to disorganized (scar tissue) is responsible for a change in the scatter, and thus the attenuation properties of the tissue. Release of proteoglycans from cartilage, with papain, has been shown to change the arrangement of the collagen fibrils in the cartilage [22]. Thus, progression of osteoarthritis could be associated with changes in the ultrasound attenuation coefficient.

Insertion loss techniques are often used to measure ultrasonic attenuation [55]. A typical arrangement for insertion loss attenuation measurement is shown in Figure 1.5. Two ultrasound pulses are needed to determine the attenuation coefficient. A reference pulse is transmitted through the coupling medium, usually water or saline, and the echo signal, $A_0(t)$, is received, as shown in Figure 1.5. A second, identical pulse is transmitted through the tissue of interest, reflected by a quartz plate and then received by the transducer, $A(t)$. An acoustically transparent membrane, such as resinite, is often used to keep the sample stationary during the measurement.

![Figure 1.5: Experimental setup for determining frequency dependent attenuation. After D'Astous & Foster (1986).](image)

These two pulses, the reference ($A_0(t)$) and the attenuated ($A(t)$), are then compared in the frequency domain to calculate the attenuation coefficient. The thickness of the tissue sample, $l$,
is determined by calculating the distance between the resilin membrane and the quartz reflector. This measurement is made during speed of sound measurements by the time of flight technique.

The frequency spectra of the two pulses, $A_0(t)$ and $A(t)$, are then obtained by Fourier transform. The frequency dependent attenuation coefficient is calculated by taking the ratio of the magnitude of the attenuated to the reference spectrum, according to Equation 1.1:

$$
\alpha(\nu) = \frac{-20}{2\ell} \log_{10} \frac{|A(\nu)|}{|A_0(\nu)|} \text{dB mm}^{-1},
$$

where $\ell$ represents the thickness of the cartilage sample in mm, and $|A(\nu)|$ and $|A_0(\nu)|$ represent the magnitudes of the frequency spectrum for the attenuated and reference pulses respectively. The logarithm is taken in order to obtain the frequency dependent attenuation in the familiar units of dB mm$^{-1}$ MHz$^{-1}$. The frequency dependent attenuation, $\alpha(\nu)$, can be modeled by the power relationship between attenuation and frequency:

$$
\alpha(\nu) = \alpha_o \nu^\gamma,
$$

where $\nu$ represents the frequency, $\alpha_o$ represents a constant coefficient, and $\gamma$ is the frequency dependence of the model [64].

**Speed of Sound**

The speed of sound is a bulk acoustic measurement of the speed at which ultrasound waves propagate through tissue. The speed of sound, $c$, in a homogeneous medium is characterized by Equation 1.3:

$$
c = \sqrt{\frac{B}{\rho}},
$$

where $B$ is the bulk modulus and $\rho$ is the density of the material. This method can be used to model the speed of sound in articular tissue, since soft tissue is composed primarily of water. More
compressible tissues, or less dense tissues have lower speeds of sound. Figure 1.6 lists the speeds of sound of various articular tissues in the body, with water and saline displayed for reference.

![Figure 1.6: Speed of sound of various human articular tissues, water and saline. Values from references 43, 65–67.](image)

As Figure 1.6 demonstrates, more elastic articular tissues such as muscle have a lower speed of sound than more incompressible articular tissues such as tendon, which is composed primarily of collagen fibrils.

Speed of sound is primarily measured using a time of flight technique. A common setup is shown in Figure 1.7, where $t_o$, $t_s$ and $t_w$ are the return times for the ultrasound pulses traveling through the medium alone, the medium with the sample in place, and the sample thickness, $l$, in water respectively. This value, $t_w$, is used to calculate the thickness, $l$, of the sample. If the speed
of sound in the medium is already known to be $c_m$, then the thickness of the sample, $l$, is given by:

$$ l = \frac{1}{2} c_m t_w. \quad (1.4) $$

The factor of one half accounts for the return trip of the pulse.

Finally, the speed of sound can be determined by using the simple distance/time relationship shown in Equation 1.5.

$$ c_t = \frac{2 l}{(t_w + t_s - t_0)} \text{ } m \text{ } s^{-1} \quad (1.5) $$

The expression on the bottom of the fraction represents the total time of transit of the sound through the tissue alone.
Tissue Characterization of Cartilage

Articular cartilage was among the first tissues characterized with ultrasound [43]. Dussik & Fritch (1958) reported on the speed of sound and attenuation of sound in bovine and human cartilage at frequencies of 1 to 5 MHz [43]. Their results established the speed of sound in cartilage at 1665 ms\(^{-1}\). They also attempted to measure the ultrasonic attenuation coefficient, using an insertion-loss technique. Their results are summarized in Figure 1.8. These data from Dussik and Fritch served as the accepted acoustic parameters for cartilage until as recently as 1990 [49].

![Figure 1.8: Frequency dependent attenuation of human and bovine cartilage data from Dussik & Fritch (1958). Data have been fitted, for frequency dependent attenuation, to Equation 1.2.](image)

With the development of higher frequency transducers capable of high resolution imaging, there was an increased interest in classifying the acoustic parameters of various tissues, including cartilage. Agemura et al. performed pulse echo reflection measurements on speed of sound and acoustic attenuation for bovine articular cartilage at 100 MHz [49]. Values for the speed of sound were determined by interference-mode imaging of the cartilage. These experiments yielded a value for the speed of sound of 1660 ± 44 m s\(^{-1}\) for 16 specimens. This value was in agreement with that of Dussik and Fritch [63]. An acoustic attenuation coefficient at 100 MHz of 92 ±15 dB mm\(^{-1}\) was
obtained by an insertion-loss technique. There was no measure of frequency dependent attenuation. Agemura et al. also performed preliminary experiments on the effects of collagen orientation on speed and attenuation, as well as the effects of various degradative agents on these two parameters. It was found the the orientation of collagen had a noticeable effect on both attenuation and speed of sound. It was also found that cartilage digested to deplete chondroitin sulphate (a proteoglycan constituent) also showed a change in both attenuation and speed of sound. The speed of sound showed an increase, while the attenuation coefficient decreased between normal samples and those depleted of chondroitin sulphate. These experiments indicated the promise of ultrasound tissue characterization in detecting changes in cartilage morphology.

