Fourier Transform Infrared Spectral Analysis of Degenerative Cartilage: An Infrared Fiber Optic Probe and Imaging Study

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A preliminary investigation into the diagnostic potential of an infrared fiber optic probe (IFOP) for evaluating degenerative human articular cartilage is described. Twelve arthritic human tibial plateaus obtained during arthroplasty were analyzed using the IFOP. Infrared spectra were obtained from IFOP contact with articular surface sites visually graded normal or degraded (Collins Scale grade 1 and grade 3, respectively). Comparisons of infrared spectral parameters (peak heights and areas) were made to elucidate spectral indicators of surface degeneration. IFOP spectral analysis revealed subtle but consistent changes between grades 1 and 3 sites. Infrared absorbance bands arising from type II collagen were observed to change with degradation. More degraded tissues exhibited increased amide II (1590–1480 cm^{-1})/1338 cm^{-1} area ratio (p = 0.034) and decreased 1238/1227 cm^{-1} peak ratio (p = 0.017); similar changes were seen with Fourier transform infrared imaging spectroscopy (FT-IRIS) analysis. Grades 1 and 3 cartilage showed consistent spectral differences in the amide II, III, and 1338 cm^{-1} regions that are likely related to type II collagen degradation that accompanies cartilage degeneration. These results suggest that it may be possible to monitor subtle changes related to early cartilage degeneration, allowing for IFOP use during arthroscopy for *in situ* determination of cartilage integrity.

Index Headings: **Infrared fiber optic probe; Osteoarthritis; Human articular cartilage; Type II collagen.**

INTRODUCTION

Osteoarthritis (OA) is a progressively disabling, complex joint disease characterized by degeneration of the articular cartilage, a poroviscoelastic connective tissue that lines the articulating ends of diarthrodial joints. Articular cartilage is made up of four spatial zones: the superficial, transitional, deep, and calcified zones. Within each zone, chondrocytes, the cells present in cartilage, are responsible for the production, organization, and maintenance of its extracellular matrix (ECM). This ECM has a composite-like structure that is primarily composed of water (70–80%) and macromolecules: type II collagen (15–25%) and proteoglycans (5–25%).[1] Each collagen molecule consists of three polypeptide chains (α1 chains) wound into a helical fibril arrangement approximately 300 nm long. The collagen fibrils function to trap and constrain the large aggregating hydrophilic proteoglycan (PG) molecules called aggrecan. Aggrecan consists of a large 2.25 kD three domain (G1,G2,G3) protein core to which polyanionic chondroitin sulfate and keratin sulfate glycosaminoglycan chains are attached. Joint trauma and or excessive wear may initiate the breakdown of collagen and aggrecan, eventually resulting in severe OA. Late stage OA can be easily observed by radiographic imaging; however, early stages of OA that involve cellular and molecular changes but with no obvious mechanical damage are much more difficult to identify. Knowledge of specific ultrastructural characteristics that accompany early stage articular surface degradation, and those that are associated with repair tissue, would be extremely important during arthroscopic procedures where crucial decisions are made regarding salvaging or removing cartilage.

Fourier transform infrared (FT-IR) spectroscopy is a convenient tool to study molecular changes associated with degenerative cartilage structure. The primary components of cartilage, type II collagen and proteoglycan, can be readily monitored by FT-IR spectroscopy.[2] *In vitro* studies on collagen include the effects of hydration on secondary structure[3] and molecular changes during self-assembly.[4] FT-IR spectroscopic studies of PGs have identified absorbance bands characteristic of specific PGs in various tissues,[5–8] including cartilage.[2]

A new alternative for infrared sampling of biological tissues is available using an infrared fiber optic probe (IFOP). This technology enables a more flexible sampling approach than has previously been utilized, and in particular, it holds promise for the evaluation of tissue changes *in situ*. In this study, an IFOP is used to evaluate spectral parameters associated with degenerative surface damage in human articular cartilage. We demonstrate the potential of the IFOP to distinguish between normal and degraded articular surface based on spectral changes, and we hypothesize that this technique may eventually be applied to the evaluation of early stage molecular changes in OA.

