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Macromolecular Concentrations in Bovine Nasal Cartilage by Fourier Transform Infrared Imaging and Principal Component Regression

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Abstract

Fourier transform infrared imaging (FT-IRI) technique and principal component regression (PCR) method were used to quantitatively determine collagen and proteoglycan concentrations in bovine nasal cartilage (BNC). An infrared spectral library was first established by obtaining eleven infrared spectra from a series of collagen and chondroitin 6-sulphate mixed in different ratios. FT-IR images were obtained from 6 μm thick sections of BNC specimens at 6.25 μm pixel size. The spectra from the FT-IR images were imported into a PCR program to obtain the relative concentrations of collagen and proteoglycan in BNC, based on the spectral library of pure chemicals. These PCR-determined concentrations agreed with the molecular concentrations determined biochemically using an enzyme digestion assay. Use of the imaging approach revealed that proteoglycan loss in the specimens occurs first at the surface of the tissue block when compared with the middle portion of the tissue block. The quantitative correlation of collagen and proteoglycan revealed that their infrared absorption peak-areas at 1338 and 1072-855 cm^{-1} can only be used as qualitative indicators of the molecular contents. The use of PCR in FT-IRI offers an accurate tool to spatially determine the distributions of macromolecular concentration in cartilage.

Keywords

Fourier-transform infrared imaging (FT-IRI); Principal component regression (PCR); cartilage; collagen; proteoglycan; concentration

Introduction

The biological and functional properties of articular cartilage depend mainly on the concentration, structure, and interaction between collagen and proteoglycan (PG), two primary compositional molecules within the extracellular matrix, and water¹. Structurally, collagen is woven into a 3-dimensional (3D) fibril network that enmeshes the negatively charged PG, allowing the tissue's absorption of positively charged ions that is osmotically balanced by an influx of water. This specific arrangement of collagen/PG/water provides articular cartilage with its compressive resistance and mechanical resiliency². Either a disruption of the collagen network or a decrease in the PG content in cartilage would result in the deterioration of the tissue's biomechanical properties, which may develop into pathological conditions such as osteoarthritis. The ability to quantitatively determine the

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concentration distributions of both collagen and PG in articular cartilage, therefore, becomes critically important to monitor the progressive degradation of tissue.

Fourier-transform infrared (FT-IR) spectroscopy is sensitive to the vibrational motions of the molecules' dipole moments in tissue specimens. Coupled with an infrared microscope, FT-IR imaging (FT-IRI) makes it possible to spatially resolve various chemical signatures with fine spatial pixel size of 6.25 μm and spectral resolution (e.g. 1–16 cm^{-1} spectral spacing in some commercial instrument), and has been used in recent years to study cartilage tissue^{3–14}. In the mid-infrared range, the useful signatures in cartilage research include amide I (stretching vibration of the C=O group, from 1700 to 1600 cm^{-1}), amide II (the N-H bending vibration coupled with the C-N stretching vibration, from 1600 to 1500 cm^{-1}), amide III (the N-H and C-C vibrations, from 1300 to 1200 cm^{-1}) and sugar (1125 to 1000 cm^{-1}). Because of the associations between these amide groups and the molecular constituents in cartilage, the anisotropies of these infrared signatures have been used to determine the orientation of collagen and PG in cartilage¹⁰, to divide articular cartilage into histological zones^{11, 12}, and to monitor the change in the collagen orientation due to external loading¹⁴. There have also been attempts to link the individual absorption peak-areas in FT-IRI to the molecular concentrations in cartilage^{4, 5, 9}. These peak-intensity/area based approaches are problematic, since the co-existence of multiple molecular components in any biological tissue often results in significant overlaps of many absorption bands in the middle infrared region¹⁵, diminishing any possibility of linking the infrared absorption directly to the molecular concentration. Due to this reason, a curve fitting method was recently used to overcome the overlap of absorption bands in PG analysis¹⁶.