Senzig and Forster (1992) performed subsequent measurements on the frequency dependent attenuation of articular cartilage, using the same experimental setup as Agemura et al. [52] in the range of 10 to 40 MHz. Measurements of acoustic attenuation using the insertion-loss technique were used to compare the variation of attenuation with regions of different load bearing in the patella. Results indicated that higher attenuation coefficients were present in regions of higher mechanical load. Senzig et al. speculated that this increase in attenuation was due to variation in morphology of load bearing areas of the cartilage, such as distribution of proteoglycan molecules or water distribution.

Further investigations of the acoustic properties of human cartilage were performed by Myers et al. (1995) [46]. The thickness of the cartilage samples was measured using a graticule and by optical examination. The speed of sound was then calculated by dividing the thickness value by the time of flight for the ultrasound, as determined by counting pixels on a video B-mode image. The resulting values for the speed of sound had a large variance, likely due to the imprecision of the measurement techniques. The mean value of the speed of sound measured in normal human
cartilage was $1658 \pm 185 \text{ m s}^{-1}$, while the mean value measured in osteoarthritic cartilage was $1581 \pm 148 \text{ m s}^{-1}$. Myers also investigated the mean concentrations of uronic acid and hydroxyproline between normal and osteoarthritic samples. Although there were differences in proteoglycan levels between groups, no correlation between proteoglycan levels and the speed of sound was found. This lack of correlation is likely due to the large variance in the mean speed of sound for the two sample groups.

Recent work in high frequency ultrasound tissue characterization has demonstrated a sensitivity in the attenuation and speed of sound to morphologic changes in cartilage [46,49]. There have also been preliminary results in comparing human cartilage with normal and osteoarthritic cartilage, indicating potential correlation between acoustic parameters and osteoarthritis [46]. However, there has been difficulty in establishing this correlation, due to poor precision in the measurement of attenuation and speed of sound in these experiments. In addition, the data in the literature on the speed of sound and frequency-dependent attenuation for normal human articular cartilage are either dated or suffer from a large variance. We believe that a precise measurement of the speed of sound, using a time of flight technique is needed, and may aid in characterization of normal and osteoarthritic articular cartilage. In addition, there is a need for an understanding of the frequency dependent attenuation in a controlled, in vitro, experimentally induced model of osteoarthritis. These experiments might lend precision to the preliminary work that has been accomplished, and demonstrate the sensitivity of ultrasonic tissue characterization to osteoarthritis-like changes in cartilage.
1.3 Histopathology of Cartilage

Histopathology is the study, at a microscopic level, of structural and functional changes in a tissue that arise with disease. Examination of thin tissue sections under light microscopy visualizes small structural components of tissue up to magnifications of approximately 1000x. Histology can be used to demonstrate differences between diseased and normal tissues, and can be enhanced by the use of coloured dyes in staining specific types of tissue.

Tissue stains are selected to visualize the molecular composition of the tissue of interest. The dyes Safranin O and Picro-Sirius Red have an affinity for the proteoglycans and collagen II fibrils of cartilage respectively, thus providing qualitative information on biochemical structure and organization [12,15].

The changes in the morphology of cartilage with osteoarthritis are clearly visualized by histopathological examination of tissue sections. Cartilage is primarily composed of a negatively charged, proteoglycan rich matrix, along with ordered arrangements of collagen fibrils. The initial stages of osteoarthritis are characterized by a loss of matrix proteoglycans, chondrocyte cloning and surface fibrillations [60].

1.3.1 Safranin O Histology

Safranin O is a cationic dye which binds selectively to polyanions, including chondroitin sulphate and keratan sulphate glycosaminoglycans, within the proteoglycan aggregate in articular cartilage [12]. Safranin O is commonly used in cartilage staining to demonstrate the proteoglycan content of the tissue. The staining intensity of Safranin O represents semi-quantitatively the proteoglycan aggregate concentration in cartilage [12]. Darker orange staining indicates a higher number of binding sites for the molecule, and thus a higher concentration, whereas absent or low colour
reflects a low concentration of glycosaminoglycans. Fast Green is a counter-stain which can be used to provide contrast in the areas of cartilage where proteoglycans are absent.

Figure 1.9 displays a representative histology slide of normal human cartilage stained with Safranin O. There is a large concentration of orange staining throughout the cartilage, indicating the presence of proteoglycans. There is also a thin superficial layer which exhibits minimal colour and the tidemark of the cartilage is defined as the staining boundary of the Safranin O at the deeper subchondral bone level of the cartilage. The lighter green staining is that of the Fast Green dye, which helps to demarcate the tidemark and also indicate regions of proteoglycan loss.

![Figure 1.9: Photomicrograph of a Safranin O stained section of human cartilage. The scale bar represents a thickness of 1 mm.](image)

Safranin O histology provides a qualitative indication of the presence of proteoglycans in cartilage [12]. Mankin et al. defined a grading scale for the severity of osteoarthritis progression based upon the histopathology of Safranin O stained cartilage, which was subsequently revised by van der Sluijs [13, 65]. These grading scales use the Safranin O stained slides and score for
osteoarthritis of the cartilage based upon the criteria listed in Table 1.1. This grading system uses a 0 - 13 scale of grading the cartilage, with zero representing healthy cartilage and 13 representing severely osteoarthritic.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Irregular surface, including fissures into the radial layer</td>
<td>1</td>
</tr>
<tr>
<td>Pannus</td>
<td>2</td>
</tr>
<tr>
<td>Superficial cartilage layers absent</td>
<td>3</td>
</tr>
<tr>
<td>Slight disorganization (cellular rows absent, some small clusters)</td>
<td>4</td>
</tr>
<tr>
<td>Fissures into calcified cartilage layer</td>
<td>5</td>
</tr>
<tr>
<td>Disorganization (clusters, chaotic structure)</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cellular abnormalities</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Hypercellularity, small clusters</td>
<td>1</td>
</tr>
<tr>
<td>Clusters</td>
<td>2</td>
</tr>
<tr>
<td>Hypocellularity</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Matrix Staining</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal/slight reduction</td>
<td>0</td>
</tr>
<tr>
<td>Staining reduced in radial layer</td>
<td>1</td>
</tr>
<tr>
<td>Reduced in interterritorial matrix</td>
<td>2</td>
</tr>
<tr>
<td>Only present in pericellular matrix</td>
<td>3</td>
</tr>
<tr>
<td>Absent</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 1.1: The Modified Mankin score for grading osteoarthritic cartilage. Reproduced from van der Sluijs (1992).

