Published in final edited form as:

Ann N Y Acad Sci. 2019 April ; 1442(1): 104–117. doi:10.1111/nyas.13934.

Spatial correlation of native and engineered cartilage components at micron resolution

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Abstract

Tissue engineering (TE) approaches are being widely investigated for repair of focal defects in articular cartilage. However, the amount and/or type of extracellular matrix (ECM) produced in engineered constructs does not always correlate with the resultant mechanical properties. This could be related to the specifics of ECM distribution throughout the construct. Here, we present data on the amount and distribution of the primary components of native and engineered cartilage (i.e., collagen, proteoglycan (PG), and water) using Fourier transform infrared imaging spectroscopy (FT-IRIS). These data permit visualization of matrix and water at 25 micron resolution throughout the tissues, and subsequent co-localization of these components using image processing methods. Native and engineered cartilage were cryosectioned at 80 μm for evaluation by FT-IRIS in the mid-infrared (MIR) and near-infrared (NIR) regions. PG distribution correlated strongly with water in native and engineered cartilage, supporting the binding of water to PG in both tissues. In addition, NIR-derived matrix peaks correlated significantly with MIR-derived collagen peaks, confirming the interpretation that these absorbances arise primarily from collagen and not PG. The combined use of MIR and NIR permits assessment of ECM and water spatial distribution at the micron level, which may aid in improved development of TE techniques.

Graphical abstract

The amount and/or type of extracellular matrix (ECM) produced in tissue-engineered cartilage constructs does not always correlate with the resultant mechanical properties, which could be related to how ECM is distributed in the construct. This paper presents data on the amount and distribution of the primary components of native and engineered cartilage (i.e., collagen, proteoglycan, and water) using Fourier transform infrared imaging spectroscopy.

Keywords

near-infrared spectroscopy; mid-infrared spectroscopy; FTIR imaging spectroscopy; engineered cartilage; articular cartilage; extracellular matrix

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Competing interests

The authors declare no competing interests.

Introduction

Articular cartilage lines the ends of long bones and aids in providing a low friction surface, along with being resistant to mechanical forces experienced within the joints. When damage to articular cartilage occurs, typically due to trauma or wear and tear from aging, this can result in the start of osteoarthritis (OA). The onset of OA can be delayed with early clinical intervention; however, there is not an accepted therapeutic method to prevent OA.¹ Tissue engineering has been extensively investigated for repair of focal defects in articular cartilage, in an attempt to prevent the onset of widespread OA.² The inability of articular cartilage to repair itself arises in large part from its avascular and aneural nature³, which has led to advanced techniques for engineering cartilage as a replacement for damaged native tissue.² However, in engineered constructs, the specific control of extracellular matrix (ECM) proteins such as proteoglycan (PG) and collagen in an optimal zonal arrangement has not yet been achieved.

Distribution of water and ECM proteins in engineered cartilage has been widely studied utilizing both destructive and non-destructive methods.^{4–7} Nonetheless, in spite of numerous studies that investigated the amount of ECM produced by engineered cartilage², relationships between the ECM and mechanical properties are not always conclusive.^{8,9} In Yousefi *et al.*⁸, near-infrared (NIR) spectra, which contain information related to water and ECM composition, were used to predict dynamic moduli and bulk biochemically-derived ECM composition with a 10–11% error compared to gold-standard methods, but were not as useful for equilibrium moduli prediction. In another study from our laboratory, the biochemical composition of polyglycolic acid (PGA)-based engineered constructs correlated with mechanical properties for some constructs, but not for constructs treated with ultrasound stimulation.¹⁰ Mauck *et al.*⁹ showed that free-swelling constructs have significantly lower equilibrium and dynamic moduli compared to dynamically loaded constructs, while variations between PG and collagen content in the two construct types were not as evident. These studies support that ECM content is not the sole factor in determining the mechanical properties of engineered cartilage. It has also been previously shown that, in addition to the amount of ECM, mechanical properties of engineered cartilage could be affected by the synthesis rates and spatial distribution of ECM^{6, 11–13} and by water content.^{14–16} These results could partially be attributable to the wide range of methods utilized to engineer cartilage, such as the use of scaffolds versus scaffold-less, or hydrogels versus fibrous scaffolds, all of which may have an effect on cell division, ECM production, and water distribution.

