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Diffusion of Paramagnetically Labeled Proteins in Cartilage: Enhancement of the 1-D NMR Imaging Technique

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Quantifying the diffusive transport of large molecules in avascular cartilage tissue is important both for planning potential pharamacological treatments and for gaining insight into the molecular-scale structure of cartilage. In this work, the diffusion coefficients of gadolinium–DTPA and Gd-labeled versions of four proteins—lysozyme, trypsinogen, ovalbumin, and bovine serum albumin (BSA) with molecular weights of 14,300, 24,000, 45,000, and 67,000, respectively—have been measured in healthy and degraded calf cartilage. The experimental technique relies on the effect of the paramagnetic on the relaxation properties of the surrounding water, combined with the time course of a 1-dimensional spatial profile of the water signal in the cartilage sample. The enhanced technique presented here does not require a prior measurement of the relaxivity of the paramagnetic compound in the sample of interest. The data are expressed as the ratio of the diffusion coefficient of a compound in cartilage to its diffusion coefficient in water. For healthy cartilage, this ratio was 0.34 ± 0.07 for Gd–DTPA, the smallest compound, and fell to 0.3 ± 0.1 for Gd–lysozyme, 0.08 ± 0.04 for Gd–trypsinogen, and 0.07 ± 0.04 for Gd–ovalbumin. Gd–BSA did not appear to enter healthy cartilage tissue beyond a surface layer. After the cartilage had been degraded by 24-h trypsinization, these ratios were 0.60 ± 0.03 for Gd–DTPA, 0.40 ± 0.08 for Gd–lysozyme, 0.42 ± 0.09 for Gd–trypsinogen, 0.16 ± 0.14 for Gd–ovalbumin, and 0.11 ± 0.05 for Gd–BSA. Thus, degradation of the cartilage led to increases in the diffusion coefficient of up to fivefold for the Gd-labeled proteins. These basic transport parameters yield insights on the nature of pore sizes and chemical–matrix interactions in the cartilage tissue and may prove diagnostically useful for identifying the degree and nature of damage to cartilage. © 2001 Academic Press

Key Words: **diffusion; cartilage; proteins; NMR; osteoarthritis.**

INTRODUCTION

Since articular cartilage contains no blood vessels throughout most of its volume, the chondrocytes within the cartilage tissue must receive nutrients and macromolecules from the articular surface by diffusive transport and/or entrainment in the fluid being transported. The quantitative evaluation of macromolecular transport through the cartilage matrix is likely to be especially important in the study and treatment of osteoarthritis. Macromolecules such as metalloproteinases, cytokines, and growth factors have been shown to play a role in cartilage degradation of osteoarthritis (*1–6*). Proposed treatments include protease inhibitors and cytokines that antagonize the action of interleukin-1. All of these molecules are proteins that must move through the cartilage matrix to exert their effect on either chondrocytes or the structural matrix. In this study, a 1-dimensional NMR imaging technique is developed and used to measure the diffusion coefficients of several protein-sized molecules in healthy and degraded cartilage.

In previous studies, the diffusivity of small molecules (molecular weight less than 200) in healthy articular cartilage has been shown to be 45 to 60% of that of the same molecule in aqueous solution (*7, 8*). This fraction is largely independent of the charge of the diffusing molecule. Degraded cartilage, whether produced by *in vitro* protease treatment or obtained from arthritic joints, exhibits an increased weight fraction of water (hydration) and an increased diffusivity (*7, 9*). Static compression of cartilage had the reverse effects, with decreased hydration and a decreased diffusivity of water. This correlation between hydration and diffusivity indicates that steric hindrance plays a large role in determining the diffusion coefficient of small molecules in cartilage. A study of the diffusion of divalent copper ion into bovine nasal cartilage found a potential interaction between the copper and the fixed charges of the cartilage matrix, leading to non-Fickian diffusion behavior (*10*). Cyclic compression does not alter the transport of small molecules significantly, as measured by the rate of absorption of the solute into cartilage (*11, 12*).