MATERIALS AND METHODS

The infrared fiber optic probe (IFOP) consists of a flexible fiber-optic bundle composed of a mid-infrared transmitting "chalcogenide" glass (RemSpec Corp, Sturbridge, MA) equipped with a mercury cadmium telluride (MCT) detector module coupled to a BRUKER spectrometer (BRUKER, Germany). The fiber-optic bundle is approximately one meter in length and transmissive over the infrared region of 4000–900 cm^{-1}. Sampling is achieved by the use of a flat-tipped, 1-mm-diameter ZnS ATR (attenuated total reflectance) crystal attached to the end of the fiber-optic bundle.

To establish the minimum sampling time required to obtain spectral consistency (due to the viscoelastic nature of cartilage), normal mature bovine knee articular cartilage explants (obtained from a local slaughter house im-

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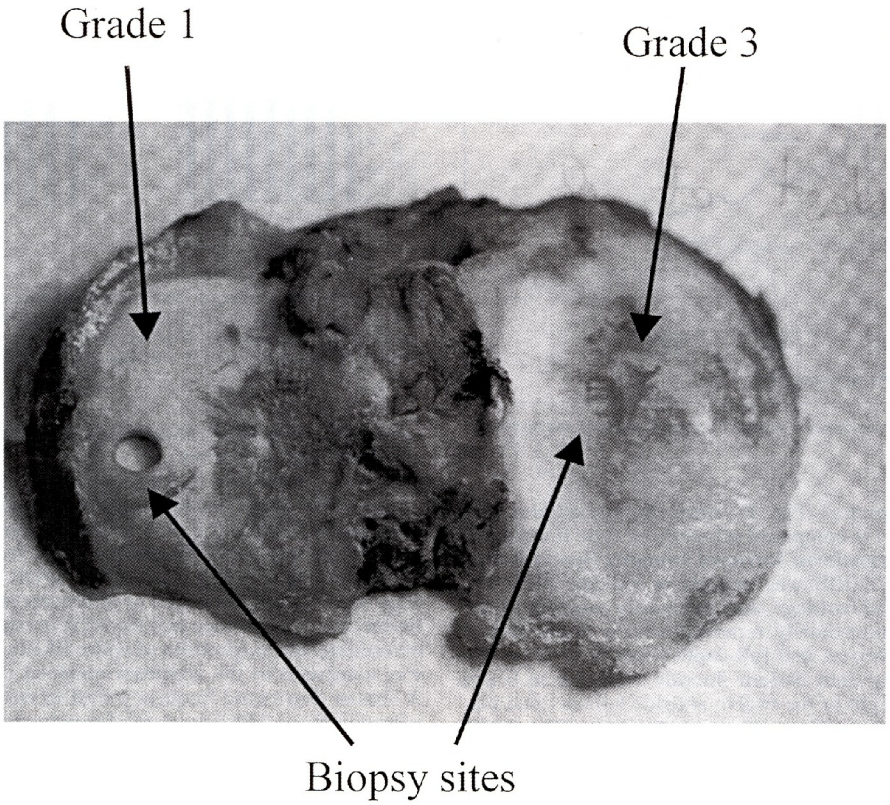

FIG. 1. Arthritic human tibial plateau with Collins scale grade 1 (nearly normal) and grade 3 (degraded) cartilage sites identified. Grade 1 and grade 3 areas were utilized for infrared fiber optic probe (IFOP) and Fourier transform infrared imaging spectroscopy (FT-IRIS) data acquisition.

