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Molecular NMR T_2 Values Can Predict Cartilage Stress-Relaxation Parameters

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Abstract

Articular cartilage lines synovial joints and functions as a low-friction deformable tissue to enable smooth and stable joint articulation. The objective of this study was to determine the relationships between cartilage stress-relaxation properties and the collagen and GAG NMR transverse relaxation times (T_2) toward understanding mechanisms of cartilage viscoelasticity. Stress-relaxation tests were performed on both cultured and enzymatically-digested bovine cartilage, followed by measurements of both the collagen and GAG T_2 using the Call-Purcell-Meiboom-Gill pulse sequence. The peak and equilibrium stresses were correlated with the GAG T_2 , and the stress-relaxation time constant was correlated with the collagen T_2 . Multiple linear regression models were successful in using the specific T_2 values to predict the stress-relaxation properties. As a model of osteoarthritis, enzymatic digestion with collagenase and testicular hyaluronidase had weak effects on T_2 values. These data present a complex picture of cartilage mechanical behavior, with cartilage stiffness associated with the GAG T_2 values and the stress-relaxation time constant associated with the collagen T_2 .

Keywords

cartilage viscoelasticity; transverse relaxation time; cartilage material properties; polymer dynamics; cartilage; flow-independent viscoelasticity; matrix viscoelasticity

Introduction

Osteoarthritis (OA) is a joint disease involving mechanical and molecular cartilage degeneration which is estimated to affect more than 10% of the population above age 60, with estimated costs greater than \$60 billion[1]. OA progression results in degradation of both the mechanical properties of cartilage[2] and the molecules comprising the extracellular matrix [3]. Understanding the roles of matrix molecules in determining cartilage mechanical properties is of major importance.

There are many known mechanisms of cartilage mechanical properties. Previous research has investigated interstitial fluid flow[4;5;6], electrostatic interactions[7;8], and fibril-

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reinforcement[9;10]. Cartilage stiffness correlates with the nuclear magnetic resonance transverse (NMR) relaxation time (T_2) measurements of bulk water both with[11;12;13] and without[14] gadolinium enhancement. From these and other studies, a conceptual model of cartilage includes a low-permeability solid phase containing collagen fibrils and negatively-charged GAGs, a fluid phase composed of water, and an ionic phase composed of positive ions. However, within this model, the contributions of specific extracellular matrix molecules to tissue-level cartilage mechanical properties are not sufficiently well-understood[15].

The theory of polymer dynamics[16;17] has been applied to cartilage[18;19]. For purely polymeric systems, polymer dynamics theory predicts an inverse relationship between the stress-relaxation time constant and T_2 . Thus we hypothesized that there would be a negative correlation between the stress-relaxation time constant and the specific molecular T_2 values of the cartilage biopolymers collagen and aggrecan.

The purpose of this study was to use NMR spectroscopy to investigate the roles of collagen and glycosaminoglycans in tissue-level cartilage stress-relaxation. This study utilized NMR spectroscopy to determine the relationships between the tissue-level stress-relaxation properties and the mobility of two cartilage molecules (collagen and GAG) as assessed by T_2 values. Such relationships between molecular properties and tissue-level behavior are important for understanding healthy cartilage function and with further research may prove useful as a clinical tool for predicting cartilage material properties.

Materials and Methods

All cartilage samples were harvested from a standardized location on the patellofemoral groove of 1-3month old calves as previously described[20] and placed into tissue culture at 37°C with 5% CO₂ with chemically-defined medium which was changed after 2 days.

Mechanical testing consisted of unconfined compression stress-relaxation tests at 5% nominal strain. Following mechanical testing, samples were immersed in 0.15M phosphate-buffered D₂O (99% atomic purity) for 1 hour, and placed in 5mm NMR tubes in the presence of 0.15M phosphate-buffered D₂O with 0.2 mM TSP, and frozen at -20°C until NMR spectroscopy. T_2 values were determined from ¹H NMR spectra using the CPMG sequence[21;22]

A stretched exponential model was fit to the cartilage stress-relaxation data (Eq. 1).

$$\sigma = (\sigma_{\text{peak}} - \sigma_{\text{eq}}) e^{-\left(\frac{t}{\tau}\right)^\beta} + \sigma_{\text{eq}} \quad [1]$$

σ_{peak} and σ_{eq} represent the peak and equilibrium stress which were defined by the experimental data. Note that the existence of σ_{eq} in the model implicitly models stress resulting from a solid, elastic component of cartilage (*e.g.* the cross-linked collagen network). τ is the time constant of stress-relaxation, related to the physical characteristics (*e.g.* temperature, polymer length, and concentration) of the system[24]. β is the stretching parameter, related to the specific type of polymer motion (*e.g.* Rouse or Reptation). This model represents stress-relaxation of polydisperse polymer systems such as cartilage[25;26]. τ and β were determined using nonlinear optimization to minimize the weighted square residuals between the model and the data. In addition to τ and β , a model-independent parameter, $\hat{D}$, was used to quantify stress-relaxation dynamics (Figure S2).