In studies of larger molecules, the diffusion coefficient of serum albumin in articular cartilage was found to be 25% of its value in aqueous solution (*9*). This finding only applied to articular cartilage with a fixed charge density (FCD) between -0.05 and -0.13 m · equiv/g. When the FCD was even more negative, the partition coefficient for serum albumin, defined as the ratio of the concentration of a solute in cartilage to the concentration in a bathing solution at equilibrium, was very low (0.01 or less), which may have made measurement of the diffusion coefficient difficult. In this same study, the partition coefficient was found to strongly depend not only on FCD, but also on the size of the solute for molecular weights ranging from 17,000 to 160,000. The diffusion coefficient was not measured for these other molecular weights.

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Another study produced estimates of the apparent diffusion coefficients of various sizes of dextran in articular cartilage which decreased with increasing Stokes radius (*13*). In contrast to small solutes, cyclic compression tended to enhance the transport of serum albumin (*12*), proteoglycan fragments, and collagen fragments (*14*) by 30 to 100%. A quantitative estimate of the effective diffusion constant was not obtained in these studies. This enhanced transport was hypothesized to arise due to increased fluid flow in the cartilage due to cyclic compression (*11, 15*). Static compression impeded the flow of larger molecules (*14*).

This study characterizes and models the transport of a range of protein sizes under varying degradation states of articular cartilage. The experimental technique involves chelating gadolinium (Gd) to the protein and then using a time-series of magnetic resonance 1-dimensional profiles to monitor the transport of the bound Gd into a cartilage sample of defined geometry. Such a technique has been described (*16*), but this previous technique required a separate determination of the relaxivity of the paramagnetic compound in the gel. The approach described here measures the relaxivity and diffusion coefficient with a single experiment. The technique is first validated by mathematical simulations and by measuring the diffusion of copper ions in agarose gel.

THEORY

Diffusion Theory

The geometry of the experiment allows transport of the paramagnetic compound from a well-mixed reservoir through just one end of the cylindrical cartilage. Thus, the concentration of the paramagnetic compound varies only in one dimension. The NMR images (1-d profiles) used to monitor the transport of the paramagnetic compound into the cartilage produce a signal intensity that depends on the water relaxation time T_1. The effect of the paramagnetic is to shorten the T_1 of surrounding water according to

$$\frac{1}{T_1} = a + bC, \qquad [1]$$

where C is the concentration of the Gd-labeled protein, a is the inverse T_1 of water in the absence of the paramagnetic, and b is the molar relaxivity. Thus, the signal intensity at a given spatial position in the sample reflects the concentration of the protein of interest at that position. By obtaining a time-series of these profiles as the paramagnetic compound moves into the cartilage, one can determine the time required for each concentration of protein to reach a given position within the sample.

The solution to the diffusion equation for free diffusion into a semi-infinite slab (sample at all locations where $x > 0$) from a constant concentration reservoir is

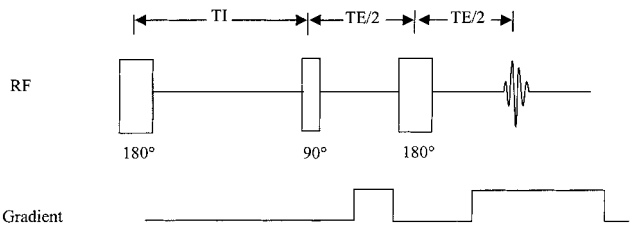

FIG. 1. Schematic of the NMR pulse sequence used for acquisition of diffusion data. The time TI is variable. The time TE is typically 7 ms.

$$C = C_0 \cdot k \cdot \mathrm{erfc}\left[\frac{x + x_0}{2\sqrt{Dt}}\right], \qquad [2]$$

where C_0 is the concentration of paramagnetic molecule in the reservoir, k is the partition coefficient for the paramagnetic (μM paramagnetic in tissue water/μM paramagnetic in reservoir), x is the distance from the solution–cartilage boundary, x_0 is a parameter to account for uncertainty in the precise location of the solution–cartilage boundary, t is the elapsed time of the experiment, and D is the diffusion coefficient. Parameter x_0 exists because the location of the solution–cartilage boundary is not precisely determined by the NMR profile, and therefore the depth of any particular NMR data point in the profile is better determined by fitting the diffusion model to the data than by choosing the boundary location. Substitution of Eq. [2] into Eq. [1] produces an equation in which T_1 depends on position in the sample (x) and time of experiment. Note that substitution of Eq. [2] into Eq. [1] produces the product $b \cdot C_0 \cdot k$, which is considered a single parameter called $P1$ for curve-fitting purposes. Equation [2] assumes a sample that is infinite in width and semi-infinite in depth. Regarding width, the small diffusion distance in the x direction (at most 1.0 mm) relative to the width of the sample (7 mm) indicates that the lateral edges of the sample will exert a minimal effect. Regarding the depth, care must be taken to only use data in which no detectable paramagnetic has reached the bottom of the sample, as detected by a decrease in the signal intensity at the bottom of the sample for the most sensitive profile. Only data obtained before this event are used for data analysis.

