A Chemometric Analysis for Evaluation of Early-Stage Cartilage Degradation by Infrared Fiber-Optic Probe Spectroscopy

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In vivo identification of early-stage cartilage degradation could positively impact disease progression in osteoarthritis, but to date remains a challenge. The primary goal of this study was to develop an infrared fiber-optic probe (IFOP) chemometric method using partial least squares (PLS1) to objectively determine the degree of cartilage degradation. Arthritic human tibial plateaus (N = 61) were obtained during knee replacement surgery and analyzed by IFOP. IFOP data were collected from multiple regions of each specimen and the cartilage graded according to the Collins Visual Grading Scale of 0, 1, 2, or 3. These grades correspond to cartilage morphology that displayed normal, swelling or softening, superficially slight fibrillation, and deeper fibrillation or serious fibrillation, respectively. The model focused on detecting early cartilage degradation and therefore utilized data from grades 0, 1, and 2. The best PLS1 calibration utilized the spectral range 1733–984 cm\(^{-1}\), and independent validation of the model utilizing 206 spectra to create a model and 105 independent test spectra resulted in a correlation between the predicted and actual Collins grade of \(R^2 = 0.8228\) with a standard error of prediction of 0.258 with a PLS1 rank of 15 PLS factors. A preliminary PLS1 calibration that utilized a cross-validation technique to investigate the possibility of correlation with histological tissue grade (33 spectra from 18 tissues) resulted in \(R^2 = 0.8408\) using only eight PLS factors, a very encouraging outcome. Thus, the groundwork for use of IFOP-based chemometric determination of early cartilage degradation has been established.

Index Headings: Fourier transform infrared spectroscopy; FT-IR spectroscopy; Infrared fiber-optic probe; IFOP; Partial least squares; PLS; Cartilage; Collagen; Osteoarthritis.

INTRODUCTION

Articular cartilage is a thin layer of tissue that covers the ends of articulating bones in arthrodial joints. It functions to shield the bone from damage and minimize the mechanical stresses during joint loading by lubricating the contacting joint surfaces.\(^1,2\) Two distinct phases comprise articular cartilage, a fluid phase and solid phase.\(^3–5\) The fluid phase contains water and solutes, i.e., ions and nutrients. The solid phase, which is porous and permeable, is primarily composed of type II collagen, proteoglycans (PG), and chondocytes.\(^6,7\) The proteoglycans are a diverse group of uniquely glycosylated proteins, also called sulfated glycoproteins, which are most abundant in the extracellular matrix of connective tissues. PGs, collagens, and their interactions with the fluid phase play an important role in the mechanical response of the tissue.

Normal cartilage has the ability to resist large forces, but cartilage disruptions, such as changes in PG and collagen structure and content, that occur during pathological conditions such as osteoarthritis (OA) lead to impaired joint motion and pain.

Despite significant improvements in our understanding and treatment of osteoarthritis (OA) over the past decade, OA continues to cause disability and impairment. It is believed there is a “window of opportunity” to control the progression of OA that exists in the early course of the disease.\(^8,9\) Therefore, early diagnosis is critical. High-resolution techniques such as arthroscopy, radiography, and magnetic resonance imaging are currently utilized to monitor cartilage degeneration, but unfortunately these clinical techniques are still relatively qualitative and to date are insensitive to early degenerative changes.\(^10–12\)

The technique of FT-IR spectroscopy is a powerful tool to study the previously described changes in the molecular structure of degenerative cartilage.\(^13\) Advantages include rapidity, simplicity, nondestructive measurement, and the ability to measure samples directly without pretreatment or labeling. Recent studies from our laboratory have utilized an infrared fiber-optic probe (IFOP) to identify spectral molecular signatures associated with degenerative collagen in harvested osteoarthritic human cartilage\(^14\) and have also linked these spectral changes to enzymatic collagen damage.\(^15\) In the current study, we seek to advance that work by development of a spectral method that can rapidly distinguish classes of cartilage degradation. The widely used approach of chemometric analysis,\(^16–19\) statistical pattern-recognition methods to identify or classify materials based on their infrared spectral characteristics, was investigated as a method to automate cartilage analyses and to facilitate diagnosis of early-stage degeneration. This was accomplished by correlation with the current clinical standard for visual grading of cartilage during operative procedures, the Collins visual grading scale.\(^20\) A partial least squares (PLS1) regression method was used to correlate variations in the spectra with the corresponding cartilage Collins visual grading scale. A preliminary assessment of the feasibility of developing a similar method based on Mankin histological grading of the tissue was also investigated.