mediately after death) were harvested and cored using a 5-mm biopsy punch. The plugs were placed in a saline-filled cylindrical bath attached to a 10 μm resolution micrometer driven z-stage at room temperature. Initial contact between the ATR crystal and the tissue surface was established via a 5-lb. load cell attached to the z-stage (the load cell uses a Wheatstone bridge, which converts load variations into a change in electric signal, which can be monitored on a load meter). Surface contact with the crystal was then maintained at a displacement of 100 micrometers, as measured by the micrometer. IR spectral changes related to tissue stress relaxation were then acquired using data acquisition software OPUS NT v.3 (BRUKER, Germany). Sampling rate was continuous for 5 minutes with a sampling collection time of 30 s/spectra at a spectral resolution of 4 cm^{-1}. A Blackman–Harris three-term apodization function and a zero filling factor of 2 were used.

Tissues. Twelve arthritic human tibial plateaus (male and female patients ages 55–83) were obtained during knee replacement surgery and analyzed using the IFOP under an IRB (Institutional Review Board)-approved protocol. Samples obtained were stored in saline and immediately delivered for IFOP analysis. Each specimen was secured on the z-stage equipped with a load cell and the fiber optic was placed in contact with sites visually identified and graded as either grossly normal (no obvious macroscopic damage) or degraded (fibrillations, clefts, or fissures present), corresponding to Collins' Scale[9,10] grade 1 and grade 3, respectively (Fig. 1).

For each site evaluated, 256 scans (1 min) were acquired, averaged, and referenced to a saline background. Spectral processing was done using OPUS NT v.3. For all spectra, a Savitzky–Golay 17-point smooth was applied. The spectra were baselined and the type II collagen absorbances (Fig. 2) were monitored in the 1690–1600 cm^{-1}, 1590–1480 cm^{-1}, 1338 cm^{-1}, and 1300–1200 cm^{-1} infrared regions. The primary molecular vibrations associated with these wavenumber absorbances are the amide I carbonyl stretch (C=O),[11–15] the amide II out-of-phase in-plane N–H deformation and C–N stretch,[11–15] CH_2 side-chain vibrations, and the amide III coupled N–H/C–H deformation vibrations,[16–18] respectively. Spectra were evaluated for changes in area and peak intensity in these regions, and area and peak intensity ratios were calculated. Ratios were utilized to avoid errors that could be incurred as a result of concentration-dependent changes in spectral absorbance. The following parameters were calculated based on absorbance area and peak intensities at specific frequencies: amide II/1338 cm^{-1} area ratio and 1238/1227 cm^{-1} peak ratio. It was assumed that negligible signal from the proteoglycan component was pre-

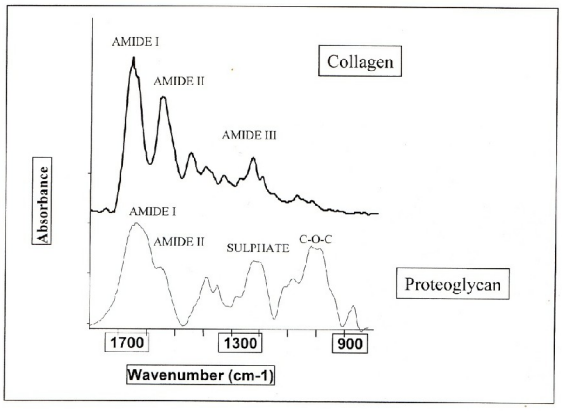

FIG. 2. Infrared absorbance spectra for type II collagen and proteoglycan.

sent in the spectra[2] (Fig. 2), since the probed area (superficial zone) is composed primarily of type II collagen.