Current histological methods to evaluate distribution of proteins throughout tissue utilize dyes that can be sensitive to preparation techniques.¹⁷ In addition, histological dyes may exhibit nonspecific staining and may be challenging for quantification of the distribution of a specific component throughout the sample. Fourier transform infrared imaging spectroscopy (FT-IRIS) is an advanced vibrational spectroscopy technique that combines an FT-IR spectrometer with an optical microscope with an array detector to produce spatially-resolved images based on the IR spectra.¹⁸ This technique does not require the use of dyes and is sensitive at the micron level, providing the ability to more precisely detect changes in distribution and relative amount of tissue components. Spectral data can be collected in the

mid-infrared (MIR) and NIR ranges with FT-IRIS to obtain quantifiable information regarding matrix proteins, such as PG and collagen, and water content. FT-IRIS has been widely used to evaluate various musculoskeletal tissues, including cartilage^{10, 19–24}, ligament^{25, 26}, and bone.^{27–37} While FT-IRIS in the MIR range has been extensively used to evaluate sample composition and the distribution and orientation of specific organic components throughout the tissue^{14, 19, 21–24, 38–40}, NIR is uniquely suited for evaluation of water at micron resolution.^{27, 41} Thus, the use of these spectral techniques together can facilitate correlations between water and ECM protein distribution, and would aid in interpreting the contribution of ECM distribution to the mechanical properties and integrity of engineered cartilage.

We hypothesize that FT-IRIS will allow for qualitative visualization of the distribution of water and ECM, and quantitative assessment of correlations between water and ECM proteins in engineered cartilage, with comparison to native juvenile cartilage as a standard. Here, methacrylated hyaluronic acid (MeHA) engineered constructs and native cartilage plugs were cryosectioned, evaluated with FT-IRIS in the mid- and near-infrared spectral ranges, and stained with Safranin O for PG content for comparison.

Methods

Methacrylated hyaluronic acid (MeHA) synthesis

MeHA was synthesized as previously reported.^{8, 42} Briefly, methacrylic anhydride (94%; FW: 154.17 kDa; Sigma; ~30-fold excess) was added dropwise to a 1 wt% HA solution (research-grade sodium hyaluronate powder (Lifecore; FW: 65 kDa) dissolved in deionized (DI) water; adjusted to a pH of 8–9 with 5 N NaOH) and the reaction was then placed in an ice bath for 6 hours. The MeHA solution was purified via dialysis (MW cutoff of 6–8 kDa) against DI water for 14 washes over 7 days. The final product was lyophilized and stored at –20°C. The degree of methacrylation on the final product was confirmed by ¹H-NMR to be ~60%. The lyophilized MeHA was dissolved in PBS (1% wt/vol) and Irgacure I2959 (Sigma) photoinitiator (PI) was added to allow for UV-mediated polymerization. The solution was sterilized using a 0.2 µm filter (Millipore).

Tissue harvest, primary chondrocyte isolation, and encapsulation

Articular cartilage for investigations of native cartilage structure was harvested from the patellofemoral groove of three juvenile bovine knees (Research 87, Boylston, MA) using a 6 mm biopsy punch (Integra® Miltex®) (*n* = 3). The knees were kept refrigerated and the cartilage plugs were harvested within 24 hours of slaughter. Plugs were stored in a solution phosphate-buffered saline and protease inhibitor at –20°C until they were cryosectioned. The remaining cartilage from the patellofemoral grooves was used for chondrocyte isolation. Cartilage was digested in a spinner flask containing 250 U/mL type II collagenase (Worthington Biochemical), 99 mL DMEM (Life Technologies), and 1 mL penicillin/streptomycin/fungizone (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, 25 µg amphotericin B; Lonza). Spinner flasks were incubated at 37°C and 5% CO₂ for 12 h. Chondrocytes were isolated from the digest solution by filtering through a 70 µm sterile nylon filter and centrifugation at 1000 g. The cell pellet was collected, reconstituted in