MATERIALS AND METHODS

Tissue Sampling. Sixty-one arthritic human tibial plateaus from male and female patients 46–87 years of age...
Fig. 1. Arthritic human tibial plateaus contain regions of tissues in varying states of degradation. The Collins visual scale grades tissues as 0, 1, 2, or 3, corresponding to (A) normal surface morphology, (B) slight superficial swelling or fibrillation, (C) deeper fibrillation, or (D) serious fibrillation or degeneration, respectively.

were obtained during knee replacement surgery under an IRB-approved protocol. Normal cartilage was obtained from donors with no documented history of joint disease, a 28-year-old female, a 58-year-old female, and a 65-year-old male immediately after death (National Disease Research Interchange, Philadelphia, PA) and were either snap-frozen in liquid nitrogen or stored immediately in DMEM solution and shipped overnight. The harvested osteoarthritic tibial plateaus were immediately taken to the lab from the operating room, and the cartilage surface was gently cleaned with 0.9% saline to remove the synovial fluid. A mid-infrared fiber optic probe (IFOP) (Remspec Corp, Sturbridge, MA) was used to collect infrared spectral data from specific regions of each specimen, visually identified and graded as Collins scale grade 0, 1, 2, or 3 corresponding to morphology that had a normal surface, slight superficial swelling or fibrillation, deeper fibrillation, or serious fibrillation or degeneration, respectively, as shown in Fig. 1. Since cartilage degenerative changes identified as Collins Grade 3 are later stage and macroscopically obvious, we limited the chemometric model to include the earlier degradative changes that are clinically more challenging to distinguish, cartilage grades 0, 1, and 2. Some tissue regions were also evaluated histologically, which involved extraction of the cartilage and underlying subchondral bone with a 5 mm diameter biopsy punch followed by processing of tissue in a mixture of 80% ethanol and 1% CPC (cetylpyridinium chloride). The fixed biopsies were embedded in paraffin, sectioned with a thickness of ~7 μm onto glass slides, stained with Alcian blue and H&E, and graded according to Mankin. The histological Mankin score is the standard grading scheme used for cartilage and incorporates grading based on structure fissuring, cell cloning, loss of proteoglycan, and tidemark integrity, where grade 0 represents normal cartilage and grade 14 represents severely degenerated cartilage. Two investigators evaluated randomized and blind-coded samples independently, and the final Mankin score was calculated as a mean of the two evaluations.

Infrared Fiber-Optic Probe Data Acquisition. The IFOP contains a flexible fiber-optic bundle composed of mid-infrared transmitting chalcogenide glass which transmits over the infrared region of 4000–900 cm$^{-1}$. The fiber
bundle is coupled to a Bruker (Billerica, MA) spectrometer with a mercury cadmium telluride (MCT) detector module. A ZnS attenuated total reflectance (ATR) crystal with a 1 mm diameter flat tip was attached to the end of the fiber-optic bundle (Fig. 2). This crystal design results in smaller displacements compared to traditional conically tipped crystals and produces negligible damage to cartilage cells and tissues. Contact between the ATR crystal and the tibial plateau cartilage was controlled by a 5-lb. load cell that uses a Wheatstone bridge, whereby load variation is converted into a change in electric signal that can be monitored on a load meter. The pressure was controlled at 0.7 lb and the infrared data were collected with a spectral resolution of 8 cm\(^{-1}\). Data collection was initiated after a period of \(\sim 60\) s of contact between the cartilage and crystal to enable the relaxation of the cartilage around the crystal tip. Spectral acquisition took approximately 1 min for 256 scans. Two to three regions from each tibial plateau were selected for sampling and three spectra/region were collected, for a total of 342 infrared spectra. The total number of spectra for each grade of cartilage was as follows: Grade 0, 27 spectra; Grade 1, 151 spectra; and Grade 2, 164 spectra.