The first group of samples (n=24, Tissue Culture) were used to determine the relationships between stress-relaxation parameters and specific molecular T_2 values. After 5 days in tissue culture, these samples were mechanically tested, equilibrated in 0.15M phosphate-buffered

D₂O, and frozen at -20°C until T_2 measurements were made. Correlation analysis was performed between each T_2 (collagen or GAG) and the stress-relaxation parameters (Peak and Equilibrium Stresses, $\hat{D}$, τ or β) using Pearson's correlation coefficient. Multiple linear regression was performed to determine if specific molecular T_2 values could predict the stress-relaxation parameters. Using linear regression, multiple regression models of the form of Eq. 2 were fit to the data:

$$Y = m_1 T_{2C} + m_2 T_{2GAG} + m_3 T_{2C} T_{2GAG} + b \quad [2]$$

In Eq. 2, Y represents a stress-relaxation parameter (Peak Stress, Equilibrium Stresses, $\hat{D}$, τ or β), m_1 represents the slope for the collagen T_2 , m_2 represents the slope for the GAG T_2 , m_3 represents the slope for the T_2 interaction term, and b represents the intercept.

The second group of cartilage samples was used to investigate mechanisms of cartilage viscoelasticity via enzymatic digestion. After 5 days of tissue culture, samples were incubated overnight in either collagenase (n=7), testicular hyaluronidase (n=7), or a control solution (n=5) and mechanically tested. The collagenase solution consisted of 5U/mL bacterial collagenase (Sigma, C5138), and the testicular hyaluronidase solution consisted of 50U/mL testicular hyaluronidase (Sigma, H3506). Both enzymes were dissolved in HBSS with 0.01% BSA. Control samples were incubated overnight in HBSS with 0.01% BSA. Following mechanical testing, samples were equilibrated in 0.15M phosphate-buffered D₂O and frozen until NMR spectroscopy. For these samples, statistical analysis was performed using student t-tests to compare the enzyme-treated groups with the control groups. All statistical analysis was performed with an *a priori* significance level of 0.05, and results are expressed as mean $\pm$ sem.

The final group of samples was used to determine whether mechanical testing affected the measured T_2 values. These samples (n=3) were subjected to 5 days of culture, equilibrated in 0.15M phosphate-buffered D₂O, and frozen prior to spectroscopy.

Results

Overall the stretched exponential models described the data well with high coefficients of determination ($R^2=0.951\pm0.001$, Figure 1). Enzymatic digestion had marked effects on stress-relaxation parameters (Figure 2 and Table S1). The peak stress was significantly smaller in the collagenase and testicular hyaluronidase than control groups (both $p\leq0.05$, Figure 2a). There were no significant changes in equilibrium stress (Figure 2b). $\hat{D}$ was significantly smaller after collagenase digestion ($p=0.03$, Figure 2c). τ was significantly larger after testicular hyaluronidase digestion and smaller after collagenase digestion (both $p\leq0.04$, Figure 2d). No significant changes in β were observed (Figure 2e).

There were significant correlations between stress-relaxation parameters and NMR T_2 values (Figure 3, Table 1). The T_2 related to the cartilage GAGs was correlated with both the peak ($r=0.186$, $p=0.04$) and equilibrium stresses ($r=0.588$, $p=0.01$) (Figure 3, a-b). The T_2 related to the cartilage collagen was correlated with $\hat{D}$ ($r=-0.567$, $p<0.01$), τ ($r=-0.399$, $p=0.05$), and β ($r=0.524$, $p=0.01$) (Figure 3, c-e). Multiple regression models were successful in using the collagen and GAG T_2 values to predict the stress-relaxation parameters (Table 2). Regressions were significant for all stress-relaxation parameters except peak stress and τ .

Enzymatic digestion had weak effects on the T_2 values. The collagen T_2 was larger in the testicular hyaluronidase group than in the control group with marginal significance ($p=0.06$, Figure 4a). The average GAG T_2 was higher in the collagenase group than in the control group, but not statistically significant (Figure 4b). In the collagenase group, both the average collagen

and GAG T_2 values were larger than controls, but statistically significant differences were not detected (Figure 4). All T_2 values for tissue culture samples not subject to mechanical testing were within the range of T_2 values for samples subjected to mechanical testing, suggesting that mechanical testing did not change the T_2 values.