Principal component regression (PCR) method is a common chemometrical method based on factor analysis, which fully utilizes all spectral data in multivariate analysis. As such, it is possible for PCR to identify the number of molecular components in a mixed sample and to calculate the component concentrations in unknown samples according to the standard spectral library¹⁷. The current investigation, the first project that correlated the quantitative biochemistry with the combined FT-IRI and PCR approach in cartilage research, aimed to determine the molecular concentrations (collagen and PG) in cartilage quantitatively. First, a set of infrared spectra was obtained from the pure chemicals of collagen (type II) and glycosaminoglycans (GAG) associated with cartilage PG (chondroitin 6-sulphate), mixed into eleven different ratios. Second, FT-IRI experiments were carried out for thin sections of bovine nasal cartilage (BNC), which was chosen because BNC is relatively homogeneous (i.e., lack of histological zones^{18, 19}) and contains PG and collagen that are both compatible with the macromolecular components in articular cartilage²⁰. Thirdly, the PCR method^{17, 21} was adapted in the analysis of the FT-IRI data, which were correlated with the bulk concentrations in BNC as determined biochemically by enzyme digestion²². Finally, the PCR results were compared with the three signature bands in the infrared spectra of BNC, the band of collagen II (which centers around 1338 cm^{-1} , assigned predominantly to the CH₂ wagging vibration of proline side chains¹⁵), the band of sugar ring vibrations (e.g., C-O-C, C-OH, C-C) around 1072 cm^{-1} ⁴ and the band of chondroitin 4-sulfate (CS4, which centers around 855 cm^{-1} , assigned to C-O-S)^{4, 23}.

Materials and Methods

Tissue preparation

Bovine nasal cartilage, obtained from a local slaughterhouse (C. Roy Inc., Yale, MI), was immersed in saline (154 mM NaCl in deionized water) and kept in a refrigerator at $-20\text{ }^{\circ}\text{C}$ before the onset of the experiment. After thawing the tissue at room temperature, one large BNC block was cut into nine consecutive specimens (Fig 1a), all having a size

approximately 2 mm × 2 mm × 2 mm. These specimens were rinsed in saline after all non-cartilaginous tissues were removed.

Table 1 summarizes the treatment conditions of these tissue blocks. Among them, the #2 block proceeded immediately to cutting without any delay; the #6 block was frozen in water at −80 °C over 24 hours, thawed, and then immersed in saline for 2 hours before cutting; the #1, #5, and #9 blocks were digested in the papain solution to determine their bulk PG concentrations; and the other four blocks were immersed in the trypsin solution ranging from 10 min to 17 hours. Among the four trypsin-treated blocks, #7 and #8 were also cut into 6 μm sections on a cryostat (Reichert HistoStat Cryotome). These thin sections were placed on MirrIR slides (Kevley Technologies, Chesterland, OH) and left to air-dry for 2 hours before the FT-IRI experiments were carried out. For all tissue blocks being cut into thin sections (#2, #6, #7, #8), several sections near the block surface were obtained (Fig 1b). Thin sections from the middle of the two treated blocks (#7 and #8) were also obtained, as shown in Fig 1c. (The labels “s” and “m” indicated the tissue sections from the surface and middle portions of the block respectively.)

FT-IR spectroscopy and imaging

Both FT-IR spectroscopic and imaging experiments were carried out on a PerkinElmer Spotlight-300 imaging system (Wellesley, MA), which consists of a FT-IR spectrometer and an infrared microscope. The infrared microscope has a 16-element liquid-nitrogen-cooled mercuric-cadmium-telluride (MCT) focal plane array detector for imaging and a single point MCT detector for spot measurement. An internal coaxial LED illumination with variable intensity is available to produce visible images, which enables the identification of the tissue region for infrared sampling. In the spectroscopic mode, pellets of potassium bromide (KBr) containing the chemicals associated with collagen and PG can be loaded directly into the macro sample chamber of the infrared spectrometer. In the imaging mode, a thin section of tissue can be placed on a moving stage of the infrared microscope, which scans the tissue section under infrared irradiation.