phosphate buffered saline (Life Technologies), and cells were encapsulated at 60 million cells/mL in MeHA (1% w/v with 0.05% w/v of PI). Gels were polymerized by UV exposure (15 min) with a 365 nm Black-Ray UV lamp (Model #UVL-56) between glass plates separated by 2 mm spacers, and sterile 4 mm diameter punches (Miltex) were used to create chondrocyte-laden hydrogel constructs. Constructs were cultured as previously described⁸ in 3 mL of chemically defined medium including 10 ng/mL TGF- β 3 (R&D Systems). Media was replaced twice a week for 6 weeks, followed by analysis of constructs.

Tissue preparation

After 6 weeks of culture, tissue-engineered HA constructs ($n = 3$) were prepared for cryosectioning and infrared imaging similar to a previous study.¹⁹ Briefly, a small amount of optimal cutting temperature (OCT) compound (Sakura Finetek) was spread on a cryomold, the tissue was stood on edge so minimal sample was exposed to OCT, and then flash frozen in liquid nitrogen. In addition, native cartilage plugs ($n = 3$) were prepared similarly, with the plugs being laid on the side so full thickness sections could be obtained. OCT has a strong spectral signal, and thus it is critical to avoid contact between OCT and areas of the samples being sectioned for FT-IRIS. Immediately after flash freezing, samples were placed in a Leica CM3050 S Cryostat (Leica Biosystems, Buffalo Grove, IL) at -20°C . Samples were mounted to the cryostat with enough OCT, while again ensuring that excess OCT would not interact with regions of the samples being sectioned. Sections were obtained at 80 μm thickness (which had been optimized in a previous study to ensure appropriate absorptivity in MIR spectra and signal-to-noise ratio in NIR spectra¹⁹) and deposited onto silicon (Si) wafers (UniversityWafer Inc., Boston, MA). Multiple sections per sample were obtained, and the section with the best structural integrity was utilized for analysis. Thus, there were three total sections evaluated per tissue type, one from each sample. Sections were air dried prior to imaging to ensure loosely-bound water was not included in spectral measurements.

Fourier transform infrared imaging spectroscopy

FT-IRIS data were collected with a Spotlight 400 imaging spectrometer (Perkin Elmer, Shelton, CT). Si wafers with tissue sections were imaged in transmittance mode in the 750–7800 cm^{-1} spectral range, allowing data to be collected in the MIR (750–4000 cm^{-1}) and NIR (4000–7800 cm^{-1}) regions simultaneously. Spectral data collection parameters were optimized separately for engineered and native cartilage to obtain high signal to noise in a reasonable amount of time. Background spectra were collected in transmittance from an area of the slide without tissue at a spectral resolution of 8 (engineered cartilage) or 16 (native cartilage) cm^{-1} and a spatial resolution of 25 μm with 45 co-added scans. Engineered cartilage sections were imaged at a spectral resolution of 8 cm^{-1} and a spatial resolution of 25 μm with 16 co-added scans, and native cartilage sections were imaged at a spectral resolution of 16 cm^{-1} and a spatial resolution of 25 μm with 16 co-added scans. The engineered cartilage data were collected at a higher spectral resolution to ensure separation of the MeHA-related absorbances from the matrix absorbances. The background spectra were collected with a greater number of co-added scans than the sample spectra to ensure low background noise and that any spectral noise arose from the sample. Data acquisition times for native and engineered cartilage were approximately 2 hours per sample. This

resulted in evaporation of the free water, and thus the majority of the spectral data was attributable to bound water. Imaging parameters were previously optimized by our lab with similar samples.¹⁹