NMR Microscopy

The T_1 measurement is accomplished using a standard inversion-recovery NMR pulse sequence (Fig. 1). The NMR signal intensity from the inversion recovery pulse sequence is given by

$$M = M_0 \cdot (e^{-\mathrm{TE}/T_2})(1 - (1 - \cos\alpha)e^{-\mathrm{TI}/T_1}), \qquad [3]$$

where M is the resultant signal intensity, M_0 is the maximum signal intensity, α is the RF pulse angle (nominally 180°, but must curve-fit for accurate value), TE is the echo time, and TI is the delay following the inversion pulse. This T_1 measure-

ment is performed with a magnetic field gradient applied along the diffusion direction so as to acquire a profile of the T_1-dependent signal intensity along the depth of the cartilage.

Substituting Eq. [2] into Eq. [1] and then substituting the result into Eq. [3] produces the equation used to fit NMR signal intensity data. The echo time TE was short (7 ms) relative to T_2 in the absence of paramagnetic (50 to 100 ms in cartilage, higher in agarose gels). In the presence of paramagnetic, the T_2 will decrease as the concentration of paramagnetic increases. However, the effect of altered T_2 as the paramagnetic diffuses will be quite minimal until high concentrations of paramagnetic are reached due to the high initial T_2 of the samples relative to TE. For example, a reduction of T_2 from 100 to 60 ms causes only a 4.6% reduction in signal intensity, which is a small change compared to the changes in signal intensity due to T_1 alterations. Therefore, due to the small effect of T_2 on signal intensity, $M_0 \cdot \exp(-\mathrm{TE}/T_2)$ is grouped into a single parameter called M_1. The full equation is then

$$M = M_1(1 - (1 - \cos\alpha)e^{-\mathrm{TI}\cdot(a+P1\cdot\mathrm{erfc}((x+x_0)/2\sqrt{D_{\mathrm{eff}}t}))}). \quad [4]$$

Prior to initiating the diffusion of the paramagnetic compound, the proton signal intensity and α at each depth of cartilage were measured. These data are used to facilitate the curve-fits of the paramagnetic diffusion data. Neither the total proton signal intensity (M_1) nor α is expected to change during the diffusion experiment.

Diffusion Measurement

The NMR technique used to estimate the diffusion constant is related to a technique previously used to monitor diffusion in gels (*16*) and cartilage (*10*). In that earlier technique, a signal intensity profile is obtained as a function of time after exposing one surface of the sample to a paramagnetic compound. The signal intensity profile that is monitored is obtained using the inversion-recovery pulse sequence at a single TI value. After taking the absolute value of the signal intensity data, the most prominent feature of such a profile is a null signal at a particular depth in the sample. According to Eq. [3], the null signal for a particular TI corresponds to a specific T_1 value and thus also corresponds to a particular concentration of the paramagnetic compound according to Eq. [1]. As the experiment progresses, the null point progresses deeper into the sample according to the diffusion equation. Note that a different choice of TI would produce a null point that moves through the cartilage at a different "rate," simply because it is tracking a greater or lesser concentration of the paramagnetic. In order to accurately quantify D from this experiment, one needs to know the concentration that is represented by this null point. Examination of Eqs. [1] and [4] reveals that relating the choice of a particular TI to a particular concentration that gives the null signal (or any other signal intensity) requires knowledge of the relaxivity b and the partition coefficient k. The approach taken in previous studies has been to perform a separate determination of the $b \cdot k$ product of the paramagnetic compound in the gel of interest. This measurement is done by equilibrating multiple gel samples in solutions of varying paramagnetic concentration and then measuring the T_1 of each sample using multiple TI values and the standard inversion-recovery experiment. In some experiments this approach may be either impossible, if the number of samples is limited or the biological experiment is performed *in vivo*, or simply inefficient due to the requirement of the separate determination of relaxivity and partition coefficients.