Fourier Transform Infrared Imaging Spectroscopy (FT-IRIS). FT-IRIS was utilized to validate the IFOP spectra. Graded cartilage sites previously sampled by the IFOP were immediately removed with a 5-mm biopsy punch, flash frozen, and full-depth slices were cryo-sectioned directly onto barium fluoride windows in 6 μm sections for eight of the twelve arthritic human tibial plateaus. A Digilab (Cambridge, MA) UMA 300A FTIR microscope with an FTS-60A step-scanning FTIR spectrometer and a 64 × 64 MCT focal plane array detector (Santa Barbara Focal Plane, Golota, CA) was used in transmission mode to acquire spectra at 8 cm^{-1} resolution under N_2 purge. Information on collagen content and distribution was obtained from a 400 × 400 μm^2 region, resulting in 4096 individual spectra for each sample. Twenty individual surface spectra were randomly chosen from the superficial zone of each sample for comparison to the IFOP data. Collins scale grade 0 (showing no degenerative morphological changes) cartilage was obtained from a deceased 19-year-old human male and used as a reference in the FT-IRIS analysis.

Statistical Analysis. For each parameter examined (amide II/peak 1338 cm^{-1} area ratio and 1238/1227 cm^{-1} peak ratio), mean values and standard deviations were obtained. A paired t-test, a standard statistical test used for comparison between two sets of data from the same subject, was used to compare differences between grade 1 and grade 3 cartilage with significance determined at $p < 0.05$.

RESULTS

Spectra of the bovine knee articular cartilage explants were obtained continuously at 30-s intervals for 5 min. Time-dependent changes in the amide II and III intensities were noted. Absorbance areas and heights for the amide II and III bands increased linearly for the first 75 s, after which there were no significant changes in the IR spectra (Figs. 3A and 3B). Based on this data, all subsequent IFOP spectral data were acquired after 75 s of contact between the IFOP and the tissue. The use of this

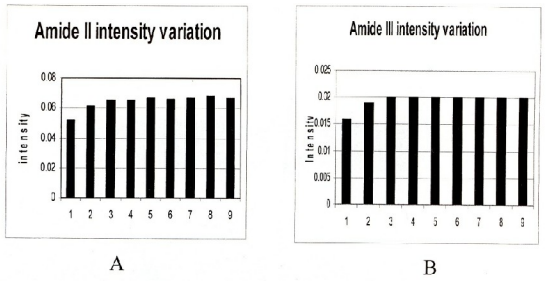

FIG. 3. IR spectral changes related to tissue relaxation in mature bovine articular cartilage during IFOP data acquisition. (**A**) Temporal variation in amide II intensity after initial IFOP contact. (**B**) Temporal variation in amide III intensity after initial IFOP contact.

information was later validated on normal mature human explants (data not shown).

Spectra of grade 1 (nearly normal) cartilage obtained by the IFOP were obtained and compared to those obtained by FT-IRIS. The principal difference between the two spectra was a shift in peak position from 1660 cm^{-1} to approximately 1630 cm^{-1} in the IFOP amide I band (Fig. 4). This was attributed to molecular interactions between the tissue surface and the ATR crystal.[19] To further investigate this, a spectrum of water taken in transmission was compared to the spectrum obtained with the IFOP. A similar downward frequency shift in the O–H bending vibration from 1640 cm^{-1} to approximately 1632 cm^{-1} was observed. Since the detailed nature of the frequency shift is not yet fully understood, the amide I band was excluded from the spectral analysis.

Infrared fiber optic probe spectral analysis of the twelve arthritic human tibial plateaus revealed subtle but relatively consistent changes between grade 1 and grade 3 sites (Fig. 5). Quantitation of these subtle differences revealed area changes in the amide II peak and in the absorbance centered at 1338 cm^{-1}. Specifically, an increased amide II/1338 cm^{-1} area ratio was found ($p = 0.034$) (Fig. 6A). Contour changes were also observed in the amide III absorbance region. Analysis of these find-

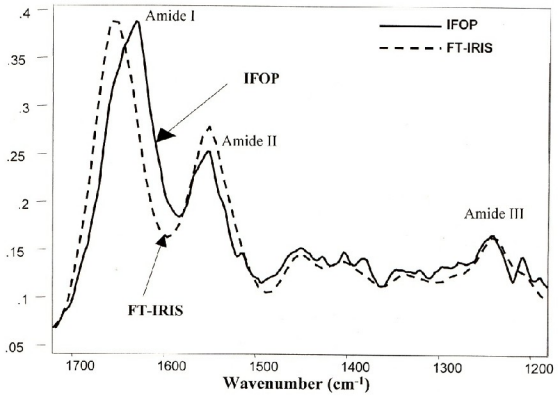

FIG. 4. Comparison between IFOP and FT-IRIS spectra showing the amide I absorbance band shift due to interactions with the ATR crystal.