Histological analysis

Serial sections of 9 μm thickness were obtained immediately after the sections utilized for IR imaging were obtained. Serial sections were used to ensure histologically stained sections could be directly compared with IR images. Sections were transferred onto Superfrost® Plus microscope slides (Fisher Scientific, Waltham, MA), covered in OCT, and stored at -80°C . Safranin O staining was performed to visualize the PG distribution within the tissues.⁴³ Histologically stained sections were digitally imaged using an Olympus BX53 microscope (Olympus, Japan) equipped with Olympus cellSens software (Olympus, Japan). Images were collected at 10 $\times$ zoom with 1 ms exposure.

Image processing

FT-IRIS images were first processed using the ISys chemical imaging analysis software (version 5.0.0.14; Malvern Instruments, Columbia, MD) to obtain compositional distributions based on the intensity of specific MIR and NIR peaks. The MIR peaks of interest were at $\sim 856\text{ cm}^{-1}$ (PG, C–O–S vibration) and 1340 cm^{-1} (collagen, side chain vibration), and the NIR peaks were at $\sim 5200\text{ cm}^{-1}$ (water, OH vibrations) and $\sim 4400, 4600,$ and 4890 cm^{-1} (matrix peaks, resulting from C–H and N–H vibrations).¹⁹ The second derivative of the spectral images were obtained using the Savitzky-Golay filter, applying 5 smoothing points for the MIR region ($\sim 1800\text{--}750\text{ cm}^{-1}$) and 31 smoothing points for the NIR region ($\sim 5500\text{--}4000\text{ cm}^{-1}$). A large number of smoothing points are frequently required in the NIR range due to absorbances with lower signal-to-noise ratio, as they arise from overtones, in contrast to the fundamental vibrations found in the MIR range. The derivatives were inverted to show positive peaks by multiplying the spectra by -1 , which is not mathematically necessary but aids in understanding the nature of the correlations.

The analysis of second derivative spectra is a very well established and widely used approach to improve the visualization of subtle and overlapping peaks of the raw spectra.^{19, 27, 44–46} The height of the peaks in the second derivative of the spectra reflect the intensity of the absorbance seen in the raw spectra and provide a direct approach to obtain quantitative or semi-quantitative data from MIR and NIR spectra. A recent paper from our group demonstrated a very strong correlation between second derivative peak heights and integrated peak areas.²⁷ Second derivative peak intensities were determined based on the height of the peaks above the zero baseline. A good compromise between optimization of signal-to-noise ratio and spectral features was obtained by applying the Savitzky-Golay algorithm using the Unscrambler X software (CAMO, Oslo, Norway). This software allows processing of the second derivatives of many spectra simultaneously, with the exact same parameters, which minimizes errors. Use of the second derivative provides an objective approach for the analysis of spectra, since once the parameters are set, it is very reproducible. Additionally, using spectral derivatives eliminates the need for correcting baseline shifts, and thus improves the accuracy of quantification.⁴⁴

Compositional images were prepared based on each of the peaks of interest, using the peak height histograms to set a threshold including all positive values of the inverted second derivative peaks. Peak intensity distributions were acquired in grayscale, with brighter pixels representing spatial regions where the selected peak, related to a specific molecular moiety, was higher.

The spatial correlation analysis between MIR and NIR images was done using ImageJ software version (v1.51w). Briefly, the pair of MIR and NIR images to be analyzed were converted to 8-bit images. A region of interest was set selecting the entire sample area and excluding the background. The images were then analyzed with the “colocalization threshold” plugin, setting the MIR image to the red channel, the NIR image to the green channel, red/green corresponding overlays to yellow, and areas with spatial correlations shown in greyscale in a separate channel. As is standard for colocalization analysis, the plugin applies the Costes *et al.* method⁴⁷ to quantify the pixel intensity spatial correlations of components in the red and green channels.

