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Improved specificity of cartilage matrix evaluation using multiexponential transverse relaxation analysis applied to pathomimetically degraded cartilage

David A. Reiter¹, Remigio A. Roque¹, Ping-Chang Lin¹, Stephen B. Doty², Nancy Pleshko³, and Richard G. Spencer¹

¹Magnetic Resonance Imaging and Spectroscopy Section, National Institute on Aging, National Institutes on Health, Baltimore, MD 21224

²Research Division, Microscopy Core Facility, Hospital for Special Surgery, New York, NY 10021

³Department of Mechanical Engineering, Temple University, Philadelphia, PA 19122

Abstract

Noninvasive early detection of specific matrix alterations in degenerative cartilage disease would be of substantial use in basic science studies and clinically, but remains an elusive goal. Recently-developed MRI methods exhibit some specificity, but require contrast agents or nonstandard pulse sequences and hardware. We present a multiexponential approach which does not require contrast agents or specialized hardware, and uses a standard multiple-echo spin-echo sequence. Experiments were performed on tissue models of degenerative cartilage using enzymes with distinct actions. MR results were validated with histologic, biochemical, and infrared spectroscopic analyses. The sulfated glycosaminoglycan (sGAG) per dry weight (dw) in bovine nasal cartilage (BNC) was 0.72 ± 0.06 mg/mg dw and was reduced through chondroitinase AC (ChAC) and collagenase digestion to 0.56 ± 0.12 and 0.58 ± 0.13 mg/mg dw, respectively. Multiexponential analysis of data obtained at 9.4T permitted identification of tissue compartments assigned to the proteoglycan (PG) component of the matrix and to bulk water. Enzymatic treatment resulted in a significant reduction in the ratio of PG-bound to free water from 0.13 ± 0.02 in control cartilage to 0.03 ± 0.02 and 0.05 ± 0.06 under ChAC and collagenase treatment, respectively. As expected, monoexponential T_2 increased with both degradation protocols, but without further specificity to the nature of the degradation. An important eventual extension of this approach may be to map articular cartilage degeneration in the clinical setting. As an initial step towards this, localized multiexponential T_2 analysis was performed on excised bovine patella. Results obtained on this articular cartilage sample were readily interpretable in terms of PG-associated and relatively free water compartments. In potential clinical applications, SNR constraints will define the threshold for detection of macromolecular compartment changes at a given spatial scale. The multiexponential approach has potential application to the early detection of cartilage degradation with use of appropriate pulse parameters under high SNR conditions.

Keywords

cartilage MRI; transverse relaxation; multiexponential T_2 ; proteoglycan; cartilage degradation

Introduction

Current approaches to the detection of cartilage degeneration with MRI (1–3) rely upon the association of MR outcome measures with the macromolecular and water content of the tissue, but exhibit limited specificity (4–7). Improved specificity is necessary for early detection of disease and has been attempted through the use of exogenous contrast agents and specialized MR pulse sequences and hardware (8–10). We describe a multiexponential T_2 analysis approach for identification of proteoglycan (PG)-associated and relatively mobile water compartments in cartilage that requires no contrast agent and uses the standard multiple echo pulse sequence.

Cartilage is composed of 65–80% water and 18–35% extracellular matrix by wet weight (11), with the predominant matrix constituents being type II collagen and PG. Collagen-associated water exhibits rapid transverse relaxation, (12, 13), with T_2 reported to range from ~30–100 μ s (14, 15). Water associated with PG and protons within the PG molecule are more mobile, with T_2 s in the range of 15–80 ms (14). The polydispersity of PG monomers and side chains is expected to result in multiple T_2 components within that range. The less bound water within cartilage exhibits longer T_2 's ~ 100 ms and contributes preferentially to the monoexponential T_2 value.

Identification of water fractions using multiexponential analysis permits a relatively direct assay of PG-associated water as a proportion of more mobile water. As in our previous work we use bovine nasal cartilage (BNC) as a model system due to its largely isotropic and homogeneous structure (16) which permits more reliable interpretation of bulk sample measurements. However, the present study greatly extends our previous work (16) in several important ways. First, we subjected cartilage to two distinct enzymatic treatments: chondroitinase AC (ChAC), acting primarily on glycosaminoglycan (GAG) chondroitin sulfate side-chains; and collagenase CLSPA, a highly purified collagenase which denatures the collagen triple helix and promotes PG loss without necessarily resulting in substantial collagen loss. Second, MR results were supported by biochemical evaluation of sulfated glycosaminoglycans (sGAG), histology of collagen and PG, and Fourier transform infrared imaging spectroscopy (FT-IRIS) to quantify the spatial distribution of collagen and PG (17). Finally, in addition to bulk measurements on BNC, we present results from an imaging experiment on native and trypsin-degraded articular cartilage from the bovine patella; trypsin cleaves attachment sites and the core protein of PG monomers resulting in PG loss. The longer TE of the imaging sequence precluded the ability to detect all of the compartments detected by the non-localized measurements of BNC, but demonstrates the ability to detect the spatial variation of PG within cartilage and indicates the potential to develop MR-based matrix component-specific imaging.