Although the grading of osteoarthritis is established, it can only provide a qualitative description of the cartilage, and is usually dependent on the user's experience. Safranin O is accepted as an excellent stain for the histopathology of the matrix and its constituents. Hence, as osteoarthritis progresses, Safranin O histology can indicate both the quality of the cartilage surface, as well as the distribution of proteoglycans throughout the tissue.
1.3.2 Quantitative Proteoglycan Assay

The degradation and release of proteoglycans is a characteristic feature of osteoarthritis which can be modeled in vitro by papain digestion of cartilage or by incubation with recombinant human interleukin-1α [18, 66]. In these experimentally induced osteoarthritis models, proteoglycan fragments including glycosaminoglycans are released into the cartilage matrix and surrounding medium. Quantifying this loss of proteoglycans in an experimental situation allows both validation of proteoglycan depletion for the model and provides information about the amount of proteoglycan loss [29]. Farndale’s dimethylmethylene blue (DMB) assay for glycosaminoglycans is a means of quantifying the concentration of these fragments released from the cartilage [29].

The dimethylmethylene blue assay is based on the affinity of the dimethylmethylene blue molecule for free glycosaminoglycans. When mixed in solution with free glycosaminoglycans, the dye molecules bind to the free glycosaminoglycan molecules in a concentration proportional to the number of free glycosaminoglycans [29]. A measure of the light absorbency at 525 nm can be correlated with a known concentration of glycosaminoglycans bound to the dimethylmethylene blue molecule, and thus provide a quantitative value for comparison. The DMB glycosaminoglycan assay involves the complete digestion of the tissues to be analyzed, and is suitable for in-vitro experiments.

This method is another useful histological correlate for investigating experimentally-induced osteoarthritis, where often tissues are cultured in vitro in a control/experimental pairing. In non-human tissues, where availability and tissue variability is more uniform, the DMB assay can provide a more quantitative histological correlate than the qualitative Safranin O stain. In tissues such as human cartilage, where morphology can vary throughout the tissue, more qualitative methods can be applied. However, in general, in order to provide a histological correlate for other experimental
findings, a quantitative correlate such as that provided by the DMB glycosaminoglycan assay should be the method of choice.

1.4 Summary

Current diagnostic imaging methods lack the sensitivity to detect osteoarthritis in its early stages, where morphological changes are subtle. One important change associated with the early stages of osteoarthritis is the loss of hydrophilic proteoglycan molecules in the cartilage. Previous studies have used high frequency ultrasound (25 MHz) to detect changes in the speed of sound in cartilage following incubation in proteoglycan degrading chemicals [46]. The correlation between proteoglycan aggregate loss in a model of early osteoarthritis and the effect of the loss on the acoustic properties of human and bovine articular cartilage are described in Chapter 2.

The acoustic properties, at 30 MHz, of human and bovine cartilage subjected to experimentally-induced models of early osteoarthritis are described in Chapter 2. The ability of high frequency tissue characterization to detect changes in the acoustic attenuation and speed of sound between control and proteoglycan depleted cartilage is also described in Chapter 2. Finally, an extension of the in-vitro studies of Chapter 2 towards the non-invasive in-vivo tissue characterization of human cartilage is outlined in Chapter 3.
Chapter 2

Acoustic Properties of Human and Bovine Cartilage

2.1 Introduction

Osteoarthritis affects the articular cartilage of weight bearing joints, and is characterized, in its early stages, by matrix degradation and a loss of proteoglycans [8]. Previous investigations using high frequency ultrasound tissue characterization have demonstrated that chemically-induced removal of matrix proteoglycans or collagen in bovine cartilage leads to changes in the frequency dependent attenuation and the speed of sound [49,52]. However, there has not yet been an investigation into the effects of matrix degradation on attenuation and speed of sound performed on human articular cartilage that has demonstrated significant results [46]. In addition, there is currently a lack of data on the frequency dependent attenuation in the 20–40 MHz range in normal human and bovine articular cartilage.

The purpose of this study was to determine the effect of matrix degradation on the speed
of sound and the frequency dependent attenuation in human and bovine articular cartilage. To determine the effects on speed of sound and attenuation, we performed ultrasound tissue characterization on two experimental models of matrix degradation: papain digestion of human articular cartilage in vitro and culture of bovine cartilage explants in interleukin-1α. Tissue explants cultured with interleukin-1α were used for the bovine cartilage to preserve the cell-extracellular matrix interactions in a controlled intact tissue system [67]. Papain was used with the human cartilage samples, because samples were procured from arthroplasty, where it was not possible to maintain the chondrocyte viability. Hence, two independent models of matrix degradation were used in this experiment. We also made measurements of the speed of sound and frequency dependent attenuation of normal human articular cartilage.

2.2 Materials and Methods

2.2.1 In Vitro Digestion of Human Articular Cartilage with Papain

Samples of "normal" cartilage used in this study were obtained at autopsy from the knees of 2 healthy 29 and 34 year old patients. This cartilage was obtained from the bone bank at Mt. Sinai Hospital (Toronto, Canada). Articular cartilage was stored at -30°C prior to sectioning. It has been shown, in various tissues, that no significant differences in the speed of sound, attenuation or backscatter were found in measurements made in a fresh sample compared with the same sample after it had been frozen and thawed [48, 49, 64]. Femoral condyles were sectioned into cartilage pieces 5 mm in width. These samples were referred to as "young normal cartilage". Seventeen young normal cartilage specimens were obtained for control vs. digested experiments. Seven of these young normal specimens served as healthy normal control specimens, while ten samples were digested in various concentrations of papain to obtain a dose response of the acoustic parameters.
to papain digestion.