Statistics

The Pearson correlation coefficients (R) and percent areas of correlation were obtained based on pixel-to-pixel comparison of spectral images generated from intensities of specific frequencies that were overlaid in ImageJ. Pairwise comparison was done by two-way analysis of variance (ANOVA) with post-hoc Fisher’s least significant difference (LSD) test, using SPSS (version 25; IBM) to assess differences between correlation of water or matrix in NIR with either PG or collagen in MIR. The two factors in place were the frequency of the MIR peak (reflecting PG or collagen), and the frequency of the NIR peak, reflecting water or matrix components. Differences were considered significant at $P < 0.05$.

Results

Samples from native and engineered cartilage presented typical features, such as a rich PG content (Fig. 1A). In contrast to the sections that were histologically stained for PG, analysis of unstained sections (Fig. 1B) with FT-IRIS (Fig. 1C) enabled exploration of the distribution and spatial correlation of different components of the matrix.

The raw FT-IRIS spectra (Fig. 2A) of the native and engineered cartilage were also typical, including the high intensity MIR peak for the amide I protein vibrations. Clear NIR peaks were observed with much lower absorbances, as expected (Fig. 2A, inset). The second derivatives of the raw spectra were used to reveal more details of subtle and overlapping peaks in the MIR (Fig. 2B) and NIR (Fig. 2C) region. The well-established MIR peaks at ~ 856 and 1340 cm^{-1} were used to obtain the spatial distribution images of PG and collagen, respectively. Spatial correlation analysis of these components showed that their distributions had opposite trends along native cartilage thickness (Fig. 3A); PG content was greater towards the deep zone and collagen content greater towards the superficial zone, presenting a weak overall spatial correlation ($R \approx 0.19$) that was most prominent in the middle zone. In engineered cartilage (Fig. 3B), distribution of PG and collagen also had a weak spatial correlation ($R \approx 0.08$) and showed somewhat opposite trends, with, for instance, a greater collagen content observed around the edges of the constructs.

To evaluate and correlate the spatial correlation of these matrix components with compositional features in the NIR spectra, FT-IRIS images were also created based on the NIR peaks at $\sim 5200\text{ cm}^{-1}$ (water) and ~ 4400 , 4600 and 4890 cm^{-1} (matrix peaks). Images based on the intensity of the MIR and NIR peaks show the distribution of specific matrix components and water in native cartilage (Fig. 4A). Correlation analysis of the NIR and MIR images enabled quantifying the area where each NIR compositional feature is spatially correlated with the PG or collagen content of native cartilage (Fig. 4B and Fig. 4C, respectively). The same approach was used to evaluate engineered cartilage (Fig. 5A–C).

We found several significant spatial correlations between NIR compositional images and the distribution of PG and collagen determined using the MIR peaks at ~ 856 and 1340 cm^{-1} , respectively. In native cartilage, the distribution of PGs presented the highest correlation coefficient with the NIR water peak at $\sim 5200\text{ cm}^{-1}$, whereas very low correlation values were found with the NIR matrix peaks at ~ 4400 , 4600 , and 4890 cm^{-1} (Fig. 6A). An opposite trend was seen with the correlation of collagen and the NIR peaks, although the differences were less pronounced (Fig. 6A). Interestingly, the NIR water and matrix peaks differentially correlated with the PG and collagen distribution obtained by MIR imaging, with the water peak having a greater correlation with PG distribution, and the matrix peaks with collagen. Quantification of the MIR image area that correlated with the NIR images confirmed the observed results, showing, for example, $\sim 90\%$ of the PG distribution correlated with the NIR water peak distribution (Fig. 6B). Additionally, it was possible to evaluate how much of the NIR images were not specifically correlated with the MIR images, further quantifying NIR areas that showed poor spatial correlation with PG or collagen distribution (Fig. 6C). Similar trends were observed in the correlation analysis of the engineered cartilage samples (Fig. 7A–C). However, the correlations of PG and collagen with NIR water did not show significant differences between them, which may be related to the hydrogel nature of the scaffolds.