The multiexponential analysis was performed using non-negative least squares (NNLS). Extensive NNLS analysis of simulated noisy relaxation data corresponding to both non-localized and localized acquisition conditions was performed to support our experimental results, since acquisition parameters, signal-to-noise ratio (SNR), and tissue T_2 properties all affect the accuracy of identified T_2 components (16, 18–20).

Methods

Sample Harvest and Preparation

BNC plugs—Plugs (8 mm diameter) were excised from the nasal septa of mature cows (Green Village Packing, Green Village, NJ, USA), moistened with Dulbecco's phosphate buffered saline (DPBS) (Invitrogen, Carlsbad, CA, USA) and stored at 4° C. Samples

subjected to MR analysis were analyzed biochemically, with parallel samples being used for histology and FT-IRIS.

Bovine patella—A patella was excised from the stifle joint of a skeletally mature young cow (~3 months old; Green Village Packing) and kept moist in DPBS at 4° C.

Enzymatic Treatment

BNC—Samples were assigned to a control group or incubated at 37° C in 2.5 mL of DPBS containing one of two enzymes. ChAC group: bath containing 0.1% bovine serum albumin and 0.4 M sodium acetate, and 0.033 U/ml of ChAC (Seikagaku Corp., Tokyo, Japan) for 1 h, or 0.1 U/ml for or 0.033 U/ml of ChAC for 2, 6, or 24 h. Collagenase group: bath containing 30 U/ml collagenase CLSPA (Worthington, Biochemical Corp., Lakewood, NJ, USA), supplemented with Ca²⁺ to a final concentration of 200 mg/mL for 4, 10, or 16 h. Four samples were assigned to each time-point. Samples were washed with DPBS solution after degradation.

Bovine Patella—The patella was cut in half axially, with one half immersed in control solution and the other in a solution containing 1 mg/ml trypsin at 37°C, for 7 hrs. The halves were then cemented back together using dental plaster to permit simultaneous imaging of the control and degraded halves.

Biochemical Analysis for sGAG Content

BNC samples were weighed wet, dried overnight in a vacuum at room temperature, and again weighed prior to digestion in buffer containing 1 mg/ml proteinase k (Sigma-Aldrich, St. Louis, MO, USA) and 100 mM ammonium acetate. Digests were analyzed using a 1,9-dimethylmethylene blue assay (DMMB) to determine sulfated glycosaminoglycan content (21). A SPECTRAmax 340PC³⁸⁴ spectrometer (Molecular Devices, Sunnyvale, CA, USA) was used to measure absorbances at 525 nm which were converted to sGAG content through comparison with chondroitin sulfate C standards (Sigma-Aldrich, St. Louis, MO, USA).

Histology

Samples were fixed in 10% neutral-buffered Formalin (Sigma-Aldrich, St. Louis, MO, USA) for approximately 12 hours with agitation, rinsed with DPBS and stored in 35% EtOH (Sigma-Aldrich, St. Louis, MO, USA). Samples were then embedded in paraffin and sliced into 6 µm sections perpendicular to the face of the discs. Sections were stained with alcian blue for proteoglycans (22) and picro-sirius red (PSR) for collagen (23).

Fourier Transform Infrared Imaging Spectroscopy (FT-IRIS)

Six micron thick sections of paraffin-embedded BNC samples were examined on MirrIR low-e glass slides (Kevley Technologies, Chesterland, Ohio). The side onto which the sample is affixed is silvered, rendering it reflective to infrared radiation, while the slide remains transparent to visible light.

Data were collected with a Spectrum Spotlight FT-IR imaging system (Perkin-Elmer, Bucks, UK) (24) with 8 cm⁻¹ spectral resolution. A mid-IR spectrum from 800 to 4000 cm⁻¹ was obtained from each 25 × 25 µm imaging pixel.

The amide I and proteoglycan sugar band areas were calculated by integrating over the ranges 1592–1744 cm⁻¹ and 960 – 1184 cm⁻¹, respectively, after baseline subtraction (17, 24) and used to generate maps of collagen and proteoglycan (ISys software v3.1, Spectral Dimensions Olney, MD, USA).

NMR and MRI Methods

Each sample was placed into an NMR tube containing Fluorinert FC-77 (catalog number F-4758, Sigma-Aldrich, St. Louis, MO, USA) to maintain sample hydration and eliminate MR signal contamination by bath solution. Data were acquired at 4° C with a 9.4 T vertical bore Bruker DMX NMR spectrometer (Bruker Biospin, GmbH, Rheinstetten, Germany).