Therefore, a modification of this technique was made by monitoring multiple TI values as the paramagnetic compound diffuses into the sample. This modification thus combines the $b \cdot k$ product measurement and the 1-dimensional diffusion experiment, both described above. In practice, the combination of these two measurements is performed by obtaining a profile at one TI value, then obtaining a profile at a second TI value, and so on until profiles at 10 TI values have been obtained. Then this sequence of sequentially obtaining profiles at 10 different TI values is repeated until the diffusion experiment is complete. This process is illustrated in Fig. 2, in which each row represents a specific TI value, and each column represents a different cycle through the list of TI values. Thus, the experiment described previously (*10*, *16*) would correspond to a single row of Fig. 2.

EXPERIMENTAL

Sample and Solution Preparation

Agarose gels were prepared by mixing 1 or 3% (w/v) agarose (Sigma, St. Louis) in 150 mM NaCl solutions at room temperature. The mixture was heated in a 100°C water bath until the agarose dissolved and then poured into a cylindrical sample holder with a diameter of 7 mm. The depth of the gel was 1 to 2 cm. After gelling, the plug was removed from the sample holder, the meniscus was trimmed from the plug, and then the entire plug was inverted and reinserted into the sample holder. This enabled the exposure of a flat surface to the bath containing the diffusing compound.

Frozen knee joints of 6- to 10-week-old calves were obtained from a local supplier (Der Dutchman, Waynesville, OH). Articular cartilage was harvested from the femoropatellar groove. A coring tool was used to create plugs of cartilage plus underlying bone of diameter 7 mm. The plugs were then placed in a lathe and then the cartilage was trimmed on the top surface (formerly exposed to synovial space) and trimmed away from the bone to create plane-parallel disks of thickness 1 to 2.5 mm. The cartilage disks were stored at −20°C until the time of the experiment. Some cartilage disks were placed in a bath containing 10 mg/ml trypsin (Sigma) for 24 h. Treatment with trypsin degrades the proteoglycan matrix, thereby reducing the