Other human articular cartilage specimens were obtained from the femoral condyles of twenty-four patients, 18 female and 6 male, undergoing total knee arthroplasty for osteoarthritis. These samples were selected from regions of the condyles that were visually judged to be the least affected by osteoarthritis. These samples were referred to as "control" cartilage. The mean age of the patients was 71 ± 7 years of age. Femoral condyles were cut into 24 pairs of samples of 5 mm width, adjacent to one another, to serve as digested and control specimens. Adjacent samples were chosen in order to provide a good control for the experiment.

For each sample pair, the experimental specimen was incubated in a solution of 0.3 % Papain (Sigma P-3125), while the control was incubated in a 0.3 % solution of 0.05 M sodium acetate (Sigma S-7899) and 0.01 % thymol (Sigma T-0501). Both specimens were incubated for 10 hours at 37°C. Following digestion, the specimens were removed from solution and washed with distilled water. A portion of each specimen underwent proteoglycan specific Safranin O staining, to provide our qualitative measure of proteoglycan loss, as described by Lillie [68]. The remainder of the specimens were stored at 5 °C in humid containers for up to 24 hours prior to ultrasonic tissue characterization.

Validation of Papain Dose for Proteoglycan Digestion Experiments

Ultrasonic tissue characterization was performed on the articular cartilage samples for papain concentrations in the range of 0.005 % to 0.5 %. The ultrasonic attenuation and speed of sound were both normalized with the intra-sample controls. The results for the attenuation coefficients at 30 MHz are arranged graphically in Figure 2.1, while the results for the speed of sound are arranged in Figure 2.2. One data point was selected to represent the attenuation for each cartilage sample. Therefore, we chose to represent the data by the mean attenuation coefficient at the centre
frequency (30 MHz) of our transducer.

On both graphs, we observed similar changes in the normalized data points for both attenuation (increase) and speed of sound (decrease), at papain concentrations greater than 0.1 %. The attenuation showed an increase of approximately 70 % in this range, while the speed of sound showed a decrease of 2 % for the same range. Hence, for our model of inducing osteoarthritis-like changes in cartilage with papain, we chose a digestion concentration of 0.3 % for further experiments with this experimental model of osteoarthritis.

![Figure 2.1: Change in the ultrasonic attenuation coefficient at 30 MHz between articular cartilage digested in papain and controls. A value of 1 represents no change from the control.](image)

2.2.2 Bovine Cartilage Explants Cultured with Interleukin-1α

Normal bovine cartilage was obtained fresh. Two stifle joints from young steers, with intact synovial capsules, were selected for use and stored at 0 °C during transport to preserve the cartilage and chondrocytes in their natural environment. A total of fifteen pairs of 3 mm diameter and 1.5 mm thick cartilage explants were isolated and prepared for culture according to the methods described by Dumont et al. - tissue culture experiments were performed in Montreal, QC [67]. Each pair had
Figure 2.2: Change in the speed of sound in the articular cartilage between control specimens and those subjected to digestion by varying concentrations of papain. A value of 1 indicates no change from the control.

one sample cultured in interleukin-1α, while the other served as a control. Experimental cartilage explants were cultured in serum-free media, along with 5 ng/ml or recombinant human interleukin-1α, while control explants were cultured in serum-free media alone. All explants were incubated at 37 °C and 5 %CO₂ for 11 days. Following incubation, 3 sample pairs were immediately subjected to mechanical testing in order to determine equilibrium and dynamic stiffness, as described by Soulhat et al. [69]. These 3 sample pairs were also assayed with Farndale’s dimethylmethylene blue method in order to determine the extent of proteoglycan removal in the cartilage explants [29]. The remaining 12 sample pairs were stored in humid containers at -30°C during transport from Montreal to Toronto. Upon arrival, samples were stored at 5 °C for 12 hours awaiting ultrasound tissue characterization.
2.2.3 Ultrasonic Tissue Characterization

The methods used for calculating the ultrasonic frequency dependent attenuation and speed of sound were based on those previously described by D'Astous and Foster [64]. The ultrasonic arrangement consisted of a radio frequency (RF) pulser (Avtech Inc., Ottawa, Canada) and a 30 MHz PVDF transducer manufactured in our laboratories, as well as electronics that have been previously described [48].

Prior to ultrasonic tissue characterization, the cartilage specimens were cut, in order to detach the articular cartilage from the underlying bone. These small cartilage plugs were placed in a sample holder, consisting of a quartz reflector plate and a resinite cover film (Borden Packaging, Canada) to hold the sample in place, as shown in Figure 2.3. The entire tissue mounting stage was kept in 1 % phosphate buffered saline (PBS) at 37°C throughout the experiment.

Data acquisition consisted of collecting a raster pattern (350 μm x 350 μm) of 64 RF lines, with 50 μm separation between individual acquisition points. The fine motion of the transducer was controlled by a PC computer and a Burleigh 7000 micro-positioning system (Burleigh Inc., Rochester NY). The RF lines were collected with a HP54201D digital oscilloscope (Hewlett Packard) and stored on hard disk for further analysis. Data analysis for calculation was performed using Matlab software using the algorithms of D'Astous and Foster [64] to calculate frequency-dependent attenuation in the 20 to 40 MHz frequency range as well as the speed of sound in cartilage specimens. Speed of sound was calculated using pulse-echo time of flight, while frequency-dependent attenuation coefficients were measured using the insertion-loss technique. Once calculated, the frequency dependent attenuation coefficients were fitted to Equation 2.1:

\[ \alpha = \alpha_o f^\gamma. \] (2.1)
Figure 2.3: Schematic illustration of tissue characterization setup.
2.2.4 Analysis of Data

Data are summarized as mean ± standard error in the mean, unless otherwise indicated. To analyze the changes in the results from the control and experimentally-degraded cartilage samples, we performed a paired Student’s t test on measurements of the frequency dependent attenuation and the speed of sound. To compare the changes in the frequency dependent attenuation between the young normal cartilage and the control cartilage specimens, we performed a Student’s t test because the two populations were not related. We considered differences significant at p < 0.05.