BNC—Relaxation data were obtained with a 10 mm Helmholtz coil using a nonlocalized CPMG pulse sequence with a two-step phase cycle scheme $90_{(x, x)}/180_{(y, -y)}$ and with sampling of a single point from the top of each echo, with TE/TR = 600 μ s/10 s, 2048 echoes, and number of excitations (NEX) = 64. SNR ranged from 17,000 to 154,000. TE was set to ~10 times the pulse length to avoid spin locking effects. Even echoes were used resulting in an effective TE = 1.2 ms and 1024 echoes.

Patella—Localized relaxation data were obtained with a 25 mm resonator using a single slice multi-echo CPMG imaging sequence with a two-step phase cycle scheme $90_{(x, -x)}/180_{(y, y)}$ and with TE/TR = 5.5 ms/5 s, 128 echoes, slice thickness = 3 mm, FOV = 3×3 cm, matrix = 128×128, and NEX = 32. The approximate SNR was 1,300. Even echoes were used resulting in an effective TE = 11 ms and 64 echoes. We acquired only a single slice through the isocenter of the coil, effectively eliminating any off-resonance saturation and thus avoiding magnetization transfer effects.

Fitting of T₂ Relaxation Data

The non-negative least squares (NNLS) approach was used for multiexponential T₂ analysis as previously described (16). The NNLS procedure makes no *a priori* assumptions about the number of relaxation components present. A set of 80 possible T₂ values were logarithmically spaced over the interval [0.1, 3000] ms. We defined an optimal NNLS regularizer, μ , by the condition that chi-squared (χ^2) following regularization was 101% of the non-regularized χ^2 (20). The resulting distribution of T₂ values was interpreted in terms of matrix composition. The first moments and associated fractions for each T₂ component were calculated. Relaxation data were also fit using a nonlinear least square algorithm in order to obtain conventional monoexponential T₂ values for comparison to the multiexponential results. All fitting routines were implemented in MATLAB (MathWorks, Natick, MA, USA).

Simulation of T₂ Relaxation Data

Analysis of simulated data using the parameters corresponding to the nonlocalized experiments, TE = 1.2 ms and 1024 echoes, as well as with TE = 11 ms and 64 echoes corresponding to the localized experiments, was performed to ensure the reliability of our results. Data were simulated based on the average experimental values for the component T₂'s and weights (see below) using the following expression (16):

$$y(n \cdot TE) = B + y_0 \sum_{m=1}^M w_m \cdot e^{-(n \cdot TE)/T_{2,m}} + \varepsilon(0, \sigma), \quad [1]$$

with $y(n \cdot TE)$ the amplitude of the n^{th} echo, B a baseline offset, y_0 the overall signal amplitude, w_m the fractional weight of the m^{th} T₂ component, and $\varepsilon(0, \sigma)$ additive Gaussian noise with mean 0 and standard deviation σ . SNR, defined as y_0/σ , was set to either 17,000 or 500, the minimum values obtained in our non-localized and localized experiments, respectively. The reliability, accuracy, and precision of the results for component T₂s and weights were evaluated over 100 trials for each SNR value. Reliability was defined as correctly identifying the number of T₂ components, accuracy was defined by percent error

of the derived T_2 s and weights, and precision was defined as the coefficient of variation (CV) of the identified component T_2 values and associated weights over the 100 trials.

Statistical Analysis

Experimental results are reported as mean $\pm$ SD. Differences between control and degraded samples were evaluated using ANOVA and post-hoc testing (SigmaStat, Systat Software Inc., San Jose, CA).

Results

Simulations

BNC showed four components in control samples, labeled C_a , C_b , C_c , and C_d . Table 1 shows the accuracy and precision, respectively, for the major relaxation components, C_c and C_d , determined from simulations based on our minimum non-localized experimental SNR of 17,000. Input values of w_c/w_d corresponded to experimentally-derived results. T_2 s and weights were accurate to within 10% of the input value with the exception of $T_{2,c}$ and w_c in the ChAC group, reflecting the small w_c value. Precision in parameter determination was within ~6–8% in all cases, except for w_c and w_d in the collagenase group, which were ~25%. This resulted from a large variation in w_a , attributed to its small value in the presence of a fifth component, C_e . These calculations were all based on our minimum experimental SNR of 17,000; when using the mean experimental SNR of 78,000, the precision for the weights from the collagenase group improved to ~14%.

Figure 1 indicates the dependence of errors in w_c and w_d as a function of their ratio. The error in the larger component, w_d does not depend on w_c/w_d over the range illustrated, while the error in the smaller component, w_c , is greatly reduced as its relative amplitude is increased.

Reliability was 96%, 100%, and 86% for the minimum non-localized SNR of 17,000 for control, ChAC, and collagenase groups, respectively. Using average, rather than minimum, SNR values, all groups showed 100% reliability, indicating the robustness of identifying the correct number of components.