**Chemometric Model.** A chemometric model was developed based on classification of cartilage degradation using the partial least squares (PLS1) regression algorithm provided as part of the QUANT II OPUS NT software (Bruker Optics, Billerica, MA). A leave-one-out cross-validation test of models built from un-preprocessed spectra was used to identify the optimal infrared spectral region based on the lowest root mean square error of cross-validation (RMSECV); the quality of the fit was also checked by evaluating the regression coefficient \(R^2\). Thirty-one spectra (exclusion rate, 31/342 = 9.1%) that were repeatedly predicted greater than 0.7 grades away from the actual Collins grade were treated as outliers and eliminated from the model. We then utilized 206 spectra as input for calibration to create the model and utilized 105 independent test spectra as input to compare the predicted grade of cartilage to the actual grade as determined visually. Several spectral regions were investigated to optimize the model. These included the OH stretching/amide A/C–H stretching region (3707–2748 cm\(^{-1}\)), amide I:C=O stretching (1733–1583 cm\(^{-1}\)), amide II:N–H bending and C–N stretching (1583–1482 cm\(^{-1}\)), side-chain bending and stretching (1482–1270 cm\(^{-1}\)), amide III:N–H bending and C–N stretching (1270–1134), and C–O stretching (1134–984 cm\(^{-1}\)). Several different preprocessing methods were also investigated, including straight-line subtraction, first derivative, second derivative, and vector normalization.

The reliability of the model was tested by evaluation of the standard error of calibration (SEC), the standard error of prediction (SEP), and the regression coefficient \(R^2\). The standard error of calibration (SEC), also called the root mean square error of calibration (RMSEC), was calculated as

\[
SEC = \sqrt{\frac{\sum_{i=1}^{n} (C_i - \hat{C}_i)^2}{n - h - 1}}
\]

where \(C_i\) is the actual Collins score, \(\hat{C}_i\) is the calibrated value, \(n\) is the number of calibration spectra, and \(h\) is the number of PLS factors. The standard error of prediction (SEP), also called the root mean square error of prediction (RMSEP), was calculated as

\[
SEP = \sqrt{\frac{\sum_{i=1}^{m} (C_i - \hat{C}_i)^2}{m}}
\]

where \(C_i\) is the actual Collins score, \(\hat{C}_i\) is the prediction value, and \(m\) is the number of prediction spectra. The regression coefficient \(R^2\) gives the percentage of variance present in the actual component values, which is reproduced in the regression. \(R^2\) approaches 100% as the fitted values approach the true value:

\[
R^2 = 1 - \frac{\sum_{i=1}^{q} (C_i - \hat{C}_i)^2}{\sum_{i=1}^{q} (\hat{C}_i - C_{mean})^2}
\]

where for calibration \(C_i\) is the actual Collins score, \(\hat{C}_i\) is the calibration value, \(q\) is the number of calibration spectra, and \(C_{mean}\) is the mean calibration value. For prediction, \(C_i\) is the actual Collins score, \(\hat{C}_i\) is the prediction, \(q\) is the number of prediction spectra, and \(C_{mean}\) is the mean prediction value.

**Statistical Methods.** One-way analysis of variance (ANOVA) followed by post hoc Bonferroni analysis was used to determine whether differences existed among mean values of actual or predicted Collins grade 0, 1, and 2. Significance was determined at the \(p < 0.05\) level.