2.3 Results

2.3.1 Acoustic Parameters of Normal Human Cartilage

The measured value of the speed of sound was 1666 ± 16 m s\(^{-1}\) for the 7 young normal samples. This value agrees with the results from the literature characterizing the speed of sound at 1665 m s\(^{-1}\) in human articular cartilage [63].

The mean attenuation coefficient of the articular cartilage, measured at 30 MHz, was 6.2 ± 0.4 dB mm\(^{-1}\). A summary of the frequency dependent attenuation is presented in Figure 2.5.

2.3.2 Ultrasonic Tissue Characterization of Papain Digested Human Cartilage

The mean speed of sound was 2 % lower in the 24 papain digested cartilage samples than in the 24 corresponding control cartilage samples (1642 ± 9 vs. 1664 ± 7 m s\(^{-1}\)). The observed mean decrease of the speed of sound was statistically significant, p = 0.03. A summary of the speed of sound data for the 24 paired control and papain digested human cartilage samples is presented in Figure 2.4.

The mean attenuation coefficient, measured at 30 MHz, was 20 % higher in the 24 proteo-
Figure 2.4: Effect of matrix degradation on speed of sound (mean ± SEM) for 24 control and papain digested human cartilage samples.

Figure 2.5: Frequency dependent attenuation (mean ± SEM) for 24 control and papain digested human cartilage samples. Frequency dependent attenuation data for 7 young normal human cartilage samples are also presented.
Figure 2.6: a) Photomicrograph of a control section of decalcified human articular cartilage stained with Safranin O. b) Photomicrograph of a papain digested section of decalcified human articular cartilage stained with Safranin O. The scale bar represents 1 mm and both images are at 2.6 x magnification.
glycan depleted cartilage samples than in the 24 corresponding control cartilage samples (8.5 ± 0.5 vs. 7.1 ± 0.4 dB mm⁻¹). The increase of the mean frequency dependent attenuation coefficient was statistically significant, p = 0.02. A summary of the frequency dependent attenuation data for the 24 paired control and papain digested human cartilage samples along with the 7 young normal cartilage samples is presented in Figure 2.5. The data for all populations were fitted to Equation 2.1 and these lines are displayed on the Figure.

Safranin O histology confirmed the removal of proteoglycans from human cartilage specimens digested with papain. Safranin O stained photomicrographs of control and papain digested human cartilage specimens are presented in Figure 2.6. Figure 2.6(b) shows a loss of Safranin O intensity, following papain digestion, associated with a loss of cartilage matrix proteoglycans [12].

2.3.3 Tissue Characterization of Bovine Cartilage Explants Cultured in Interleukin-1α

The mean speed of sound was 2 % lower in the 12 interleukin-1 cultured cartilage samples than in the 12 corresponding control cartilage samples (1631 ± 17 vs. 1666 ± 8 m s⁻¹). The observed mean decrease of the speed of sound was statistically significant, p = 0.04. A summary of the speed of sound data for the 12 paired control and proteoglycan depleted bovine cartilage samples is presented in Figure 2.7.

The mean attenuation coefficient, measured at 30 MHz, was 30 % higher in the 12 interleukin-1 cultured cartilage samples than in the 12 corresponding control cartilage samples (9.1 ± 1.0 vs. 6.8 ± 1.2 dB mm⁻¹). The observed mean increase of the frequency dependent attenuation was statistically significant, p = 0.04. A summary of the frequency dependent attenuation data for the 12 paired control and proteoglycan depleted bovine cartilage samples is presented in Figure 2.8.
Figure 2.7: Effect of matrix degradation on the speed of sound (mean ± SEM) in 12 control and interleukin-1 cultured bovine cartilage samples.

Figure 2.8: Frequency dependent attenuation (mean ± SEM) for 12 control and interleukin-1 cultured bovine cartilage samples.
To use the culture of bovine cartilage in interleukin-1 as an independent model of matrix degradation and proteoglycan loss, we needed to validate the effects of the interleukin-1 on releasing cartilage matrix proteoglycans. The weights of glycosaminoglycans present in control specimens and digested specimens were measured and are presented in Table 2.1. There was a mean loss of 80% of the proteoglycan content following digestion. This observation confirmed the effectiveness of our interleukin-1 model of matrix degradation.

<table>
<thead>
<tr>
<th>GAG Content (µg)</th>
<th>Control</th>
<th>Interleukin-1 Cultured</th>
</tr>
</thead>
<tbody>
<tr>
<td>680 ± 260</td>
<td>155 ± 55</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1: Proteoglycan content (mean ± SEM) in 3 control and 3 interleukin-1 cultured samples of bovine cartilage assayed by Farndale's dimethylmethylene blue method [29].

### 2.4 Discussion

Our experiments in human and bovine articular cartilage show that matrix degradation and proteoglycan loss result in a decrease in the speed of sound and an increase in the frequency dependent attenuation. We used two independent experimental models of osteoarthritis: digestion of human cartilage with papain and culture of bovine cartilage with interleukin-1α. Both models function by primarily degrading the proteoglycans of the articular cartilage, although the mechanism of degradation for each method is different [22,27]. A mean decrease in the speed of sound of 2% (p < 0.05) was observed in paired samples of human (n = 24) and bovine cartilage (n = 12) depleted of matrix proteoglycans, while a mean increase of 20 to 30% (p < 0.05) was observed in the frequency dependent attenuation. These observations support our hypothesis that the matrix proteoglycans and collagen of articular cartilage play an important role in determining the acoustic attenuation and speed of sound within articular cartilage.
The 2 % (p < 0.05) decrease observed in our speed of sound data for human and bovine cartilage, following matrix degradation and proteoglycan loss, is in agreement with the observation of Myers et al. (1995). Previous investigators reported a 4 % mean decrease in the speed of sound when comparing osteoarthritic and normal human cartilage specimens; however, they did not attempt to establish a significant correlation between the speed of sound in normal and osteoarthritic cartilage specimens [46]. Conversely, the data available for bovine cartilage indicate a slight increase in the speed of sound after depleting bovine cartilage of chondroitin sulfate, a glycosaminoglycan, or collagen [49]. The observed decrease in speed of sound, following matrix degradation, suggests it may be related to the function of the proteoglycan aggregate in articular cartilage, which acts to oppose the tensile forces of the collagen fibrils [1]. Following matrix degradation and proteoglycan loss, this swelling force is removed and the cartilage softens. Consequently, this softening of the cartilage might translate into an increase in the compressibility. The speed of sound within a medium is related to its compressibility by Equation 2.2:

\[
c = \sqrt{\frac{1}{\rho \kappa}},
\]