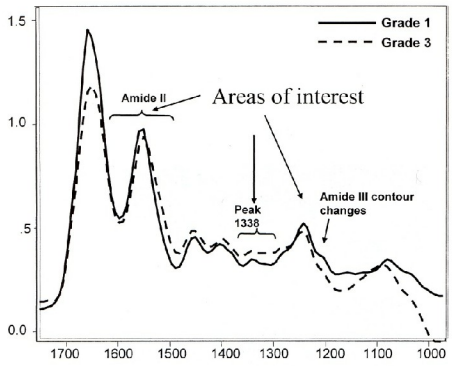

FIG. 5. Spectral comparison between grade 1 and grade 3 articular cartilage using IFOP data acquired at the articular surface.

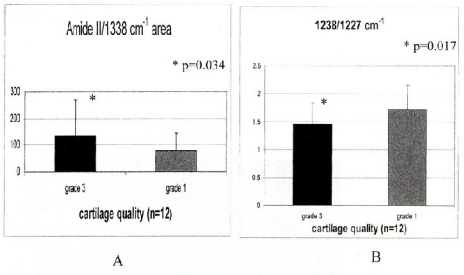

FIG. 6. IFOP-determined spectral changes between grade 1 and grade 3 articular cartilage. (**A**) IFOP amide II/1338 cm^{-1} area ratio comparison. (**B**) IFOP amide III 1238/1227 cm^{-1} intensity ratio comparison. (*) A paired t-test was used to determine significant differences at $p < 0.05$.

ings revealed a decreased 1238/1227 cm^{-1} peak intensity ratio ($p = 0.017$) for the more degraded tissues (Fig. 6B).

Fourier Transform Infrared Imaging (FT-IRIS). FT-IRIS analysis revealed similar changes between grade 1 and grade 3 sites for the two aforementioned parameters. The amide II/1338 cm^{-1} area ratio increased ($p < 0.001$) and the 1238/1227 cm^{-1} peak ratio decreased ($p < 0.001$) between grade 1 and grade 3 surface sites (Figs. 7A and 7B). All the changes observed between grade 1 and grade 3 sites with FT-IRIS analysis were also referenced to Collins' scale grade 0 cartilage (normal cartilage) that showed no degenerative morphological changes (Figs. 7A and 7B). In this tissue, the amide II/1338 cm^{-1} area ratio was lower than that of grade 1 tissue, and the 1238/1227 cm^{-1} peak ratio was greater than that of grade 1 tissue.

DISCUSSION

In this study we investigated the use of an infrared fiber optic probe (IFOP) as a tool for detecting molecular changes in osteoarthritic human tibial plateaus. The analysis demonstrated that the IFOP is sensitive to changes related to the degradation of the articular surface in the superficial zone of cartilage. Comparisons were made between nearly normal (grade 1) and severely degraded cartilage (grade 3), and although these changes were subtle, these results do suggest that it may be possible to monitor *in vivo* changes related to cartilage degeneration with a fiber-optic system. For the cartilage analyzed from the twelve human tibial plateaus in this study, comparison of grade 1 and grade 3 sites showed spectral changes in the amide II and III and 1338 cm^{-1} region. A previous study from our laboratory[2] established that spectra obtained from the superficial zone of articular cartilage by FT-IRIS arise primarily from type II collagen, and thus it is reasonable to attribute the spectral changes found by IFOP to changes in the environment of type II collagen molecules.