**RESULTS**

Figure 3 illustrates a typical IFOP spectrum of cartilage acquired from a Collins grade 1 region from a human tibial plateau. Several different spectral regions were investigated by the PLS1 method and the sources of the molecular vibrations in each frequency range are listed in Table I. Qualitatively, the most obvious progressions of spectral changes correlated with visual cartilage grade were in the water OH stretching absorbance (broad feature centered at 3200–3300 cm\(^{-1}\)) and in the 1338 cm\(^{-1}\) region (Fig. 4). We can see that the water content increased as cartilage grade increased from 0 through grade 3, a finding likely related to the disruption of the collagen network and consistent with previous studies. The 1338 cm\(^{-1}\) collagen absorbance that arises from the CH\(_2\)
side-chain vibrations has previously been shown to decrease in intensity as the collagen denatures. 14, 24

The effects of different selections of spectral regions on regression coefficients from the PLS cross-validation model are also listed in Table I. The combination of regions B through F, 1736–984 cm⁻¹, gave the best regression coefficient of 0.8185, and this range was selected for use in building subsequent PLS1 models. Models for validation were built using 206 spectra for the calibration set and 105 independent spectra as a test set. The best results for spectral preprocessing were obtained using a straight-line subtraction, \( R^2 = 0.8185 \) (Table II).

When a PLS1 calibration model is based on a data set that exhibits even a moderate degree of correlation, it is expected that the RMSECV (in a leave-one-out cross-validation of the model) will decrease and the regression coefficient will increase as the rank (or the number of factors) of the PLS1 model increases, towards a limit where the RMSECV reaches a steady value (as shown in Fig. 5B). This is because each succeeding factor captures a decreasing amount of the spectral variation that shows a statistically significant association with cartilage degradation as measured by the Collins grade. Beyond a certain rank, the factors begin to model irrelevant aspects of the spectra, notably various types of noise. Most chemometric software will automatically “recommend” an optimum rank to be used in a given calibration, usually based on the rate of change in the RMSECV. In the present case the Quant2 recommendation was 18 factors, but we chose to use 15 factors based on a less conservative threshold for rate of change of RMSECV. The correlation between the calibrated values and the actual Collins grading scores was \( R^2 = 0.8185 \) with a standard error of calibration of 0.260. The model predicted 292 out of 311 spectra within ±0.5 of a Collins grade level, resulting in 93.8% correct. The predicted percentage for each grade is listed in Table III.

When the test set of 105 independent spectra was predicted against a chemometric model created with 206 spectra using a rank of 15, the correlation factor was 0.8228 and the RMSEP was 0.258 (Fig. 6). This result confirms the existence of some level of correlation between the mid-infrared spectrum and the quality of the cartilage in early stages of degeneration. In this model, we can predict 95.2% of the spectra within ±0.5 of a Collins grade level. The predicted percentage for each grade is shown in Table IV. In addition, each mean predicted value of grade 0, 1, and 2 was significantly different from the other predicted values.

In an attempt to further characterize, and possibly

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**TABLE I. Regression coefficients \( R^2 \) obtained with different spectral regions (PLS cross-validation method utilizing 311 human tibial plateau cartilage spectra).**

<table>
<thead>
<tr>
<th>Selected spectral region (cm⁻¹)</th>
<th>Molecular vibrations</th>
<th>Regression coefficient ( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 3707–2748</td>
<td>–OH stretching, amide A and C–H stretching (water, collagen, PG)</td>
<td>0.3309</td>
</tr>
<tr>
<td>B 1733–1583</td>
<td>Amide I C=O stretching (collagen, PG) and –OH bending (water)</td>
<td>0.5492</td>
</tr>
<tr>
<td>C 1583–1482</td>
<td>Amide II N–H bending, C–N stretching (collagen, PG)</td>
<td>0.3917</td>
</tr>
<tr>
<td>D 1482–1270</td>
<td>Side-chain bending and stretching (collagen)</td>
<td>0.5711</td>
</tr>
<tr>
<td>E 1270–1134</td>
<td>Amide III N–H bending, C–N stretching (collagen, PG)</td>
<td>0.3608</td>
</tr>
<tr>
<td>F 1134–984</td>
<td>C–O stretching (PG)</td>
<td>0.1624</td>
</tr>
<tr>
<td>Combined A–F</td>
<td>3707–984</td>
<td>0.5250</td>
</tr>
<tr>
<td>Combined B–F</td>
<td>1733–984</td>
<td>0.8185</td>
</tr>
<tr>
<td>Combined D–F</td>
<td>1482–984</td>
<td>0.7435</td>
</tr>
</tbody>
</table>