(2.2)

where \( c \) refers to the speed of sound, \( \rho \) is the density of the medium, and \( \kappa \) represents the compressibility of the medium. Hence, as tissue becomes more compressible, we would expect the speed of sound to decrease. We observed this decrease in speed of sound in human and bovine cartilage samples, while the frequency dependent attenuation consistently increased following experimentally-induced matrix degradation.

Our results in human and bovine cartilage demonstrated that experimentally-induced matrix degradation resulted in a 20 to 30 % (p < 0.05) increase in the frequency dependent attenuation. A previous investigation by Agemura et al. (1990) compared attenuation coefficients after depleting adult bovine cartilage proteoglycans [49,52]. Contrary to our observations, their work performed
at 100 MHz showed a decrease in the attenuation coefficient following proteoglycan depletion. Figure 2.5 presents the frequency dependent attenuation for young normal, control and papain digested human cartilage, and Table 2.2 presents the best fits of our measurements with Equation 2.1. The attenuation coefficient of the control samples, which were obtained from osteoarthritic patients during total knee arthroplasties, was consistently higher than the attenuation for young normal cartilage. Similarly, the papain digested cartilage had a consistently higher attenuation than that of the control specimens. The observed increase between matrix degraded and control cartilage specimens was statistically significant (p < 0.05), as was the observed increase between young normal and control samples (p < 0.05) when verified with an unpaired Student's t test. We suggest that the control human cartilage samples had a frequency dependent attenuation higher than the young normal samples because they had already undergone some osteoarthritic changes prior to removal during knee arthroplasty. Hence, the control cartilage samples may have already suffered some matrix degradation and collagen alteration. Thus, these samples may represent an intermediate stage in matrix degradation between young normal cartilage and papain digested articular cartilage. These observations suggest that the frequency dependent attenuation is sensitive to the concentration of matrix constituents in the articular cartilage.

The mean frequency dependent attenuation results from the human and bovine articular cartilage specimens were plotted graphically in Figures 2.6 and 2.9 respectively. The data on these graphs were then fitted with Equation 2.1 to model the frequency dependence of the mean attenuation values. In Figure 2.6, the human data agrees well with the predicted response of the model. However, in Figure 2.9, the data does not appear to fit the predicted frequency dependence as well as the human data fits. We believe this is a result of the low sample size (n = 12) that was analyzed. An increased sample size from more animals would likely reduce the discrepancy
between the mean attenuation coefficient data and the predicted frequency dependent response.

<table>
<thead>
<tr>
<th>Frequency Dependence</th>
<th>Young Normal $\alpha_n(f) = 0.059 \ f^{1.37}$</th>
<th>Control $\alpha_c(f) = 0.112 \ f^{1.23}$</th>
<th>Proteoglycan Depleted $\alpha_d(f) = 0.216 \ f^{1.08}$</th>
</tr>
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Table 2.2: Frequency dependent attenuation for young normal, control, and papain digested human cartilage. The exponent value represents the frequency dependent term, $\gamma$.

The attenuation mechanism is primarily comprised of scattering and absorption interactions of ultrasound with tissue [55]. We suggest that the observed increase in the attenuation coefficient associated with matrix degradation may arise from changes to the orientation of the collagen fibril network. Williams et al. (1996) observed a loss of matrix proteoglycans as well as an alteration in the spatial orientation of collagen following intra-articular papain injection to cause matrix degradation in rabbits [22]. Their observations showed that the collagen fibrils changed from an anisotropic, orderly, structure, to a more random, isotropic, organization. The collapse of the collagen fibrils was attributed to the loss of the ability of the proteoglycans to ‘inflate’ the cartilage and oppose the tensile forces of the collagen fibrils. Previous investigations of the ultrasound characterization of cartilage have indicated a correlation between the orientation of collagen and the attenuation at 100 MHz [49]. We suggest that the change in collagen orientation associated with matrix degradation and proteoglycan loss, from our experimental models of osteoarthritis, may contribute to the observed increase in the attenuation coefficient of ultrasound. Although we believe the increase in attenuation arises from the alteration in collagen orientation, it is not clear whether the increase arises from changes in scattering or absorption of the ultrasound.

Previous experiments have reported the speed of sound in normal human cartilage to be 1665 m s$^{-1}$ [43,46]. Our results of 1666 $\pm$ 16 m s$^{-1}$ agree. The frequency dependent attenuation of normal human cartilage had not previously been reported in the range of 20 to 40 MHz. Our data,
when fitted to 2.1, yielded a frequency dependence described by: \( \alpha = 0.059 f^{1.4} \text{dB/mm MHz}^{-1} \), and a mean attenuation coefficient at 30 MHz of 6.2 \( \pm \) 0.4 dB mm\(^{-1}\).

In summary, the experimentally-induced degradation of the matrix in human and bovine articular cartilage increases the attenuation coefficient by 20 to 30 \% (p < 0.05) and decreases the speed of sound by 2 \% (p < 0.05). These changes are consistent in two independent models of matrix degradation in both human and bovine articular cartilage samples. The ultrasonic properties of articular cartilage may be sensitive to the concentration of matrix constituents, such as proteoglycans and collagen. Because matrix degradation and proteoglycan loss occurs in the early stages of osteoarthritis, we believe that ultrasound tissue characterization may potentially have a role in the assessment of articular cartilage during osteoarthritis. Future work will examine the feasibility of measuring and detecting these changes in an in vivo model of early osteoarthritis.
Chapter 3

Future Work

3.1 Low Frequency Tissue Characterization of Articular Cartilage

The implications of our results and the research necessary to determine the feasibility of the development of a non-invasive cartilage assessment tool will be described in this chapter. The experimental requirements for the development of such a device are described. We envision a non-invasive probe eventually to be used to perform non-invasive ultrasound attenuation measurements on articular cartilage for the assessment of osteoarthritis.