Simulations with SNR and acquisition parameters representative of localized bovine patella images showed reliability of 100%, with accuracy and precision of component T_2 s of 12% and 5%, respectively, and accuracy and precision of magnetization fractions of 19% and 6% (data not shown).

Bovine Nasal Cartilage

No significant differences were found in biochemically- or FT-IRIS-derived matrix components in BNC between treatment durations. Results for each enzyme are presented in Table 2. Water content per wet weight increased with degradation by ChAC while both enzymatic treatments resulted in a statistically significant reduction in biochemically-determined sGAG per dry weight. FT-IRIS analysis was consistent with these results. FT-IRIS-determined collagen content was not reduced significantly in either of the degradation groups. The PG-to-collagen ratio showed a trend to decrease with ChAC ($p = 0.14$) and collagenase ($p = 0.17$) treatment.

Figure 2 shows representative alcian blue and PSR staining. Digestion with ChAC and collagenase resulted in moderate and substantially non-uniform loss of PG.

Corresponding FT-IRIS images (Fig. 2) again indicate that the distribution of PG and collagen were fairly uniform in the control tissue, with modest regional variation of PG loss

seen in the ChAC-degraded sample, consistent with the alcian blue staining. The collagenase-treated sample shows regions of more substantial PG loss in both the alcian blue and FT-IRIS images. Increased collagen concentration in these regions is evident in both the PSR and FT-IRIS images; we attribute this to local collapse of collagen matrix in the absence of PG.

Representative monoexponential T_2 s and multiexponential T_2 distributions are also shown in Figure 2. Monoexponential T_2 increased similarly for each intervention indicating sensitivity but lack of specificity to degradation. In contrast, multiexponential analysis permits separate visualization of the most mobile water component (C_d) and of the more restricted, PG-associated, C_c . The ratio w_c/w_d decreased in the ChAC-treated sample due primarily to a decrease in w_c . Collagenase treatment showed a T_2 distribution similar to that of ChAC, with the emergence of an additional slowly relaxing C_e .

Table 3. shows results of monoexponential T_2 fits and select multiexponential T_2 s and fractions; as with biochemistry, these results did not significantly differ by treatment duration so further analysis was performed on the basis of treatment type only. Monoexponential T_2 increased significantly in all degradation groups. Multiexponential analysis yielded reliable identification of up to 5 components, as illustrated by the representative results shown in Fig. 2. We show averaged values for the three more slowly relaxing components, C_c , C_d , and C_e , in Table 3, and provide illustrative results in the fifth column of Figure 2. The rapidly relaxing components C_a and C_b are described more qualitatively in the text. $T_{2,c}$ was unchanged in ChAC- and collagenase-treated samples; since neither of these are expected to increase PG side-chain mobility, this supports the assignment of C_c to side-chain associated water. The small, slowly relaxing C_e developed in all of the samples exposed to collagenase for longer than 4 hours. $T_{2,d}$ increased significantly with ChAC and showed a trend ($p = 0.058$) towards an increase with collagenase treatment, consistent with increased mobility of the water pool loosely associated with matrix macromolecules. The ratio w_c/w_d was reduced in the ChAC- and collagenase-treated samples.

C_a and C_b consistently appeared in all control samples with T_2 s of $\sim 830 \mu s$ and $\sim 11 ms$, and weights of ~ 0.025 and ~ 0.034 , respectively. The appearance of C_a and C_b in treated samples was more variable.

C_b was detected in only 3 of 16 samples treated with ChAC; values for w_b were approximately half as large as in controls, while $T_{2,b}$ was comparable to control values. This consistent finding of C_b in controls, and its much reduced presence in ChAC-treated samples, suggests the association of C_b with PG side-chain water. Values for $T_{2,a}$ and w_a in the ChAC group were similar to control values, suggesting that this component is associated with PGs that are not readily affected by ChAC.

In collagenase-treated samples, w_a and $T_{2,a}$ increased with duration of enzyme exposure. Consistent with histology and FT-IRIS findings, collagenase resulted in moderate PG loss without loss of collagen, reflecting the known action of purified collagenase to denature collagen (25). This suggests that C_a is related to the mobility of collagen, consistent with an association of C_a with small PGs anchored in the collagen molecule. C_b was detected in 9 of 12 samples, with $T_{2,b}$ increasing, and w_b decreasing, with treatment duration. Given the reduction in PG in these samples, this further supports the association of C_b with PG side-chain water.