The infrared spectra of the pure chemical components were obtained from the commercial products of type II collagen (Elastin Products Company, MO) and chondroitin 6-sulfate (CS6) (Sigma-Aldrich, MO). Fibrillous collagen was processed into powder by resolving, grinding and drying before pelleting with KBr. Both products were dry-mixed in 10 different ratios (Collagen/CS6 = 2.54, 1.56, 1.36, 1.24, 1.09, 0.98, 0.86, 0.60, 0.40, 0 mg/mg) and pressed into 10 mm pellets with KBr. These 10 pellets were measured spectroscopically to obtain the reference infrared spectra, with a spectral range of 4000–744 cm^{−1} at a wavenumber spacing of 8 cm^{−1}. 128 averages were used in the spectroscopy to improve the signal-to-noise ratio to 2000 (peak-to-peak). These spectra of two principal components of collagen and CS6 were used to construct a standard library by using a chemometrical software, Spectrum Quant+, from PerkinElmer. Note that BNC contains dominantly chondroitin 4-sulfate (CS4)^{20, 24, 25}, while our standard chemical was CS6. Although they have some differences in spectral shape between 1000–744 cm^{−1}, CS4 and CS6 both show identical infrared spectra within the range of 4000–1000 cm^{−1}^{23, 26}. For this reason, the spectral region between 1000–744 cm^{−1} was not included in the PCR calculations by using a “blank” function in the software package. Full cross validations were carried out between the actual chemical concentrations and the PCR calculated concentrations, where an excellent correlation was found (e.g., the collagen correlation in Fig 1d).

In the infrared imaging experiments, a thin section of the specimen was mounted on the movable stage of the infrared microscope and remained fixed during the scanning. The imaging data were collected at a 6.25 μm pixel size and an 8 cm^{−1} wavenumber spacing

over a spectral range of 4000–744 cm^{-1} . No baseline correction was performed on the raw data¹⁷. During the image analysis, a rectangular region (approximately ~ 50 μm in width and 100 μm in length) was selected, and the spectra in the region were averaged to obtain one target spectrum using the function of “co-add spectra” in the PerkinElmer’s Spotlight software. One target spectrum is shown in Fig 1e. These target infrared spectra from the region-of-interest (ROI) were introduced into the Spectrum Quant+ software with the PCR algorithm to calculate the concentrations of collagen and PG in the specimens.

Biochemical determination of PG

A modified DMMB (1,9-dimethylmethylene blue) protocol²⁷ was used to determine the bulk chemical concentrations in the specimens. Briefly, a Blyscan dye reagent (BioColor, Northern Ireland) was used to determine the specific binding to PG, where the absorbance values were measured at 656nm using Shimadzu UV-1700 spectrophotometer. Standard curves of dye absorbance dependence on the PG concentration were made by assaying the known concentrations of CS4. The PG loss in the trypsin solution was calculated by linear regression based on the measured aliquot of PG content and the standard curve, which is calculated as mg/ml in the blocks. The decrease in PG concentration was obtained by comparing the absorbance of its aliquot volumes of the sample solutions with that of papain solutions of BNC blocks²⁷.

A trypsin solution of 0.05 mg/ml concentration was prepared by dissolving trypsin in a digestion buffer reagent at pH 7.6²⁸. A papain solution of 1 mg/ml concentration was prepared by dissolving papain in its digestion buffer reagent at 60 °C for 30 min at pH 6.5²⁷. Seven specimen blocks were treated by these solutions, summarized in Table 1. The #1, #5 and #9 blocks were separately immersed in the papain solutions at 65 °C until they were thoroughly dissolved. The #3, #4, #7 and #8 blocks were immersed in the trypsin solution for 10 min, 1 hour, 30 min and 17 hours, respectively. After the digestion/treatment, aliquot volumes of 5–20 μl of the digestion solutions were taken for spectrophotometric measurement of PG using the method described previously in this section.

Statistical analysis

Data groups were evaluated for significant differences using commercial software KaleidaGraph (v4.0, Synergy Software, Reading, PA) in three sets of statistical analysis. First, each tissue PG was compared with the reference using the nonparametric Wilcoxon-Mann-Whitney test. Second, each group of three ROI orientations was analyzed together using the Kruskal-Wallis test. Finally, within each group, three sets of ROI were tested among themselves using the Wilcoxon-Mann-Whitney test. In both Wilcoxon-Mann-Whitney and Kruskal-Wallis tests, if the p-value is below 0.05, the conclusion is that there is a difference between the groups.