Discussion

Here, we have shown that FT-IRIS is a powerful technique for analysis of the distribution of ECM proteins and water throughout engineered and native cartilage in a quantifiable manner. In the current study, visual analysis of the histological data provided inadequate resolution regarding the distribution of PG, as seen by the minimal variation in Safranin O intensity distribution. Although other groups have quantified histological PG distribution using sections from 5 to 10 microns thick with this method^{48–50}, it would be challenging to correlate such analysis with the distribution of other matrix components or with water. FT-IRIS is more sensitive to slight variations in intensities and provides a quantifiable image with more conclusive data regarding the distribution. In addition, FT-IRIS detects water in the NIR range, allowing the correlation of the intensity of PG and collagen distribution to water distribution at micron resolution, which is a novel application. Previously we have demonstrated that the NIR water absorbances in native cartilage correlated with the amount of gravimetrically determined water⁴¹, but have not approached this for tissue-engineered cartilage. Correlations between overall ECM protein content and water content have been shown previously^{8, 14, 51–53}; however, this is the first study to show spatial correlations between the distribution of those components in cartilage tissue sections. Our data

demonstrate that water correlates to PG to a much greater extent than to collagen, which we can interpret to mean that water in both native and engineered cartilage is bound to PG to a greater extent. This is in agreement with many other studies that have investigated this, though not at the spatial level described here.^{54, 55} These interesting results may be better explained by the chemical structures of PG, collagen, and water.

Articular cartilage exists as a biphasic tissue with water contributing to the fluid phase and ECM contributing to the solid phase. PG consists of a protein core linked covalently with sulfated glycosaminoglycan (sGAG) chains. The absorbance seen in the MIR spectra at $\sim 856\text{ cm}^{-1}$ arises from the C–O–S vibrations of the sGAG chains. The intensity of this peak is directly correlated with the content of PG in cartilage²⁴, and it has been used previously to quantify the spatial distribution of PG in engineered cartilage.¹⁹ sGAGs are glycosylated proteins and make up 10–15% of the wet weight of articular cartilage.⁵⁴ These sulfated glycosaminoglycans (chondroitin sulfates, keratan sulfates, and heparan sulfates) are polysaccharides composed of disaccharide units characteristically containing repeats of a hexosamine and a hexuronic acid residue. sGAGs are highly polar and function as water attractants. It is this large number of sGAG side chains that contribute to PGs being a rich source of negative charges within the ECM, and allows for the attraction of water molecules.⁵⁵ Similarly, collagen (90–95% of which is type II) makes up of 60% of the dry weight of the articular cartilage and consists of three polypeptide chains (glycine, proline and hydroxyproline) wound into a triple helix.⁵⁴ The presence of hydroxyproline in collagen allows for intra- and inter-chain hydrogen bonding between carbonyl groups in the collagen fibrils, providing stability to the collagen structure and also sites for the interaction with water.⁵⁶ In total, water contributes about 80% of articular cartilage wet weight, out of which $\sim 30\%$ is bound within intrafibrillar space within the collagen.⁵⁴ The remaining water is confined in the interfibrillar space of ECM (occupied by PGs) and pore space.⁵⁴ In NIR spectroscopy, the lower frequency 5200 cm^{-1} vibration that was assessed here reflects primarily bound water, whereas free or more loosely bound water is reflected by the higher frequency 6890 cm^{-1} vibration⁴¹ that was not investigated here. However, the 5200 cm^{-1} absorbance does have a loosely bound component as well, but that did not contribute to this analysis, as samples were air-dried.