Bovine Articular Cartilage

Localized multiexponential T_2 results are shown in Figure 3, where average signal intensity within the indicated regions of interest (ROIs) were used for mono- and multiexponential T_2 analysis. Surface ROIs show larger $T_{2,mono}$ in the trypsin-treated cartilage while deeper ROIs showed little difference, reflecting surface damage. Multiexponential T_2 analysis showed two components, corresponding to C_c and C_d in BNC. In control patella, the differences (Δ) between the surface and deeper ROI were substantially larger for the more slowly relaxing component ($\Delta = 61$ ms) compared to the rapidly relaxing component ($\Delta = 5$ ms), indicating the importance of free water in defining the bulk T_2 of cartilage. The corresponding differences between the trypsin-treated ROIs were $\Delta = 114$ ms and $\Delta = 51$ ms, respectively, showing the increased sensitivity of multiexponential T_2 analysis to degradation. When comparing the surface tissue between trypsin-treated and control tissue, multiexponential T_2 analysis also showed large differences between the rapidly relaxing ($\Delta = 56$ ms) and slowly relaxing ($\Delta = 51$ ms) components.

Discussion

Assessment of cartilage status through measurement of monoexponential T_2 s has important limitations. For example, T_2 differences and changes are seen in a wide variety of settings, as a function of gender, joint loading, pathology, and distance from articular surface (6, 26–29). However, since monoexponential T_2 analysis provides only a single outcome parameter, the specific and potentially differentiating features of these cannot be assessed. In contrast, multiexponential analysis provides several T_2 values and corresponding weights that can be interpreted in terms of microstructure such as water compartmentation, macromolecular fragmentation, and tissue porosity. Although absolute quantification of component T_2 s and weights is of interest, a significant amount of information is available even from observation of approximate values, as we have demonstrated. Further, monoexponential T_2 analysis is highly non-specific and depends on hydration, macromolecular content, and tissue anisotropy, with comparable T_2 changes having been detected in many disparate settings (5, 26–28, 30, 31). In contrast, multiexponential analysis provides much more specific insight into matrix status. In addition, the applicability of this approach is by no means limited to articular cartilage, but can be extended to other cartilaginous tissue. In particular, multiexponential T_2 analysis has provided substantial additional insight into the decrease in monoexponential T_2 seen with degeneration of the intervertebral disc (32).

Previous investigations of multiexponential T_2 decay in articular cartilage have used non-localized measurements (33, 34). However, the layered organization of articular cartilage complicates the interpretation of these results in terms of co-localized tissue compartments. Therefore, here and previously (16) we have emphasized the analysis of BNC, which lacks significant layered organization but has comparable matrix composition to articular cartilage. In addition, we have previously reported maps of the PG-associated water compartment in BNC and patellar cartilage, and illustrated the correspondence of these results with FT-IRIS maps and with known properties of articular cartilage (35). However, an important application of the multiexponential analysis presented here will be in defining the matrix components of degenerative articular cartilage. Therefore, we also applied a localized version of multiexponential analysis to control and degraded bovine patellar cartilage through acquisition of multi-echo imaging data as a demonstration of this capability. Our results were in full accord with the known properties of this articular cartilage and with the properties of trypsin.

Simulations

Simulation analysis is essential to assess the reliability of identified components (16, 20) and the effect of regularization (36). Accuracy depends upon SNR, echo time, number of echoes, relative component amplitudes and T_2 s (20). Analysis of simulated multiexponential T_2 decay data allowed us to identify acquisition parameters permitting identification of T_2 components to a specified degree of accuracy. We believe that simulations are critical for reliable interpretation of multiexponential results.

Several approaches have been used to evaluate the influence of acquisition parameters on biexponential fit quality in tissue (18, 19). Our simulation results are consistent with previous studies. For example, fitting errors in the larger, more slowly relaxing component, C_d , were smaller than in C_c in control and ChAC samples.

Based on experimental results, we used 4, 3, and 5 input components respectively in simulations of control, ChAC, and collagenase groups. The reliability and accuracy of the NNLS analysis decreased with increasing number of input components, with markedly lower precision seen in the collagenase group for the quantification of w_c and w_d compared to controls. Similarly, the 5th relaxation component seen in the collagenase group limited the ability to reliably characterize w_c and w_d in comparison to the control group. The accuracy of the simulations for the ChAC group was limited due to the relatively small magnetization fraction w_c . This effect is illustrated in Figure 1, showing a rapid decrease in the accuracy of w_c with decreasing w_c/w_d .

Bovine Nasal Cartilage

Use of different enzymes permitted us to evaluate the specificity of multiexponential T_2 analysis. ChAC resulted in a loss of ~21% sGAG as measured biochemically, but, consistent with its known action, no apparent loss of collagen. Nonuniformity of PG loss throughout the sample was demonstrated by histology and FT-IRIS. This would be expected to result in isolated compartments of relatively slowly relaxing water. Indeed, the T_2 distribution showed a decrease or loss of w_b and decreased w_c/w_d , consistent with the incorporation of C_b into a compartment of more mobile water, such as C_c or C_d , and a decreased ratio of PG-bound to free water.