The spectral trends observed by IFOP analysis were corroborated in our FT-IRIS analysis. With decreasing tissue quality we also observed significantly higher ($p < 0.001$) amide II/1338 cm^{-1} area ratios and significantly lower ($p < 0.001$) 1238/1227 cm^{-1} peak ratios. This was reflected in the FT-IR image (Fig. 8), which showed much higher amide II/1338 cm^{-1} area values in the superficial zone of the grade 3 cartilage as compared to the grade 1. It can also be observed in this image that there is a gradual decrease in this parameter from the superficial zone through the mid-zone, in support of the concept that collagen degradation is initiated at the articular surface. Interestingly, the differences between the grade 1 and grade 3 cartilage were more pronounced in the FT-IRIS data compared to the IFOP data. It is likely that this is attributable to the greater signal-to-noise ratio in the imaging data. The comparison of the grade 1 and grade 3 data to normal or grade 0 cartilage makes these findings even more compelling (Fig. 7), as it further demonstrates the relationship between the spectral parameters and cartilage quality.

Within articular cartilage, type II collagen represents 90–95% of the total collagen. Molecular changes in collagen architecture have been reported to be among the earliest changes in degenerative joint disease.[20] Specific matrix metalloproteinases (MMPs) are upregulated by chondrocytes, thereby initiating the degradation of the type II collagen fibrils.[1,21–23] This enzymatic activity (primarily due to the MMPs 1-, 8-, and 13-collagenases) result in the cleavage of the collagen triple helix structure at a single site, thus producing approximately ¼- and ¾-length alpha (∝) chain fragments.[24] Over time, these molecular changes gradually lead to further degradation and failure of the extracellular fibrillar network due to the

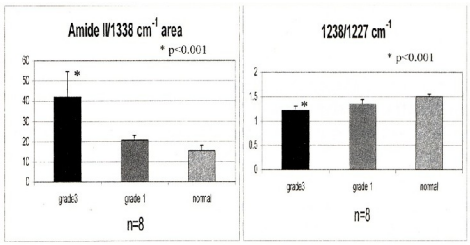

FIG. 7. FT-IRIS determined spectral changes between grade 1 and grade 3 articular cartilage. (**A**) FT-IRIS amide II/peak 1338 cm^{-1} area ratio comparison. (**B**) FT-IRIS amide III 1238/1227 cm^{-1} intensity ratio comparison. (*) A paired t-test was used to determine significant differences at $p < 0.05$ between grade 1 and grade 3.

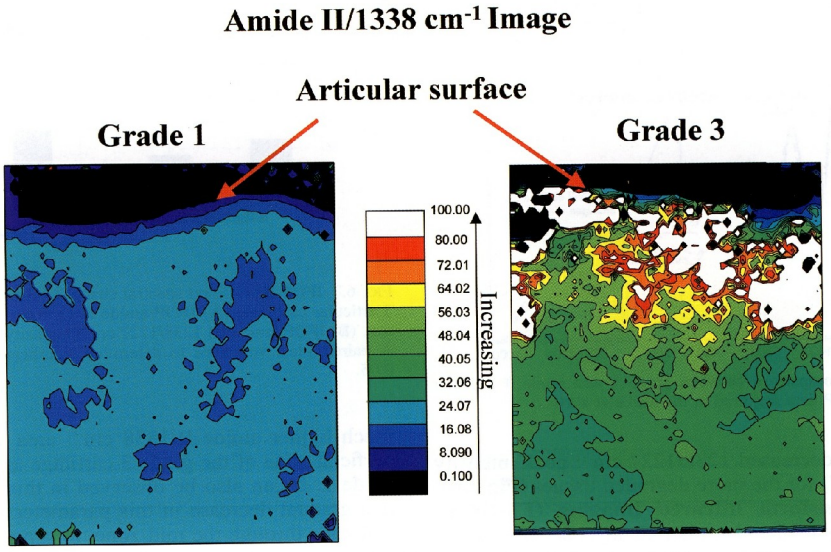

FIG. 8. Image of FT-IRIS determined amide II/1338 cm^{-1} area ratio of grade 1 and grade 3 human articular cartilage.