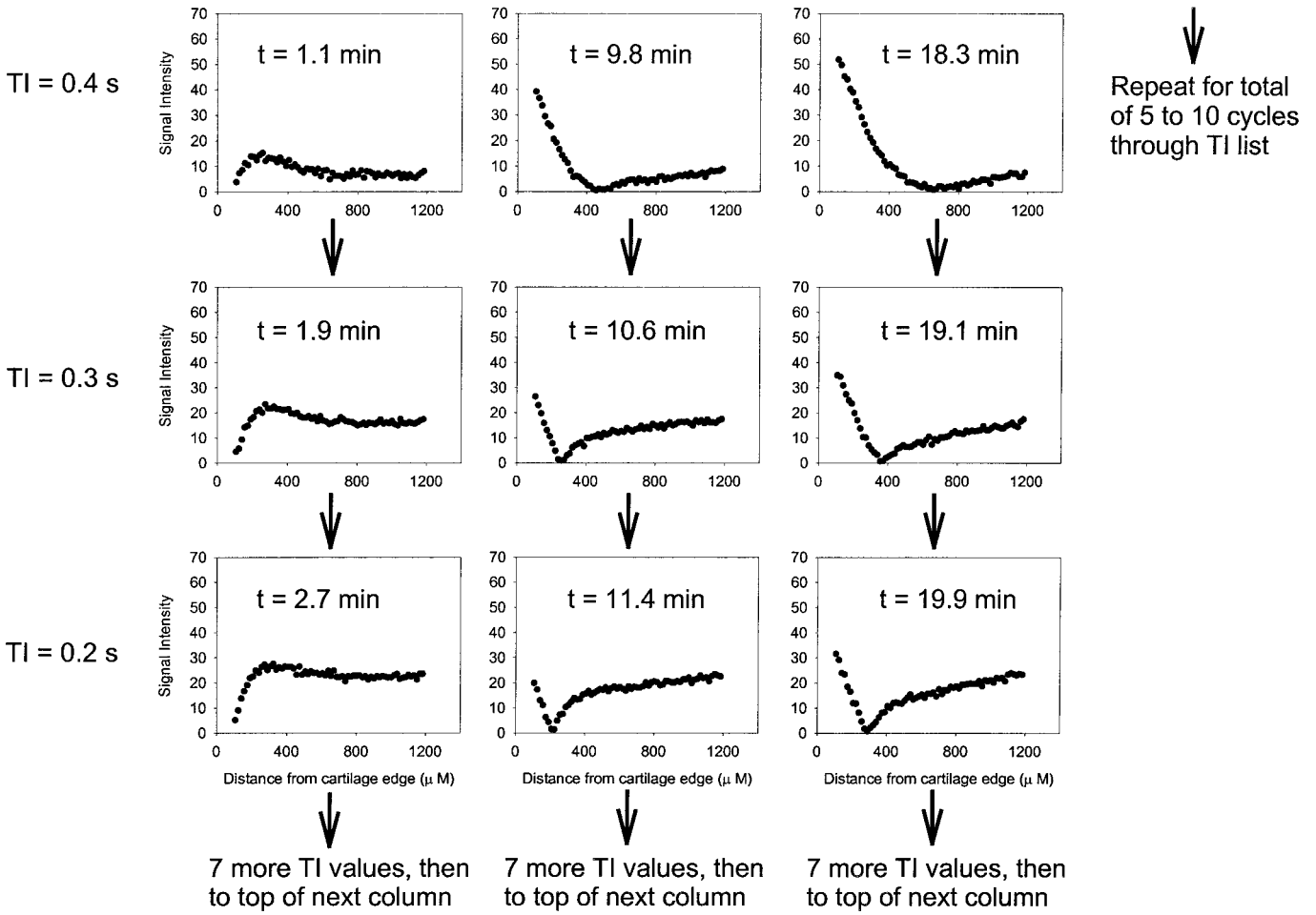

FIG. 2. Sequence of experiments to perform the diffusion measurement. Starting at the top left, a profile is obtained for each of the TI values (moving down the first column) until all TI values have been used. Then the cycle is repeated until a sufficient motion of paramagnetic into the sample is detected, typically 5 to 10 cycles through the TI list. The times printed inside each subgraph are the average times (for the eight acquisitions) for each profile after initiating diffusion with the paramagnetic. Each row is labeled with the TI value that is used for all subgraphs in that row. Note that the null point moves more rapidly for TI = 0.4 s than for TI = 0.2 s. This is due to the fact that a TI of 0.4 s is tracking a lower concentration of paramagnetic than the TI of 0.2 s. These plots were taken from an experiment in which Gd–lysozyme diffuses into healthy cartilage.

FCD and simulating a degenerative state of the cartilage (*7*). The 24-h exposure to trypsin leads to a significant loss of proteoglycans from the cartilage tissue and thereby produces severely degraded cartilage.

The paramagnetic atom gadolinium was attached to several proteins using a previously published method (*17*). The procedure used to prepare Gd–bovine serum albumin (BSA) is briefly described. To 200 ml of a 0.1 M solution of $NaHCO_3$, BSA (MW 67 kDa, Sigma) is added such that its concentration is 75 μM. Then 1.5 mM cyclic DTPA anhydride (DTPAA, Aldrich Chemical Co., Milwaukee, WI) is added. The solution is briefly stirred and then allowed to sit for 5 min to allow covalent bonding of DTPA to the protein. After the 5-min duration, 1.0 M acetic acid is added until the pH becomes 5.6. Then 2.3 mM gadolinium chloride hexahydrate ($GdCl_3$, Aldrich Chemical Co.) is added and mixed, and the solution is allowed to stand for 30 min to allow chelation of the gadolinium. The resulting solution is transferred to dialysis tubing (6000–8000 MW cutoff, Spectra/Por1, Fisher Scientific) and dialyzed against 2 L of 0.15 M NaCl plus 0.025 M Tris at pH 8.0 for 24 h to remove unchelated gadolinium from the solution. A second dialysis is then performed against a new 2-L NaCl plus Tris solution for 24 h. A similar procedure was used to chelate Gd to lysozyme (MW 14 kDa), trypsinogen (MW 24 kDa), and ovalbumin (MW 45 kDa) (all proteins from Sigma). For lysozyme, the concentrations were: protein, 180 μM; DTPAA, 7 mM; and $GdCl_3$, 8.7 mM. For trypsinogen, the concentrations were: protein, 100 μM; DTPAA, 2.91 mM; and $GdCl_3$, 4.17 mM. For ovalbumin, the concentrations were: protein, 150 μM; DTPAA, 3.0 mM; and $GdCl_3$, 5.2 mM. The T_1 values of the final Gd–protein chelate solutions used for the diffusion experiments were as follows: Gd–BSA, 0.15 to 0.29 s; Gd–lysozyme, 0.10 to 0.29 s; Gd–trypsinogen, 0.15 to 0.24 s; Gd–ovalbumin, 0.10 to 0.23 s.