Collagenase denatures the collagen helix rendering it more susceptible to other proteinases (25). The purified collagenase we used contained a minimal amount of exogenous proteinases, so loss of collagen was not expected, as was confirmed by FT-IRIS measurements. However, the disruption of the collagen helix would be expected to result in loss of PG, as was observed (37). The loss of PG with collagenase treatment was predominantly seen as reduced sGAG near sample surfaces in the alcian blue and FT-IRIS images in contrast to PG loss in the ChAC-treated samples which was distributed throughout the sample (Fig. 2). The results for collagenase treatment showed a reduction in w_c/w_d , that is, in the ratio of PG-associated water to relatively free water, similar to that resulting from ChAC degradation, but additionally, a more slowly relaxing C_e was seen with collagenase (Fig. 2, Table 3). This is consistent with the development of a pool of water within PG-depleted regions of matrix that is only weakly interacting with surrounding denatured collagen. Finally, the increase in T_{2a} with collagenase was not observed with ChAC, suggesting a relationship between C_a and collagen mobility, based on the known action of collagenase to denature collagen (25).

Zheng et al. have also recently evaluated T_2 relaxation in BNC (38), finding a single water compartment, in contrast to the multiple components reported in our work. However, Zheng et al. performed their studies on cartilage that underwent a cycle of freezing and thawing prior to NMR measurements, in contrast to our use of fresh samples. However, as noted by

several authors (39–41) freezing and thawing can have profound effects on tissue. We specifically studied the effect of freezing and thawing in the context of T_2 relaxation and found that it consistently and substantially reduced the size of w_c , the dominant PG-associated compartment, by up to 50% (42), resulting in a component fraction of ~3%. This is comparable to the cut-off used by Zheng et al. for excluding T_2 components (38). This suggests the importance of careful sample handling, and, in particular, avoidance of frozen storage, when evaluating water compartments in cartilage.

Bovine patellar cartilage

The longer TE required for spin-echo imaging as compared to non-localized measurements leads to attenuation or loss of T_2 components with $T_2 < TE$, and indeed, C_a and C_b were not observed in the patella with our effective TE of 11 ms. Simulations demonstrated the ability to accurately detect the components corresponding to C_c and C_d in BNC. These are arguably the most important, as they reflect the majority of the PG-bound and relatively mobile water components.

We found consistency between the localized multiexponential T_2 results obtained on the patella and the non-localized results for BNC in control samples. Multiexponential T_2 analysis revealed two peaks in patellar tissue with magnetization fractions and T_2 s comparable to those of C_c and C_d in BNC. The ratio w_c/w_d in control tissue was generally lower in the patella, consistent with the lower PG content in articular cartilage.

In the control patella, $T_{2,mono}$ is larger in the superficial ROI than in the deeper tissue; this is consistent with previous observations of the spatial variation in T_2 across articular cartilage (43). Multiexponential T_2 analysis shows that this difference is due to the slowly relaxing T_2 component, with little depth dependence seen between the T_2 s of the rapidly relaxing components. Further, changes in the rapidly relaxing T_2 component upon degradation, generally not discernable in monoexponential analysis, are evident in the superficial ROIs, with the amplitude and T_2 of the rapidly relaxing component greatly increasing with digestion. These results highlight the improved specificity of multiexponential T_2 analysis as compared to conventional monoexponential analysis.

Furthermore, multiexponential T_2 analysis shows improved sensitivity to degradation. While the monoexponential T_2 increased with degradation in the bovine patella, the increase in the T_2 of the dominant, slowly-relaxing superficial ROI relaxation component was substantially larger, and the relative increase in the rapid T_2 component was much larger still. This improved sensitivity may be particularly important for detection of the early stages of cartilage degeneration, for which the changes in $T_{2,mono}$ are notably small (44).

Limitations

A number of complexities remain in relating multiexponential T_2 -defined magnetization fractions to cartilage matrix. Difficulty in detecting of rapidly relaxing components, with $T_2 < TE$, limits the identification of collagen-associated water in localized multiexponential T_2 measurements. For instance, changes in collagen-bound water fraction have been observed in collagen-based parchments using solid-state probes which afford much shorter pulse lengths and echo times (45). Shorter echo spacing may also be addressed by implementation of projection-based imaging methods, again with suitable NMR probes. In addition, the effect of chemical exchange between compartments could modify apparent T_2 s and compartment sizes, to an extent varying with degradation (46, 47). However, this does not prevent the identification of macromolecular and water compartments. A potential limitation to localized multiexponential T_2 measurements is their markedly lower effective SNR as compared to bulk measurements. However, we have demonstrated that with careful selection

of acquisition parameters based on simulations, meaningful results can be obtained. The simulations presented here correspond to our experimental conditions and the tissue types under investigation. In other settings, additional simulations corresponding to the relevant spectroscopic or imaging parameters and tissue characteristics must again be performed. In addition, we note that while the use of multiple enzymatic treatments allowed for preliminary assignment of T_2 components to particular macromolecular and water compartments in this study, confirmation of these assignments is ongoing.