Evaluation of Cartilage Tissue Characterization at Low Frequencies

Although ultrasound at high frequencies can provide improved resolution of tissue structures, penetration depth is limited by attenuation. The penetration depth is determined by the attenuation coefficient of the tissue or group of tissues being examined. The attenuation of ultrasound increases exponentially with frequency. Thus, as the attenuation increases, the penetration depth of the ultrasound beam decreases proportionally. Lower frequency ultrasound (10–15 MHz) offers improved penetration through tissue over higher frequencies. Figure 3.1 presents the frequency dependent
attenuation coefficient of skeletal muscle in the range of 10 to 50 MHz [70]. Muscle is the principal attenuating tissue found between the skin and the articular cartilage of the knee.

![Figure 3.1: A log-log plot of the attenuation coefficient for skeletal muscle vs. frequency. Data fitted from Bhagat et al. [75].](image)

The depth of penetration becomes a more important parameter to consider as cartilage evaluation moves into an in-vivo environment. The anatomy of the knee is such that the cartilage on the femoral condyles can be located between 2 and 4 cm from the surface of the skin – this distance is variable. Signal penetration of this magnitude is achieved using lower frequency ultrasound systems. Currently, investigations on the tissue characterization of articular cartilage have worked in the frequency ranges of 1–5 and 25–100 MHz [43,46,49,52]. Previous work by Myers et al. at 25 MHz indicated an ability to differentiate between normal and osteoarthritic cartilage by speed of sound [46]. These findings suggest that ultrasound tissue characterization at frequencies lower than 25 MHz, where attenuation in tissue is reduced, might also be able to differentiate between normal and diseased articular cartilage. Hence, an important implication is a need to find a frequency that is low enough to provide enough penetration for the signal to reach the cartilage of the knee in
vivo, and still be able to detect changes in the attenuation coefficient.

We propose to evaluate the ability of ultrasound tissue characterization, performed at frequencies of 25, 20, 15 and 10 MHz, to detect changes in the attenuation coefficient of human cartilage subjected to experimentally-induced matrix degradation with papain. Although lower frequencies allow greater depth of penetration for ultrasound, this is achieved at a cost of resolution. An optimal frequency would allow for 2 to 4 cm of penetration through the tissue overlying the cartilage of the knee, and have an adequate resolution to detect attenuation changes in the cartilage arising from matrix degradation.

Evaluation of In-Vivo vs. In-Vitro Methods of Attenuation Coefficient Measurement

Previous investigations of the acoustic properties of normal and chemically altered articular cartilage have focused on in-vitro measurements of the attenuation coefficient [43,46,49,52]. The most accurate measurements of ultrasonic attenuation are made with a fixed path length experimental design, where the tissue of interest is placed in between an ultrasound transducer and a reflector separated by a known distance [55]. Most in-vivo measurements of the frequency dependent attenuation provide a statistical estimate of the attenuation coefficient by modeling the decay of the reflected signal through the tissue of interest [71].

The in-vivo, spectral differences, method described by Kuc and Schwartz is useful for anatomically inaccessible tissues, such as articular cartilage. The method compares two windows of the backscattered signal, a fixed length apart, from the tissue of interest. Forster implemented this method and compared the signal spectra for skin using the following equation:

\[ \alpha = \ln(G_1(f)/G_2(f))/4 \times d \]  

where \( \alpha \) is the attenuation coefficient, \( G_1 \) and \( G_2 \) represent the frequency spectra of the signals
and \( d \) is the distance between the two signal windows [72]. The decay in signal intensity over an estimated path length provides the frequency dependence of the attenuation for the tissue [73]. The path length is estimated by using tabulated values of the speed of sound in the tissue of interest to convert time difference into distance, and is a source of error in the measurement [71]. The method proposed by Kuc and Schwartz has been demonstrated to measure the attenuation of liver tissue both \textit{in vitro} and \textit{in vivo} with good agreement [73]. Similarly, Forster et al. have also demonstrated good agreement between \textit{in-vivo} measurements of skin and tabulated values [72].

![In-Vitro Setup](image1.png)

![In-Vivo Setup](image2.png)

Figure 3.2: Schematic illustrating \textit{in-vitro} and \textit{in-vivo} attenuation tissue characterization setups in human articular cartilage.

Since there have been no previously published investigations of the \textit{in-vivo} measurement of attenuation in cartilage, it is necessary to perform a study comparing the attenuation evaluated \textit{in vivo} to the attenuation evaluated using the reference, \textit{in-vitro}, measurement. We envision comparing normal specimens of human articular cartilage, first \textit{in vivo}, using a method similar to Forster et al., and then removing the section of tissue previously characterized and analyzing
CHAPTER 3. FUTURE WORK

this in vitro. The proposed experimental setup is presented in Figure 3.2. This experiment would need to be performed on multiple samples of articular cartilage at varying low frequencies, while the in-vitro setup would use 30 MHz measurements as a reference. The experimental goal would be to demonstrate no significant difference and good reproducibility between measurements of the attenuation in vitro and in vivo in articular cartilage.

In-Vivo Tissue Characterization of Osteoarthritic and Normal Human Cartilage Using a Model of the Knee

Before attempting to make in-vivo tissue characterization measurements of the attenuation in human knees, a model would be needed to understand the contributions of the overlying tissues to the attenuation measurement. In a human knee, the articular cartilage is bathed in synovial fluid, encapsulated in the joint capsule, which has overlying layers of muscle, fat and skin. Thus, there are a variety of tissues with varying acoustic properties between the surface of the skin and the articular cartilage which we would want to characterize. These tissues must all be penetrated by the ultrasound beam for in vivo imaging and tissue characterization of cartilage. In previous investigations, articular cartilage has been imaged in vivo by ultrasound at 5 MHz with reasonable success [53,54]. At these low frequencies, articular cartilage appears as a homogeneous tissue in the ultrasound image, with reflections originating from the articular surface and the subchondral bone. It is hoped that tissue characterization might be performed at a higher frequency, which has adequate axial resolution as well as provide the necessary penetration to detect morphological changes in articular cartilage resulting from proteoglycan loss and matrix degradation.