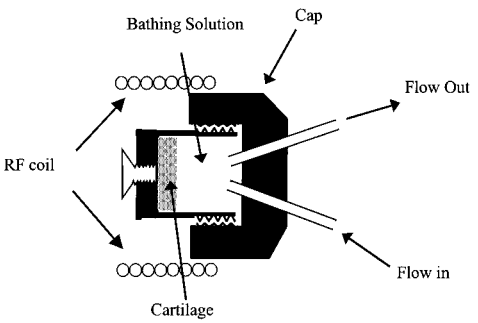

FIG. 3. Schematic for NMR cartilage sample holder. The holder has an inner diameter of the same dimension as the cartilage disk diameter, so that the cartilage fits snugly in the holder. The screw on the bottom is removed during disk insertion, to allow air to escape, and then screwed back in after the disk is in place. A cap on the holder is screwed on and makes a seal with the base using an O-ring. The cap also contains two tubes (id = 1.1 mm) running through it, one for flow into the chamber above the cartilage disk and one for flow out of the chamber. The total volume of the chamber above the cartilage disk is approximately 0.2 cm^3, with a small variation introduced by variation in the thickness of the cartilage sample. No fluid resides under the cartilage sample. The entire sample holder was placed horizontally in the magnet. The location of the 2-cm RF coil (schematically depicted as a solenoid, but actually a saddle coil) is also shown.

In addition, diffusion experiments were performed with GdDTPA (Magnevist, Berlex Laboratories, Montville, NJ). The final solution used in diffusion experiments is 5 mM GdDTPA in 150 mM NaCl.

NMR

The cartilage disks or agarose gel plugs were placed in a sample holder as shown and described in Fig. 3. A peristaltic pump, sitting outside the magnet, withdrew fluid from a nearby reservoir. The reservoir volume was typically 50 mL. The pump then pushed fluid through the inflow tube in the sample holder cap. The fluid pushed out of the outflow tube in the sample holder cap drained back into the reservoir. The rate of flow into (and out of) the chamber was set at 1 ml/s. The primary purpose of the perfusion system is to keep the bathing solution well-mixed. This fixes the boundary conditions for obtaining solutions to the diffusion equations. A secondary purpose of the perfusion system is to enable changes to the bathing/perfusion solution by changing a reservoir outside the magnet. This is a routine procedure in the diffusion experiment, since initially the perfusion is performed without the Gd-labeled protein for initial cartilage characterization, and then the bathing solution is abruptly switched to one containing the Gd-labeled protein to begin the diffusion measurement. The solution flowing in and out of the chamber above the cartilage disk was changed by turning off the pump, swapping the inflow line to a new reservoir, and then restarting the pump. When sufficient time had elapsed for the old solution in the tubing to be purged, the outflow from the tubing was placed in the new reservoir to reestablish the recirculating system.

The NMR experiments were performed on a 2.35-T Bruker Biospec spectroscopy/imaging system (Bruker Instruments, Inc., Billerica, MA). The 6-cm-diameter gradients are custom-built and capable of achieving 20 G/cm. The 2-cm-diameter radiofrequency probe is a custom-built saddle coil tuned to ^1H. For sodium and water measurements needed to calculate FCD (*18*), a double RF coil was created. The inner 2-cm-diameter saddle coil was tuned to ^{23}Na, and the outer 2.3-cm-diameter saddle coil was tuned to ^1H. The experiments were all performed at room temperature, which ranged from 22 to 24.5°C.