Results

Biochemical analysis

The results from the biochemical analysis are summarized in Table 1. The amount of PG dissolved in the papain solution was determined spectrophotometrically by measuring the visible absorbance and averaging 3 solution samples for each block²⁷. These measurements were converted into the tissue PG loss in wet-weight, using 1.12 g/ml as the density of cartilage²⁹. Averaging the PG concentrations in three totally dissolved blocks (#1, #5 and #9), the mean PG concentration in BNC was found to be 116.57 ± 5.32 mg/ml, which was consistent both with our previous measurements^{27, 29} and relevant literature³⁰. A logarithmic relationship was found by fitting these PG losses with the treatment times in Table 1, which has a fitting coefficient R^2 of 0.985 (fitting not shown). This logarithmic

trend is similar to that of BNC in guanidium chloride ³¹, suggesting that PG loss in trypsin solution increases nonlinearly with immersing time.

The effect of tissue freezing

Figure 2a shows the visible image of the ROI from the #2 section, which did not undergo any treatment before cutting/imaging. The infrared images and peak-area profiles of the two components (collagen represented by the 1338 cm^{-1} band and PG represented by the 855 cm^{-1} band) are shown in Fig 2b and 2c. The absorption values in both FT-IR images are rather homogeneous, represented by the uniform colors in the images (Fig 2b) and the flat lines in the peak-area profiles (Fig 2c). Fig 2d shows 16 independent calculations using the PCR method, across the 2s tissue section in a resolution step of $\sim 50\text{ }\mu\text{m}$ at the same locations where the peak-area profiles were extracted in Fig 2c. Since the tissue section was dry, the sum of the collagen and PG concentrations was assumed to represent 100% of the tissue's weight due to the relatively small amount of cells within cartilage ($\sim 1\%$). The mean and the standard deviation of the collagen and PG concentrations were $48.65\pm 3.12\%$ and $51.35\pm 3.12\%$ respectively, which agrees with the literature ^{20, 31–34}. Note that although both profiles of PCR calculation (Fig 2d) and infrared peak-area (Fig 2c) showed a similar uniform distribution of the collagen and PG in BNC, the absorption values of the 1338 and 855 cm^{-1} peak-areas were not calibrated and hence could not be converted to the chemical concentration (the mean values were 7.61 and 4.84 in Fig 2c).

Nearly identical results were obtained from the #6 block (not shown), which was frozen at -80°C for 24 hours, and then thawed in saline. This result indicates that the process of freezing and thawing BNC does not cause the loss of PG into the solution. Table 2 summarized the mean averages and standard deviations of the collagen and PG concentrations calculated by the PCR method for the #2, #6, #7 and #8 specimens.

Tissue penetration in trypsin treatment

From the #7 and #8 BNC blocks that were immersed in trypsin solution for 30 min and 17 hours respectively, several thin sections were cut from the surface and middle portions of the blocks. The corresponding visible images are shown in Fig 3. For each tissue section, three ROIs were imaged in the FT-IRI experiment: the edge of the section, the center portion of the section, and a horizontal region that bridges the two regions, as illustrated in Fig 3.

The FT-IR images at 1338 or 855 cm^{-1} of the three ROIs on the surface sections (7s and 8s) are shown in Fig 4, together with the PCR results. The images and the profiles were plotted on the same tissue location scale. The actual numbers of the tissue location in microns on these figures were not important, as they merely reflected the location of the tissue section on the moving stage of the FT-IRI instrument. Both the FT-IR absorption images and the PCR analysis showed that the collagen and PG concentrations exhibited spatial variations larger than the untreated specimens (Fig 2). This included several locations where the collagen concentration went over 90%. The means and the standard deviations of the collagen and PG concentrations in the 7s section were $75.81\pm 5.30\%$ and $24.19\pm 5.30\%$ respectively for the edge ROI (N=27), $87.29\pm 6.70\%$ and $12.71\pm 6.70\%$ respectively for the horizontal ROI (N=17), and $88.68\pm 4.23\%$ and $11.32\pm 4.23\%$ respectively for the center ROI (N=25). The PG concentrations in these analyses were reduced significantly from the control values (Fig 2), due to the 30 min trypsin treatment.