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**TABLE II. Regression coefficients \( R^2 \) obtained with different spectral preprocessing methods (PLS cross-validation method utilizing 311 human tibial plateau cartilage spectra).**

<table>
<thead>
<tr>
<th>Spectral preprocessing method</th>
<th>Regression coefficient ( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>No preprocessing</td>
<td>0.7860</td>
</tr>
<tr>
<td>Straight-line subtraction</td>
<td>0.8185</td>
</tr>
<tr>
<td>Vector normalization</td>
<td>0.7654</td>
</tr>
<tr>
<td>1st derivative</td>
<td>0.6838</td>
</tr>
<tr>
<td>2nd derivative</td>
<td>0.7725</td>
</tr>
<tr>
<td>Straight line + 1st derivative</td>
<td>0.6929</td>
</tr>
<tr>
<td>Vector normalization + 1st derivative</td>
<td>0.6697</td>
</tr>
</tbody>
</table>
eventually improve, the predictive ability of the mid-infrared spectrum versus cartilage quality, histological evaluation of a subset of the cartilage samples was performed. This resulted in a correlation of $R^2 = 0.8408$ between true Mankin histological grade data and PLS1-predicted grade using only eight factors (Fig. 7). Thirty-two out of 33 spectra (97%) were predicted within ±2 true histological grades, and 25 out of 33 spectra (76%) were predicted within ±1 true histological grades. While this is a limited test with only 33 spectra from 18 Mankin-graded tissues, it gives a strong indication that the mid-infrared spectra of cartilage will correlate with the histological Mankin grade of the tissue as well as with the Collins visual grade.

### DISCUSSION

The current study lays promising groundwork for the use of mid-infrared spectroscopy to determine the degree of cartilage degradation by correlation of infrared spectra with the Collins visual grade or with the Mankin histological grade in early-stage degradation. The development of this IFOP-based methodology has several advantages over the current standard visual grading method by providing a rapid (less than one minute for data acquisition), objective determination of cartilage quality that can be performed in a minimally invasive fashion in conjunction with arthroscopy. The Collins scale is essentially an ordinal scale based on four ranks of degradation, and the results described above illustrate that there is a statistically significant correlation between the IFOP spectrum and the Collins grade. With further work to develop and test the histology-based calibration, it may be possible to develop a minimally invasive cartilage evaluation technique that is usable in the operation room and that could be superior to visual evaluations using the Collins scale.

The use of noninvasive or minimally invasive techniques for evaluation of cartilage quality is very desirable from a clinical point of early intervention and of assessment of repair cartilage. Arthroscopy, a minimally invasive technique, is frequently employed to visually determine cartilage quality with osteoarthritic conditions of the knee in conjunction with debridement and lavage procedures to remove damaged cartilage. It is estimated that at least 65,000 such procedures are performed each year in the United States. Nevertheless, the effectiveness of these procedures is controversial and the precise mechanism by which relief is provided has not been established conclusively. It is possible that since arthroscopic visual grading accounts only for the surface physical appearance of the cartilage, there may be tissue that visually appears normal but actually has some early-stage pathology. Thus, incomplete removal of damaged tissue may occur. The apparent ability of the IFOP chemometric method to discern cartilage in an early stage of degeneration, even prior to visual changes, is likely attributable to the detection of subtle molecular changes in the cartilage structure.

Magnetic resonance imaging (MRI) is well known as a reliable and accurate noninvasive diagnostic tool for the assessment of mid- to later-stage osteoarthritis. To date,
MRI techniques have been utilized clinically primarily for assessing cartilage volume and thickness, with controversial results surrounding its use for clinical evaluation of cartilage composition. Only with a high field strength magnet, such as that used for in vitro and in vivo animal studies by Xia et al. and by Wachsmuth et al., can quantitative information on structural or compositional cartilage changes be provided. However, non-invasive assessment of early cartilage degeneration by this method is still not possible in a clinical setting.