It was interesting to note that in the engineered cartilage, not all the water was associated with matrix, while in the native cartilage, almost the entire water pool was associated with matrix. This is because MeHA hydrogel forms the scaffold for the engineered tissue, and bound water exists in the hydrogel. This is important to keep in mind when assessing water content of these constructs, as not all the water would contribute to their mechanical properties. It was also interesting to note that the NIR matrix absorbances investigated were associated primarily with collagen, which had been observed in an earlier study¹⁹, and not associated with PG. Thus, when applying NIR spectroscopy to assess PG in engineered constructs, the water peak will better reflect PG content, but not exclusively, if the scaffold is a hydrogel.

The relationship between the amount and spatial distribution of ECM components with the mechanical properties of cartilage is still not fully understood. The molecules under investigation in the present study are the three components of cartilage that would have the

greatest contribution to its mechanical properties: collagen, PG, and water. Thus, the information described here may aid future research by improving the understanding of the relationship between ECM, water, and the mechanical properties of cartilage. Previous investigations and discussions have brought these relationships into question, and it has been understood that the distribution and rate of synthesis of ECM may contribute more or just as much to the mechanical properties of cartilage as the overall net ECM and water.^{6, 9, 11–13}

Although the results of this study are promising for understanding matrix and water distribution in engineered tissues, there were also several limitations. One limitation is the sample size, with $n = 3$ per group, which may be of concern given the potential variability in native tissue and in maturation of engineered constructs. However, since we correlated variables within the same samples, we are confident that the three samples in each group provided an adequate representation of the variability that would have been seen with a larger sample size. Utilization of 80 μm sections is unconventional in the MIR range, because the depth of penetration of the IR radiation is typically only several microns. However, this is also dependent on the specific absorptivity coefficient of the vibration being studied, as shown by a previous study in our lab where the effects of various sample thicknesses on the signal-to-noise ratio and linearity of absorbance was assessed.¹⁹ Data showed that the absorbance of amide I and II bands reached saturation due to attenuation of the signal, but the MIR bands at 856 cm^{-1} and 1340 cm^{-1} , which have substantially smaller absorptivity coefficients, and all of the NIR bands did not reach saturation. An additional limitation relates to the ability to accurately detect water in tissue sections, as water may evaporate as the data is collected, in particular the loosely bound water.^{41, 57} This is a concern because the 5200 cm^{-1} peak has contributions from both loosely and tightly bound water⁴¹, and remaining free water may contribute to the uncorrelated water that has been seen throughout this study. However, given that the data collection time was nearly two hours total, it is very likely that the water measured was tightly bound water, as any residual loosely bound water would have evaporated during data collection.

In conclusion, FT-IRIS in the near- and mid-infrared ranges is capable of detecting variation of ECM proteins and water at the micron level, and such data can be used to determine spatial correlations between PG, collagen, and water in engineered and native cartilage tissues. Current methods of evaluating the distribution of ECM are sensitive to tissue preservation and section preparation techniques¹⁷, and there is a need for more sensitive and quantifiable methods to obtain more definitive observations. Understanding the distribution of water and ECM proteins throughout engineered cartilage, as well as their spatial relationship, will provide insight for tissue engineers to better understand the development of engineered constructs and their related mechanical properties, along with advancing techniques to improve the controlled development of engineered cartilage.

Acknowledgments

The work was supported the NIH grant R01 AR AR056145 (NP). All authors critically reviewed the manuscript and provided scientific input. JPK, WQ, SK, and NP conceived the idea and design of the study. NP supervised the study, and JPK, WQ, and SK conducted experiments and collected data. JPK and SK conducted cell culture experiments, JPK cryosectioned and conducted histological analysis, and WQ collected and processed IR imaging data. All authors participated in data interpretation and writing the manuscript.

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Figure 1.

Native and engineered cartilage samples. (A) Safranin O staining showing rich proteoglycan content. (B) Unstained sections of 80 μm thickness for FT-IRIS analysis. (C) FT-IRIS images showing the overall protein content distribution throughout the samples based on the amide I peak ($\text{C}=\text{O}$ stretching vibrations of the peptide bonds).