More severe loss of PG was observed in the 8s section, which underwent a 17 hour trypsin treatment. The results are also shown in Fig 4 and summarized in Table 2. In this set of data from the 8s section, the variation in PCR analysis became smaller, indicating a more

homogenous specimen. Also interesting is the fact that even after a 17-hour treatment in trypsin, there was still some residual PG left in the tissue.

The FT-IR images at 1338 or 855 cm^{-1} of the three ROIs of the middle sections of the same tissue blocks (7m and 8m) are shown in Fig 5 and also summarized in Table 2, together with the PCR results. Comparing with the images in Fig 4, the infrared absorptions in these middle sections are more homogenous. The increased homogeneity of the middle sections is also reflected in the smaller errors of the PCR analysis. The results of the 30 min treatment (7m) show similar PG concentrations to untreated tissue (2s and 6s), while the 17 hour treatment (8m) showed lower PG concentrations.

The correlation between FT-IRI and biochemistry

The mean values of the collagen and PG concentrations from the 24 independent PCR calculations in the 2s and 6s sections were $47.95 \pm 3.21\%$ and $52.05 \pm 3.21\%$ respectively. Using them as controls, and assuming that the collagen concentration in the specimens was constant during the trypsin treatment, all PCR calculations of the relative PG concentrations were converted into the percentage concentration of PG in the specimens, plotted in Fig 6 with the statistical results and summarized in Table 2. Three sets of statistical analysis were carried out. First, each tissue PG was compared with the control using the nonparametric Wilcoxon-Mann-Whitney test. Apart from the two values (7m Edge ROI and 7m Horizontal ROI), all tissue PG values were significantly different from the control ($p < 0.0001$). Second, each group of three ROI orientations was analyzed together using the Kruskal-Wallis test. The small p-values ($p < 0.0058$) show that there were significant differences within each group. Finally, within each group, three sets of ROI were tested among themselves using the Wilcoxon-Mann-Whitney test. Among the 12 tests, four pairs were not significant ($p > 0.05$), marked with the arrow brackets in Fig 6.

To test whether the peak-area values in the FT-IRI experiments can be interpreted directly as concentrations of collagen and PG in the specimen, the ratios of the collagen and PG from PCR (which had a profile similar to Fig 2c) were compared with the ratios of the 1338cm^{-1} -peak-area and 855cm^{-1} -peak-area (which had a profile similar to Fig 2d) in the treated specimens (sections 7m and 8m, Edge ROI). The results of 1072 cm^{-1} band were also summarized in Table 3. It is clear that (Table 3) the peak-area ratios did not agree with the PCR ratios, which were verified by the biochemical determinations. This result demonstrates that the peak-area ratios can be used as a qualitative indicator for the molecular distribution in cartilage; however, they cannot be used as the quantitative measures for the molecular concentration in cartilage.

Discussion

In using the PCR method to analyze the FT-IRI data, this project has obtained three important results. First, the FT-IRI-PCR method can quantitatively determine the distribution of molecular concentrations in BNC. Second, the degradation process of a tissue block by the trypsin protocol is logarithmic in time and heterogeneous in space, depending upon the treatment time (and protocol). Third, the peak-areas in infrared spectra cannot be used as the quantitative measures of molecular concentrations in cartilage, even if the peak-area is selected at the specific wavenumber of the chemical.

Quantitative Determination of Molecular Concentrations by FT-IRI-PCR method

By comparing our results to biochemistry measurement, this project demonstrates that the FT-IRI-PCR method can be used to determine the concentrations of macromolecules in cartilage quantitatively (Table 1). The percentage concentrations of collagen II and PG from

both methods were in excellent agreement with the understanding that collagen and PG are distributed nearly homogeneously¹⁹ and in approximately equal amounts in BNC^{20, 31–34}. Several biochemical studies^{33, 34} reported that between the concentrations of collagen and PG in BNC, PG concentration was slightly high, which was confirmed by the FT-IR-PCR analysis in this report (Table 2).