Discussion

The objectives of this study were to determine (1) the relationships between mechanical properties and T_2 values of specific cartilage extracellular matrix components and (2) if enzymatic digestion affected these properties.

We observed significant correlations between the stress-relaxation parameters and both collagen and GAG T_2 values (Figure 3, Table 1). Furthermore, NMR parameters were successful in predicting the mechanical properties of equilibrium stress, β , and $\hat{D}$ in linear regression models (Table 2). *In vivo* cartilage function involves deformation[27] and osteoarthritis is a debilitating cartilage disease intimately associated with degradation of both cartilage molecules and mechanical properties[28]. These results demonstrate that tissue-level cartilage mechanical properties can be predicted from specific cartilage T_2 values.

In these experiments, the GAG T_2 correlated with the peak and equilibrium stresses, which represent the dynamic and equilibrium stiffness of cartilage, respectively. The collagen T_2 correlated with the time-dependent stress-relaxation parameters τ , $\hat{D}$, and β . These results suggest that GAG degradation may lead to increased cartilage deformation due to the decreased stiffness and that collagen degradation may affect the time-dependence of cartilage load bearing. However, the extent to which enzymatic degradation of one component (*e.g.* collagen by collagenase) affects the mechanical and NMR behavior of another component (*e.g.* GAG) is unclear. Future studies may determine if these relationships apply to osteoarthritic cartilage.

Future studies may expand this work toward prediction of *in vivo* mechanical properties utilizing magnetic resonance spectroscopy. This may prove useful both in a clinical setting both for understanding the onset and progression of osteoarthritis (*e.g.* by determining changes in cartilage molecular and mechanical properties in conjunction with changes in OA symptoms) and in basic science for further understanding how specific molecules contribute to tissue-level cartilage mechanical behavior.

The present observations of negative correlations between transverse NMR relaxation and both τ and $\hat{D}$ support polymeric mechanisms in cartilage viscoelasticity. Polymer theory predicts this negative correlation[17]. Dynamical averaging caused by polymer connectivity results in slower transverse NMR relaxation as polymer relaxation proceeds faster[29]: less-mobile polymer chains have slower mechanical relaxation but faster T_2 relaxation than more-mobile chains. We observed significant negative correlations between the collagen T_2 and both τ and $\hat{D}$ (Figure 3, Table 1) which support polymer dynamics as a mechanism of cartilage viscoelasticity. Surprisingly, we did not find significant correlations between the GAG T_2 and either $\hat{D}$ or τ . This may be caused by multiple factors. First, GAGs comprise only about 5% of cartilage on a wet-weight basis whereas collagen accounts for approximately 20% [30], and the relatively low GAG ^1H NMR signal compared with collagen may mask a GAG contribution. Second, cartilage is an inhomogeneous solid where signal from non-GAG protons likely convolutes the observed GAG resonance. Finally and most interesting, there may not be a correlation between the GAG T_2 and $\hat{D}$ or τ . Both $\hat{D}$ and τ measure the tissue-level rate of stress-relaxation, but neither has been related to specific cartilage molecular components. Future research is needed to better define the roles of specific cartilage molecules in contributing to τ and $\hat{D}$ and the relationships between specific molecular T_2 's and their relative contributions to τ and $\hat{D}$.

The equilibrium stress was positively correlated with the GAG T_2 (Figure 3). This is consistent with previous work demonstrating that equilibrium cartilage mechanical behavior is largely due to electrostatic interactions between the highly anionic GAG chains[31].

Enzymatic digestion with collagenase and testicular hyaluronidase resulted in changes in peak stress, τ , $\hat{D}$, and collagen T_2 (Figures 2-3, Tables S1-S2). The decreases in τ and $\hat{D}$ upon enzymatic digestion are readily predicted by polymer theory: shorter molecules are more mobile resulting in faster stress-relaxation[24]. There are two interpretations of the observed increase in collagen T_2 for testicular-hyaluronidase-digested samples relative to both collagenase-digested samples and controls. First, it is possible that testicular hyaluronidase digestion affected non-collagen protons and contributed to changes in collagen T_2 . Second, digestion of the cartilage GAGs likely contributes to a decreased “prestress” in the collagen network[5; 31]. Such a decrease allows greater collagen mobility resulting in increased in collagen T_2 . The latter interpretation is supported by the observation that the average GAG T_2 is higher in the collagenase group than in both testicular hyaluronidase and control groups.