A suitable model might comprise an average thickness of each tissue type (skin, fat and muscle) placed in a saline bath in a layering similar to the anatomical organization of the knee. A femoral condyle with cartilage attached to underlying subchondral bone would be placed underneath
the layered tissue. The femoral condyles would be chosen from both normal human patients at autopsy, and osteoarthritic patients from knee arthroplasty. Thus, using the same model, the ability of the system to differentiate between normal and osteoarthritic human cartilage would be assessed.

We hypothesize that the ultrasound would penetrate the overlying tissues and be reflected by the subchondral bone of the cartilage. The received signal would provide a measure of the backscattered ultrasound from the tissue structures and the articular cartilage. Using the method described by Forster et al., we would analyze successive windows of the backscatter signal, and calculate the attenuation in articular cartilage using Equation 3.1 [72]. The distance between the signal windows would be calculated by multiplying the delay time between windows by the speed of sound previously measured in human articular cartilage presented in Chapter 2.

Layers of fat might be difficult to construct, and the use of a phantom with acoustic properties similar to those of fat might be justified. This would first have to be determined experimentally. Skin and muscle could be substituted with bovine tissue, as they have very similar acoustic properties to their human counterparts and are easier to obtain [63]. The exact thickness of the tissue layers used would be experimentally determined by examination and averaging of several human patients. These thickness values could be measured during either autopsy or total knee arthroplasty procedures. The knee model would be an essential component to the fine tuning of the in-vivo tissue characterization method. The model would enable assessment of the contributions of individual and multiple tissue layers to the acoustic signal, and might differentiate between normal and osteoarthritic human articular cartilage under in-vivo conditions.

We anticipate that performing the tissue characterization of normal and osteoarthritic human articular cartilage using this crude model will allow for the selection of optimal characterization frequencies. It is possible that the ideal frequency for measuring the attenuation of cartilage in
CHAPTER 3. FUTURE WORK

saline alone will not be the ideal frequency for characterizing cartilage in the knee. This would be most likely arise due to the acoustically irregular layers of tissue between the skin and the cartilage. Each tissue would alter the original ultrasound signal through independent attenuation processes. Thus, the knee model and characterization method will have to be designed to account for signal irregularities arising from the various layers. The possible contributions from each tissue layer are not well understood and would need to be explored further.

Non-Invasive Tissue Characterization of Articular Cartilage

A possible endpoint of this proposed work would involve the development of an ultrasonic probe for the in-vivo assessment of articular cartilage matrix degeneration by serial evaluation of the ultrasonic attenuation coefficient. Currently, the non-invasive evaluation of articular cartilage in osteoarthritis is limited to magnetic resonance imaging, ultrasound, plain film radiography and patient feedback; however, these methods all exhibit difficulty in detecting changes associated with early osteoarthritis [1]. A useful non-invasive method would require the resolution and ability to distinguish changes in articular cartilage corresponding to early osteoarthritis and should also provide this information quantitatively. The measure of the attenuation coefficient by ultrasound tissue characterization has shown the ability to resolve experimentally-induced matrix degradation under in-vitro conditions, and suggests that it might play a role in management of early osteoarthritis.

Following the completion of the previously described work, which would contribute towards the development of a non-invasive probe, we would attempt to measure the attenuation coefficient under in-vivo conditions. A possible initial experimental method might involve the investigation of a group of patients who have presented with either early symptoms of osteoarthritis, or are at a high risk of developing the disease – such individuals would have several initial measurements of ultrasonic attenuation of their cartilage performed. These patients would then be monitored with
several readings of their cartilage at regular intervals, as osteoarthritis progressed. The resulting changes in the attenuation coefficient over time could be analyzed. It is hoped that these changes would correlate with the advancement of osteoarthritis in the individuals. The envisioned use of this non-invasive cartilage characterization system would involve monitoring the articular cartilage in an individual, and potentially using changes in the ultrasonic attenuation to as a correlate with early stages of osteoarthritis.

We envision that eventually a small, hand-held, ultrasound probe could be developed for non-invasive diagnosis of the cartilage of the knee. These probes would be applied to the surface of the knee in full flexion, an examination position previously described by Aisen et al., to obtain an attenuation measurement from the femoral condyles [53]. Measurements would be taken as part of a regular physical examination after sexual maturity is reached. Because the macroscopic composition of the cartilage is relatively unaltered after maturity [1], the attenuation for the cartilage would be expected to remain at a normalized level until the cartilage was affected by early stages of disease. Following detection of a significant change in attenuation, corresponding to a change in the morphology of the cartilage, the physician would have the option of managing the progressing osteoarthritis at an early stage with drug or other treatments currently available for the management of osteoarthritis.

### 3.2 Concluding Remarks

At present, there is difficulty in detecting the early stages of osteoarthritis, as a result of the complexity of the disease and limitations in current diagnostic imaging modalities. Early stages of osteoarthritis are characterized primarily by edema and loss of matrix proteoglycans. Current diagnostic modalities such as X-ray and magnetic resonance are effective at identifying the later
stages of osteoarthritis, but exhibit difficulty in detecting the morphological changes in cartilage associated with early osteoarthritis. A sensitive, non-invasive or minimally invasive, diagnostic modality which could identify changes in the morphology of articular cartilage associate with early osteoarthritis is desirable.

The experiments described above have demonstrated the ability of high frequency ultrasound to detect an experimentally-induced degradation of the cartilage matrix through changes in the acoustic attenuation and speed of sound. We presented an overview of the potential for the development of a non-invasive method to measure the acoustic attenuation of the femoral cartilage.
Bibliography