Two interesting observations in this project are worth pointing out. First, it was found that even after 17 hours of treatment in trypsin, only 86% of PG were extracted from tissue (Table 1). This result is consistent with a number of studies in the literature^{18, 31, 32} that have reported the exhaustive extraction of PG could only be in the range of approximately 75% – 90%. The residual PG have been attributed to corresponding to pericellular basophilia or pericellular matrix granules, as seen by light or electron microscopes¹⁸. In this project we had tried to correlate the ‘hot spots’ in the FT-IR images (e.g., the red and yellow spots in Fig 2b) with the cells in the visible images of the same tissue sections (e.g., Fig 2a). We found that all ‘hot spots’ could be traced back to the cells in the visible images; but not all visible cells would result in the ‘hot spots’ in the FT-IR images. Additional study is needed to identify the condition/environment that retains PG in the pericellular structure, preferably with a higher spatial resolution³⁵. Overall, the existence of residual PG near the pericellular matrix increases the variation of the concentration from the PCR method.

Second, this work shows that BNC does not lose PG into the storage solution after being frozen and thawed. This is different from the case of articular cartilage, which will lose up to 56% of PG into the storage solution after being thawed³⁶. Given the fact that both BNC and articular cartilage share the same molecular constituents in composition (water, proteoglycan, collagen), the difference may be due to the structural differences between the tissues and that BNC lacks the histological zones evident in articular cartilage. In addition, BNC has an increased amount of PG aggregation, compared to articular cartilage, which may serve to restrict PG loss. It is worth investigating the causes of this distinct difference in the tissue’s ability to retain PG after being frozen and thawed.

Two experimental issues should be noted here. First, the PCR analysis in this project assumed that the sum of collagen and PG represented 100% of the dry tissue weight. In reality, cartilage also contains some minor components, such as chondrocytes and other minor molecules/proteins. Since the sums of all PG/collagen/water in human articular cartilage are age-dependent (approximately 96.5% – 98.7%)³⁷ and the noncollagenous proteins in BNC are about 8%³⁴, the assumption in our PCR analysis is reasonable. Second, to minimize the influence of small differences between the spectra of CS4 and CS6^{23, 26}, the PCR calculation did not include the wavenumbers below 1000 cm⁻¹. This exclusion, however, should have little effect on the accuracy of the calculation, since a number of significant spectral bands associated with the vibrations of sugar and sulfates occur within the range of wavenumbers being analyzed by the PCR (above 1000 cm⁻¹). The results of 1072 and 855 cm⁻¹ in Table 3 support and verify this approach.

The penetration of trypsin into the tissue during digestion

Although BNC has a relatively homogenous structure, the use of imaging in our project enables the identification of heterogeneities in a tissue’s PG concentration. As evident from the data summarized in Fig 6 and Table 2, the degradation of the tissue by trypsin is time-dependent, non-linear, and spatially heterogeneous. These observations in BNC are consistent with the similar results found in articular cartilage, where the appearance of a ‘penetration front’ inside the tissue can be observed in both the ‘active’ degradation by the trypsin method³⁸ or the ‘passive’ loss by soaking a tissue exposed to freeze-thaw in saline³⁶. Clearly, the penetration front in articular cartilage and the lack of such a front in nasal

cartilage must be related to the collagen fibril structure, the interaction between the fibrils and PG fragments, and the speeds at which these fragments diffuse out of the tissue.

Comparison between qualitative and quantitative concentration determination

By correlating the PG concentration between the FT-IRI-PCR method and trypsin digestion, this project has demonstrated the accuracy and quantitative nature of the FT-IRI-PCR method. It is also evident that although the FT-IR absorption at 1338 and 855 cm^{-1} have been considered as the signature bands representing collagen and PG respectively^{3–5, 9, 15, 26}, and although the profiles of these two peak-area absorptions (e.g., Fig 2c) resembled the profiles of collagen and PG concentrations from PCR (e.g., Fig 2d), the infrared absorption values of these peak-areas can only provide the qualitative determination on collagen and PG contents in cartilage (Table 3). This inability to correlate absorption to concentration is mainly due to the fact that the absorption intensity in infrared can be strongly affected by a number of experimental factors in data acquisition and data analysis, e.g., different infrared absorptivity of sample, choices of baseline correction, and overlaps among molecular bands.