The stretched exponential model parameter τ exhibited different effects between collagenase and testicular hyaluronidase digestion (Figure 2, Table S1). These effects are not yet fully understood. The absence of changes in τ and $\hat{D}$ due to testicular hyaluronidase digestion is puzzling, and future studies are required to investigate this.

When considering these results, some limitations must be considered. First, full-thickness cartilage structure and material properties have been previously shown to be spatially heterogeneous[32]. To minimize these sample inhomogeneities, we used middle zone cartilage samples which are more homogeneous than full-thickness samples[33]. Second, cartilage is a fluid-filled solid with broad NMR linewidths. These broad resonances result from both the restricted molecular motion associated with solids and from the large number of distinct cartilage protons contributing to the ^1H NMR signal[34]. The result of the broad resonances is that specific resonances, and therefore T_2 values, of the collagen and GAG protons are convoluted with those of multiple other protons. While these effects undoubtedly affect our results, collagen and GAG are by far the most abundant molecules of the cartilage extracellular matrix[30]. Furthermore, previous studies have assigned the peaks used herein to the glycine associated with collagen and the N-acyl methyl proton associated with the glycosaminoglycans [23].

In summary, we observed significant correlations between parameters describing cartilage mechanical behavior and specific cartilage T_2 values. The specific collagen and GAG T_2 values successfully predicted most stress-relaxation parameters in linear regression models. Testicular hyaluronidase digestion resulted in an increase in collagen T_2 . These results are consistent with a polymer dynamics as a mechanism of cartilage flow-independent viscoelasticity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Representative Stress-Relaxation Data with Model Fit. This dataset has the median model fit ($R^2 = 0.9504$). Overall the models described the data well with high coefficients of determination ($R^2 = 0.9513 \pm 0.001$). Note that data were downsampled for visual display.

Figure 2.

Stress-Relaxation Results. * indicates significant difference ($p \leq 0.05$) relative to control group.

(a) The peak stress was significantly smaller in the collagenase and testicular hyaluronidase than control groups (both $p \leq 0.05$). (b) There were no significant changes in equilibrium stress. (c) $\hat{D}$ was significantly smaller after collagenase digestion ($p = 0.03$). (d) τ was significantly larger after testicular hyaluronidase digestion and smaller after collagenase digestion (both $p \leq 0.04$). (e) No significant changes in β were observed.

Figure 3.

Significant Correlations between Stress-Relaxation Parameters and Transverse Magnetization Relaxation Times. The T_2 related to the cartilage GAGs was correlated with both the (a) peak ($r=0.186$, $p=0.04$) and (b) equilibrium stresses ($r=0.588$, $p=0.01$). The T_2 related to the cartilage collagen was correlated with (c) $\hat{D}$ ($r=-0.567$, $p<0.01$), (d) τ ($r=0.399$, $p=0.05$), and (e) β ($r=0.524$, $p=0.01$).

Figure 4.

$\hat{D}$ and T_2 values for the enzymatic digestion experiment. $\hat{D}$ was significantly smaller in the collagenase group than in the control group ($p=0.03$). (a) The collagen T_2 was larger in the testicular hyaluronidase group than in the control group with marginal significance ($p=0.06$). (b) The average GAG T_2 was higher in the collagenase group than in the control group, but not statistically significant.

Table 1
Correlations between stress-relaxation parameters and T2 values. Significant correlation coefficients are denoted in **bold**.

	Peak Stress		Equilibrium Stress		τ		β		D'	
	r	p	r	p	r	p	r	p	r	p
Collagen T ₂	0.052	0.81	-0.141	0.51	-0.399	0.05	0.524	0.01	-0.567	0.00
GAG T ₂	0.186	0.04	0.588	0.00	-0.188	0.38	-0.246	0.25	-0.091	0.67

Table 2
Multiple Linear Regression Parameters using T2 values to predict stress-relaxation parameters. **Boldface** type indicates significant multiple regression.

	Intercept	Collagen T_2 Slope $\times 1000$	GAG T_2 Slope $\times 1000$	Interaction Slope $\times 1000$	R^2	P-value
σ_{peak}	0.56	1.72	2.05	-0.01	0.047	0.804
σ_{equil}	0.14	0.45	1.25	0.00	0.439	0.008
τ	6.48	-43.01	-15.55	0.14	0.187	0.237
β	0.42	3.29	-0.09	-0.01	0.433	0.009
$\dot{D}$	9.08	-61.65	-13.11	0.16	0.334	0.04