Another advantage of the use of mid-infrared spectroscopy for cartilage evaluation is that spectral features can be directly examined to assess changes in tissue components in combination with multivariate methods. Recently, the evaluation of cartilage degradation by IFOP-derived spectral features from harvested human osteoarthritic cartilage was carried out in our laboratory. In the current study, the finding of a decreased 1338 cm⁻¹ band with degeneration of cartilage (Fig. 4) is consistent with those previous results. Moreover, a qualitative increase in the presence of water in more severely degenerated cartilage was found in the current study, an observation not previously described by IFOP determination. It is well known that the cartilage tissue undergoes biochemical alterations, including a loss of PGs and a concomitant increase in water content, as OA progresses. The water increase has been attributed to swelling or to breakdown of the collagen network that may occur before macroscopic structural changes appear, and could provide a possibility for early detection of OA by IFOP. However, the fact that exclusion of the water OH stretching region near 3300 cm⁻¹ from our calibration model resulted in higher correlation coefficients (Table I) suggests that there are other factors that contribute to that spectral feature, such as the amide A/C–H stretching vibrations that overlaps with that broad spectral envelope. There is also an absorbance from the water O–H bending vibration that overlaps with the amide I and amide II spectral regions, as described in our earlier study. In contrast to the water O–H stretching vibration region, however, inclusion of this lower frequency spectral range was critical in the current chemometric analysis. This indicates that changes in this spectral region, whether attributable to water or to the amide protein absorbances, were correlated with cartilage quality. From this data, we cannot conclude definitively which of these, water or protein (primarily collagen), change in a correlative fashion with cartilage quality, but it is likely that there are contributions from both components. Thus, further studies to evaluate the possible use of IFOP data to determine water content in cartilage as an indicator of pathology are certainly warranted.

The PLS1 regression method can be used as a “full spectrum method”, using information from all frequencies included in the spectral range, and the chemometric model developed should improve with an increasing number of data points if we assume that each point in the spectrum contains relevant information. However, in many real-world cases spectral noise or additional, irrelevant, components present in the samples may degrade the PLS1 model. In the current study, it was necessary to limit the frequency range to the most information-rich range of the spectrum to obtain the most useful result. The best PLS1 calibration utilized the spectral ranges 1733–984 cm⁻¹, a region that contains molecular information from both primary components of cartilage, collagen, and proteoglycan. The rank of the PLS1 model was another important factor in optimization. In our model, a small number of PLS1 factors resulted in a higher error of prediction, and addition of factors lowered the error substantially. It is also well known that in some studies, the system can be over-modeled with unnecessary increases in PLS1 factors. This results in a diminished robustness of the model. Though in most cases less than 10 factors are needed in a chemometric model, in biological systems as many as 14–16 factors have been used to calibrate the model due to the complex nature of biological samples. In the current study, in which we use 15 factors, the standard error of prediction, 0.258, is still low enough to allow for unambiguous assignment of a Collins grade to each spectrum, in spite of the scatter in the data. Ideally, we would like to predict the grade of cartilage with a SEP lower than 0.1, especially in the early stages of degeneration. We believe, however, that this limitation in our model arises from the limitation of the standard we are using, i.e., the Collins visual scale, which is somewhat subjective.

While the results discussed above are generally encouraging, there are, as mentioned, limitations to the utility of the calibrations described in this paper. It has been possible to show that there are statistically significant differences that can be used to distinguish one visual grade from another, and the method is advantageous in its objectivity, but the Collins grading system is limited to four classification grades at the macroscopic level. We have therefore turned our attention to the possibility of using histological evaluation of cartilage, a more detailed classification system, as the primary method against which spectroscopic measurements will be calibrated.
ity to determine the histological grade of cartilage by a simple, in vivo test would be of great utility. Furthermore, the results from the Quant2 model using the histological grade as the primary calibration parameter strongly suggested that it will be possible to develop robust and useful calibrations using histological data.

In summary, the current study has clearly laid the groundwork for use of the IFOP-based determination of cartilage quality for in vivo quantification of early cartilage matrix degradation. The continued development of a minimally invasive method that could be used for early OA detection, and possibly for the monitoring of repair tissue, has great clinical value towards the management of OA and other joint diseases.

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