In recent years, several approaches have been used in FT-IRI to determine the molecular components in cartilages, including Euclidean distance mapping and least square analysis³, peak ratio intensity measurement^{4, 5, 9}, and partial least square (PLS) analysis⁷. Due to the significant overlaps among the infrared bands of collagen, PG and other proteins in tissue, most of the intensity/area-based methods (such as the peak-area profiles in this report) can only provide a qualitative estimation of collagen and PG contents in cartilages. Any quantitative determination of the molecular concentrations requires the establishment of a pure chemical spectrum library and the use of factor analysis in chemometrics, such as PCR or PLS²¹. We have also used the PLS approach during this project and found that the PLS results are significantly correlated with the PCR results, with the errors not more than 1% between the two methods (data not shown).

In conclusion, this combined FT-IRI-PCR and biochemistry project successfully determined the concentration distributions of principal molecular components in BNC quantitatively, which was, to the best of our knowledge, the first such study in cartilage imaging research. The loss of PG was found to increase logarithmically with increased immersion time in trypsin. The tissue digestion by trypsin was found to be heterogeneous and depended upon the depth from the surface of the tissue block. The distribution profiles of the collagen and PG concentrations by PCR are similar to those of the peak areas at 1338 to 1072/855 cm^{-1} ; however, the latter can only be used as the qualitative measures of the molecular concentrations. Given the quantitative and spatially resolved nature of this combined FT-IRI and PCR technique, this approach has the potential to become an important analytical tool to resolve the molecular concentrations in articular cartilage. The ability of this technique to measure changes in molecular concentration and fibril structures may prove clinically important in identifying early signs of tissue degradation, such as collagen disruption and PG loss associated with pathological conditions in osteoarthritis.

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Fig. 1.

(a) The schematic diagram for the nine BNC blocks. (b) The tissue sections from the surface of the #2, #6, #7 and #8 blocks were labeled as 2s, 6s, 7s and 8s, respectively. (c) The tissue sections from the middle portion of two blocks (#7 and #8) were labeled as 7m and 8m respectively. (d) The correlation between the actual collagen concentration and the PCR calculated collagen concentration. (e) An infrared spectrum from one FT-IR image of BNC. Two arrows point to two signature bands centered at 1338 and 855 cm^{-1} , which were also shown in the inserts.

Fig. 2.

(a) Visible image of the ROI from the 2s section (size = $800 \times 220 \mu\text{m}$). (b) The FT-IR absorption images at the bands of 1338 cm^{-1} and 855 cm^{-1} , plotted on the intensity limits of 0–0.6 and 0–0.4 respectively. (c) The profiles of the infrared images shown in (b). (d) The concentration profiles of the collagen and PG by the PCR method, based on the same images of the 2s section. The horizontal scale is the reading from the micrometer in the instrument. Its absolute values have no significance (depending upon the position of the tissue section on the sample stage) but can be used to correlate between the images and profiles.

Fig. 3.

Visible images of the surface and middle sections of the #7 BNC block (30 min trypsin immersion) and the #8 block (17 hour trypsin immersion). Three ROIs were selected from each tissue section, marked with three rectangles and labeled as edge (black), horizontal (red) and center (blue), respectively.

Fig. 4.

The FT-IRI results from the surface sections of the #7 and #8 blocks shown in Fig 3. The FT-IR images at 855 cm^{-1} and 1338 cm^{-1} were shown above each PCR concentrations of the collagen and PG. All 855 cm^{-1} images were plotted using the same intensity limits of 0 and 0.3; the 1338 cm^{-1} images for the 7s ROIs were plotted using the limits of 0 and 0.4; the 1338 cm^{-1} images for the 8s ROIs were plotted using the limits of 0 and 0.2. Each data point in the profiles was calculated independently by the PCR algorithm for every $\sim 50\text{ }\mu\text{m}$ location displacement in the FT-IR images. The horizontal scales were the micrometer readings in the instrument.

Fig. 5.

The FT-IRI results from the middle sections of the #7 and #8 blocks shown in Fig 3. The FT-IR images at 855 cm^{-1} and 1338 cm^{-1} were shown above each PCR concentration of collagen and PG. All 855 cm^{-1} images were plotted using the same intensity limits of 0 and 0.3; the 1338 cm^{-1} images were plotted using the limits of 0 and 0.6. Each data point in the profiles were calculated independently by the PCR algorithm for every 100 μm location displacement in the FT-IR images. The horizontal scales were the micrometer readings from the instrument.