We have previously reported simulation results corresponding to in vivo imaging conditions (16) that demonstrate accuracy and precision adequate to detect the differences in w_c/w_d observed in the present work. Based on this, we expect that our multiexponential approach can be extended to a full imaging implementation, with of course SNR constraints defining the threshold for detection of macromolecular compartment sizes at a given spatial scale. In general, simulations can provide specific guidance on the smallest reliably detected compartment size change, and, for imaging, the spatial resolution achievable, under specified scan time constraints and experimental conditions, including probe sensitivity and field strength.

Finally, while the influence of sample orientation on monoexponential T_2 has been well-documented in articular cartilage (48), the present work has not addressed the effect of articular cartilage orientation on T_2 distribution. The effect of this on the ability to resolve multiple components and accurately quantify the respective magnetization fractions remains open.

Conclusions

Multiexponential T_2 analysis is a new approach to non-invasive analysis of cartilage that does not require the use of contrast agents, specialized hardware, or a specialized pulse sequence. Multiexponential T_2 characterization of cartilage matrix in control and enzymatically degraded cartilage was consistent with biochemical, histologic, and FT-IRIS studies. Our findings indicate the greatly improved specificity of multiexponential T_2 analysis of cartilage as compared to the conventional monoexponential analysis, and show the utility of this approach for noninvasive cartilage assessment in basic science studies. In addition to bulk analysis of cartilage samples, we have demonstrated that multiexponential analysis is capable of detecting localized matrix changes. This supports efforts to extend the technique to clinical detection and characterization of cartilage pathology; the success of this extension will depend upon available SNR and the spatial resolution required.

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Abbreviations

PG	proteoglycan
BNC	bovine nasal cartilage
ChAC	chondroitinase AC

GAG	glycosaminoglycan
sGAG	sulfated glycosaminoglycans
FT-IRIS	Fourier transform infrared imaging spectroscopy
NNLS	non-negative least squares
SNR	signal-to-noise ratio
DPBS	Dulbecco's phosphate buffered saline
DMMB	1,9-dimethylmethylene blue assay
PSR	picro-sirius red
FT-IR	Fourier transform infrared
mid-IR	mid- infrared
CPMG	Carr-Purcell-Meiboom-Gill experiment
NEX	number of excitations
CV	coefficient of variation
SD	standard deviation
ANOVA	analysis of variance
C_a, C_b, C_c, C_d, and C_e	observed multiexponential T ₂ components
w_a, w_b, w_c, w_d, and w_e	magnetization fractions associated with observed T ₂ components
ROIs	regions of interest

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Figure 1.

Simulated percent error in weights as a function of the ratio w_c/w_d . Simulations used T_2 s and weights from ChAC samples as input parameters, and the minimum experimental SNR of 17,000. The error in the larger component, w_d , is roughly constant as a function of w_c/w_d while the error in w_c diminishes in magnitude as its proportion increases.

Figure 2.

Columns from left to right: representative alcian blue staining for GAG in control and degraded cartilage, picro-sirius red staining for collagen, FT-IRIS images representing the integrated intensity under wavenumbers $960\text{--}1184\text{ cm}^{-1}$ corresponding to a proteoglycan resonance, FT-IRIS images representing the integrated intensity under wavenumbers $1592\text{--}1744\text{ cm}^{-1}$ corresponding to the amide I collagen resonance, and T_2 distributions. As shown, the monoexponential T_2 values determined for degraded samples were similar, but the multiexponential T_2 distributions demonstrate unique differences among the enzymes as described in the text. Scale bars in the control alcian blue and control FT-IRIS PG panels represent 1 mm.

Figure 3.

a) Sagittal MR image of bovine patella with indicated regions of interest (ROIs) encompassing more superficial and deeper regions in control (left) and trypsin-treated (right) tissue. The articular surface of the sample was oriented normal to the B_0 field. Scale bar represents 5 mm. b) T_2 distributions from the control and trypsin tissue ROIs with the corresponding monoexponential values indicated by a vertical line. Comparison of surface ROIs shows a much larger increase of $(80 - 24)/24 = 233\%$ in the rapidly relaxing T_2 component in comparison to the monoexponential T_2 increase $(154 - 125)/125 = 23\%$. An increase of $(186 - 135)/135 = 38\%$ was also observed in the dominant, slowly-relaxing component. In the control tissue, the differences between the surface and deeper ROI were $\Delta = 29 - 24 = 5$ ms in the rapidly relaxing component and $\Delta = 135 - 74 = 61$ ms in the slowly relaxing component, which, given the $\Delta = 55$ ms difference in monoexponential T_2 s, indicates the dominant role of free water in defining the bulk T_2 of cartilage. The corresponding differences between the ROIs in the trypsin-treated cartilage were $\Delta = 51$ ms (for the rapidly relaxing component) and $\Delta = 114$ ms (for the slowly relaxing component), showing the increased sensitivity of multiexponential T_2 analysis to degradation.