Figure 2. FT-IRIS spectra. (A) Raw spectra. Data were collected from 750–7800 cm⁻¹, allowing the evaluation of MIR (750–4000 cm⁻¹) and NIR (4000–7800 cm⁻¹) peaks from exactly the same region in the same spectrum. The high intensity MIR peak for amide I protein vibrations is highlighted. The inset shows the low intensity NIR peaks. (B) Second derivative spectra showing MIR peaks. The MIR peaks of interest were at ~856 cm⁻¹ (proteoglycan, C–O–S vibration) and 1340 cm⁻¹ (collagen, side chain vibration). (C) Second derivative spectra showing NIR peaks. The NIR peaks of interest were at ~5200 cm⁻¹ (water) and ~4400, 4600, and 4890 cm⁻¹ (matrix peaks, resulting from C–H and N–H vibrations).

Figure 3.

Spatial correlation analysis of the distribution of proteoglycan and collagen. (A) In native cartilage, proteoglycan and collagen showed opposite trends along the tissue thickness, having a weak overall correlation coefficient ($R \approx 0.19$), but a clear correlation area primarily in the middle zone. (B) In engineered cartilage, a weak spatial correlation ($R \approx 0.08$) was also found, with somewhat opposite distribution trends between proteoglycan and collagen.

Figure 4.

Spatial correlation analysis of NIR compositional images with the distribution of proteoglycan and collagen in native cartilage. (A) FT-IRIS images based on MIR and NIR peaks (in red and green, respectively) illustrating the content distribution of specific matrix components. Analysis of the spatial correlation between NIR images and the distribution of (B) proteoglycans and (C) collagen shows the pixel intensity overlays in yellow and area of significant correlation in grayscale. It is shown, for instance, that the correlation of the proteoglycan content is clearly greater with the NIR water peak at $\sim 5200\text{ cm}^{-1}$, compared to the NIR matrix peaks at ~ 4400 , 4600 , and 4890 cm^{-1} .

Figure 5.

Spatial correlation analysis of NIR compositional images with the distribution of proteoglycan and collagen in engineered cartilage. (A) FT-IRIS images based on MIR and NIR peaks, illustrating the distribution of specific matrix components in red (MIR) and green (NIR). Analysis of the spatial correlation between NIR images and the distribution of (B) proteoglycans and (C) collagen shows the pixel intensity overlays in yellow and area of significant correlation in grayscale. It is shown, for instance, that the correlation of the proteoglycan is greater with the NIR water peak at $\sim 5200\text{ cm}^{-1}$ than with the NIR matrix peaks at ~ 4400 , 4600 , and 4890 cm^{-1} .

Figure 6.

Quantitative outcomes of the spatial correlation analysis of NIR compositional images with the distribution of proteoglycan and collagen in native cartilage. (A) Pearson correlation coefficient (R). (B) Percent areas of the MIR images that are spatially correlated with NIR images. (C) Percent areas of the NIR images that do not correlate with the MIR images. The NIR water and matrix images were differentially correlated with proteoglycans and collagen: the water peak at ~5200 cm⁻¹ had a greater correlation with proteoglycan content, and the matrix peaks ~4400, 4600 and 4890 cm⁻¹ had a greater correlation with collagen content. # *P* < 0.05 when compared to the corresponding correlation using the NIR water image. * *P* < 0.05 comparing the correlation of the same NIR image with proteoglycan and collagen.

Figure 7.

Quantitative outcomes of the spatial correlation analysis of NIR compositional images with the distribution of proteoglycan and collagen in engineered cartilage. (A) Pearson correlation coefficient (R). (B) Percent areas of the MIR images that are spatially correlated with NIR images. (C) Percent areas of the NIR images that do not correlate with the MIR images. The results showed a similar trend to that observed for native cartilage (Fig. 6), with the NIR water and matrix images differentially correlated with proteoglycan and collagen content. # $P < 0.05$ when compared to the corresponding correlation using the NIR water image. * $P < 0.05$ comparing the correlation of the same NIR image with proteoglycan and collagen.