Fig. 6.

The summary of all PG concentrations in BNC by the PCR method (the mean value $\pm$ standard deviation), together with the statistical results on the data. (The label in the plot indicates the data group, e.g., “7s-c” means the data from block #7, surface section, central ROI.) The symbol “***” represents a significant difference ($p < 0.0001$) from the control (the average of 24 calculations in the sections 2s and 6s). The statistical analysis for each treatment group by the Kruskal-Wallis test was given above the straight line. Any nonsignificant correlation between two elements within a group is indicated by the pair of arrows.

Table 1

The experimental conditions and the PG concentrations determined biochemically. The last column lists the PG concentrations determined by the PCR calculation. The density of BNC was set at 1.12 g/ml. (w.w. = wet weight)

Block #	Treatment	Tissue w.w. (mg)	Biochemical Analysis			FT-IRL-PCR Analysis	
			Dissolved PG in digestion solution (µg)	Dissolved PG as Tissue PG loss (w.w.) (mg/ml)	Tissue PG loss (%)	Tissue PG loss (%)	
1	Papain, 2 h	8.2	828.65	113.19	100±5.26		
5	Papain, 2 h	9.8	1073.61	122.70			
9	Papain, 2 h	6.3	640.28	113.83			
3	Trypsin, 10 min	12.2	199.40	18.31	15.71±1.24	14.69±5.89	
7	Trypsin, 30 min	9.9	385.21	43.58	37.39±1.03	38.22±5.62	
4	Trypsin, 60 min	9.6	505.43	58.97	50.59±5.37		
8	Trypsin, 17 h	10.0	898.25	100.60	86.30±5.09	78.08±3.81	
2	No	-					
6	-80°C, 24 h	-					

Table 2

The concentrations of collagen and PG in BNC by the PCR method. The columns of "Relative Col./PG" were calculated directly by the PCR method. The 24 independent PCR calculations in the 2s and 6s sections were averaged to yield the mean concentrations of the relative collagen and PG in BNC tissue, which were $47.95 \pm 3.21\%$ and $52.05 \pm 3.21\%$ respectively. These two relative concentrations were used to scale the PG concentrations in the other tissue sections into the PG concentration in tissue, based on the assumption that the collagen was constant during the treatment.

Section	Mean Percentage Concentration of Collagen/PG in BNC (std.) by FT-IRI-PCR									
	Edge ROI					Horizontal ROI				
	N	Relative Col./PG	Tissue PG	N	Relative Col./PG	Tissue PG	N	Relative Col./PG	Tissue PG	Tissue PG
2s	16	48.65/51.35 (3.12)	52.05 (3.21)							
6s	8	46.56/53.44 (3.13)								
7s	27	75.81/24.19 (5.30)	15.30 (3.35)	17	87.29/12.71 (6.70)	6.98 (3.68)	25	88.68/11.32 (4.23)		6.12 (2.29)
8s	16	92.59/7.41 (4.60)	3.84 (2.38)	21	96.35/3.65 (2.40)	1.82 (1.20)	20	91.28/8.72 (4.50)		4.58 (2.36)
7m	9	46.80/53.20 (1.59)	54.51 (1.63)	10	47.62/52.38 (2.48)	52.74 (2.50)	9	45.57/54.43 (1.51)		57.27 (1.59)
8m	12	70.52/29.48 (3.02)	20.05 (2.05)	9	69.44/30.56 (2.39)	21.10 (1.65)	11	73.75/26.25 (5.21)		17.07 (3.39)

Table 3

Comparison of the PCR Collagen/PG ratios and the infrared peak-area ratios

	Concentration Ratio of Collagen/PG in FT-IRI-PCR	Absorption Peak-Area Ratios in FT-IRI			
		1338/855 cm^{-1}		1338/1072 cm^{-1}	
		No baseline correction	Baseline corrected	No baseline correction	Baseline corrected
7m edge (30 min)	0.88±0.06	2.23±0.54	0.47±0.17	0.17±0.01	0.01±0.01
8m edge (17 hour)	2.43±0.44	3.13±0.36	15.94±7.23	0.33±0.04	0.06±0.01