Table 1
Estimated accuracy and precision of T₂s and associated weights expressed as percent error

Condition		T _{2,e}	T _{2,d}	T _{2,e}	w _c	w _d	w _e
Control	Input value	31.46	86.45	----	10.77	83.36	----
	Accuracy	-8.11	-0.57	----	-4.56	1.14	----
	Precision	0.76	0.03	----	0.53	0.29	----
ChAC	Input Value	33.08	110.87	----	2.87	94.00	----
	Accuracy	-16.15	-0.39	----	-23.97	0.30	----
	Precision	0.53	0.01	----	0.37	0.16	----
Collagenase	Input Value	27.84	101.36	440.14	6.12	87.02	2.17
	Accuracy	-1.70	0.01	0.67	-9.67	-10.04	-3.32
	Precision	5.61	0.07	3.02	23.64	25.47	8.00

Each sample condition was simulated with 100 noise realizations based on the minimum experimental SNR of 17,000 and using the appropriate group average T₂s and associated weights as input values. Accuracy is defined as percent error. Precision is reported as the coefficient of variation (CV), defined as the standard deviation over 100 noise realizations divided by the corresponding input parameter value × 100. Input simulation values for the T₂ components in ms and their (% magnetization) were as follows. Control: C_d = 0.83 (2.49), C_b = 10.68 (3.38), C_c = 31.46 (10.77), C_d = 86.45 (83.36); ChAC: C_d = 0.87 (3.13), C_c = 33.08 (2.87), C_d = 110.87 (94.00); and collagenase: C_d = 1.43 (2.62), C_b = 12.79 (2.07), C_c = 27.84 (6.12), C_d = 101.36 (87.02), C_e = 440.14 (2.17).

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Table 2

BNC matrix component quantification using biochemistry and FT-IRIS

Sample condition	water (mg/mg w.w.)	sGAG (mg sGAG/mg d.w.)	FT-IRIS PG	FT-IRIS Collagen	FT-IRIS Ratio
Control	0.78 ± 0.007 (n = 4)	0.72 ± 0.056 (n = 4)	2844 ± 134 (n = 2)	2934 ± 91 (n = 2)	0.96 ± 0.063 (n = 2)
ChAC	0.79 ± 0.009 (n = 16) ^a	0.56 ± 0.12 (n = 16) ^a	2536 ± 424 (n = 7)	3082 ± 151 (n = 7)	0.82 ± 0.13 (n = 7) ^b
Collagenase	0.78 ± 0.015 (n = 12)	0.58 ± 0.13 (n = 12) ^a	2500 ± 402 (n = 4)	3110 ± 160 (n = 4)	0.82 ± 0.14 (n = 4) ^c

Biochemical and FT-IRIS results (mean ± SD). Percent water is expressed as a fraction of total sample wet weight (w.w.) and sGAG is expressed as a fraction of dry sample weight (d.w.). FT-IRIS results show the integrated area under the wavenumber range 960–1185 cm⁻¹, associated with proteoglycan; 1592–1744 cm⁻¹, associated with the amide I peak of collagen; and the ratio of the proteoglycan to collagen.

^a indicates p < 0.05,

^b indicates a trend with p = 0.14, and

^c indicates a trend with p = 0.17 for comparison of treatments to the control group. Both ChAC and collagenase groups contain samples from all digestion durations.

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Table 3

Mono- and multiexponential T₂ analysis of control and degraded BNC

Sample condition	T _{2,mono} (ms)	T _{2,c} (ms)	T _{2,d} (ms)	w _c /w _d	T _{2,i} (ms)	w _e
Control (n = 4)	82 ± 5	31 ± 1	86 ± 7	0.13 ± 0.02		
ChAC (n = 16)	109 ± 11 ^a	33 ± 6	111 ± 12 ^a	0.03 ± 0.02 ^a		
Collagenase (n = 12)	99 ± 15 ^a	28 ± 10	101 ± 14 ^b	0.05 ± 0.06 ^a	440 ± 225	0.02 ± 0.02

T₂ values obtained from monoexponential fits (T_{2,mono}) or multiexponential fits (T_{2,i}) with corresponding weights (mean ± SD) from NNLS analysis of control and degraded cartilage.

^a, indicates p < 0.05, and

^b, indicates a trend with p = 0.058 for comparisons between treatment and control groups. Note that quantitative comparison of individual compartment weights between treatment groups requires the assumption that net tissue water is unchanged with degradation (see text), so that the ratio of w_c/w_d is reported instead.