unraveling of the collagen molecule. These destructive biochemical changes that occur within diseased cartilage translate into observable infrared parameters. The 1338 cm^{-1} collagen absorbance arises from CH_2 side-chain vibrations, is sensitive to the order of the triple helix, and has previously been shown to decrease in intensity as the collagen denatures.[15,25] The decrease in the area of this band would account for the increased amide II/1338 cm^{-1} area ratio in diseased states. The change in the complex amide III absorbance contour with collagen degradation was quantitated by a decrease in the 1238/1227 cm^{-1} peak intensity ratio. Although it is known that the amide III absorbance envelope arises from the coupled N–H/C–H deformation vibrations within the amide bond, assignment of specific molecular motions to exact frequencies has proved challenging due to the highly complex nature of this vibrational mode.[13,25,26] It is reasonable to assume that as degenerative changes progress, alterations in the environment of the collagen amide bonds would occur and be manifested as intensity or frequency shift changes in the infrared spectra of cartilage. Thus, it is likely that the spectral changes observed are associated with the unraveling of the surface collagen triple helix structure due to enzymatic activity.

One of the primary challenges in the management of osteoarthritis is the inability to detect early molecular changes in cartilage. To date, there is no universally accepted methodology for early stage OA detection. Current techniques under investigation include diffraction-enhanced X-ray imaging (DEI), optical coherence tomography (OCT), high-frequency ultrasound, and magnetic resonance imaging (MRI), each of which presents its own challenge in instrumentation, interpretation of results, sampling resolution, and practicality. Diffraction-enhanced X-ray imaging is a new noninvasive technique with a significantly greater imaging contrast compared to regular X-ray images,[27] but it is still far from being utilized clinically due to instrument limitations related to the required high-energy monochromatic X-rays (synchrotron radiation).[28] OCT is a new method of high-resolution imaging based on the intensity of back-reflected infrared radiation;[20,29,30] however, there are many uncertainties related to cartilage scoring and data interpretation with OCT. High-frequency ultrasound is a minimally invasive technique that uses ultrasound to characterize the *in situ* mechanical properties of articular cartilage.[31–34] The primary limitation with this method lies with accurately correlating mechanical properties with stages of cartilage degeneration. Similarly, the relationship between molecular changes and mechanical stiffness as detected by an arthroscopic cartilage stiffness tester now commercially available, the ACTAEON™ Probe (OsteoBiologics, Inc.), is still under investigation. MRI is the most widely used clinical tool for diagnosis and treatment of articular cartilage disorders.[35] The superiority of MRI exists in its ability to evaluate cartilage noninvasively; however, limited spatial resolution and ambiguities still surround the interpretation of MRI examination, making it difficult to reproducibly measure early cartilage changes.[36–38]

Fourier transform infrared spectroscopy presents us with a valuable new technique for the diagnosis and management of OA. Nonetheless, there are inherent limitations associated with the use of the IFOP, including limited spatial resolution due to the ATR crystal tip size (~1 mm) and the need for more precise data analysis methods. The latter issue is currently being addressed through the development of chemometric multivariate data analysis techniques. Development of such a method and correlation with histologically defined tissues will enable more accurate prediction of spectral parameters associated with cartilage integrity. Thus, the IFOP analysis will provide a minimally invasive, nondestructive technique and may ultimately allow for early detection of molecular changes in osteoarthritis. Ideally, it would be used during

arthroscopy for *in situ* determination of articular cartilage surface integrity and could also be used to assess the quality of tissue-engineered cartilage both before and after implantation. FT-IR systems are readily available, offering superior cost effectiveness and portability, with a high degree of precision and accuracy. It is possible that with further studies on the earlier stages of cartilage degradation, the IFOP may develop into a highly reliable tool for monitoring and treating OA and other joint diseases.

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