For agarose experiments, the reservoir concentration of $CuSO_4$ was 15 mM. Typically, 40 or 80 profiles were acquired during diffusion times of 60 to 90 min. Ten different TI delays (0.1, 0.3, 0.5, 0.7, 0.9, 1.0, 1.5, 4.0, 6.0, and 15.0 s) were used for each experiment. Thus, during a typical agarose experiment, 4 or 8 cycles through the TI delay list were completed.

For cartilage experiments, 5 to 10 cycles through the list of 10 TI values are performed, resulting in 50 to 100 profiles being acquired during a 0.5- to 3-h diffusion period. The 10 TI values, in the order actually used when acquiring data, were 0.4, 0.3, 0.25, 0.2, 0.15, 0.1, 0.5, 0.6, 1.5, and 5.0 s. At each profile, 4 or 8 acquisitions were averaged, with a 5-s delay between acquisitions. The profiles contain 512 data points, corresponding to 512 different spatial locations within the 6-mm NMR field of view. The time TE used for all experiments was 7 ms.

Data Analysis

Simulations of the experiment were generated using Eq. [4]. Gaussian noise, with a standard deviation of 1 or 5% of M_1, was added to the profiles. Ten data sets were created for each noise level. Data analysis of this simulated data set was performed in a manner identical to experimental data sets, as described below.

The absolute value of M is used both in curve-fits and in plots to maintain consistency with previous reports using the null point technique (*10*, *16*). Thus the parameters which are determined by curve-fitting are M_1, α, a, $P1$, D, and x_0. These parameters are determined by two data sets: (1) the T_1 profile obtained before exposing the sample to the paramagnetic compound; and (2) the diffusion data set with 50 to 100 profiles after exposure to the paramagnetic compound. The first data set, the preparamagnetic T_1 profile, was used to estimate M_1 and α at each depth of cartilage using Eq. [3] with the M_1 substitution. Both of these parameters are expected to remain constant even after exposing the sample to paramagnetic. The second data set, the set of NMR signal intensity profiles acquired at multiple times after initiation of paramagnetic exposure, is then used in a curve fit of Eq. [4] to the data. The four parameters determined are a, $P1$, D, and x_0. These equations have been implemented in MATLAB on a PC and are fit to the data using a nonlinear least-squares optimization.

TABLE 1
Simulation of the Diffusion Experiment in Agarose Gel

	True value (no noise)	1% noise	5% noise
a $(s)^{-1}$	0.40	0.4 ± 0.0	0.4 ± 0.0
$P1$ $(s)^{-1}$	9.6	9.6 ± 0.2	9.2 ± 0.3
D (10^{-6} cm^2/s)	5.00	5.02 ± 0.03	5.0 ± 0.1

TABLE 2
Experimental Diffusivity of $CuSO_4$ in Agarose

	1% agarose	3% agarose
a $(s)^{-1}$	0.38 ± 0.02	0.41 ± 0.03
$P1$ $(s)^{-1}$	9.8 ± 0.2	10.4 ± 0.4
D (10^{-6} cm^2/s)	6.4 ± 0.4	5.6 ± 0.2

RESULTS

Simulations

Table 1 lists the true and estimated values for simulated noise levels of 1 and 5% (standard deviation as a percentage of maximum signal intensity) for the diffusion experiment. The simulations indicate that the technique accurately determines both $P1$ and the diffusion constant in a single series of experiments and that it is quite insensitive to random noise in the NMR signal. The random noise in agarose gel experiments was about 0.5%, while the random noise in cartilage experiments was 2 to 5%.

Agarose Gel

To validate the NMR technique, the diffusivity of $CuSO_4$ in agarose, which has been studied in other published reports (*16, 19*), was measured using procedures and geometry identical to that used for cartilage. Figure 4 depicts the complete profile across the gel for several different TI delays of an experiment. The curves in Fig. 4 were actually obtained fairly close in time to each other, within 5 min, and are from a single "column" of Fig. 2. As time progresses in the experiment, each null point for each TI value will progress from left to right according to the diffusion equations. Subsequent curve-fitting to estimate D and $P1$ was performed only on a subsection of the complete profile, as indicated by the region between the vertical dashed lines in Fig. 4. This was to avoid signal intensity aberrations near the boundary between the gel and the reservoir and between the gel and the bottom of the sample. These edge effects are visible as a "notch" in the profile in Fig. 4 near the zero position point and are due to differences in the magnetic susceptibility between fluid and cartilage.

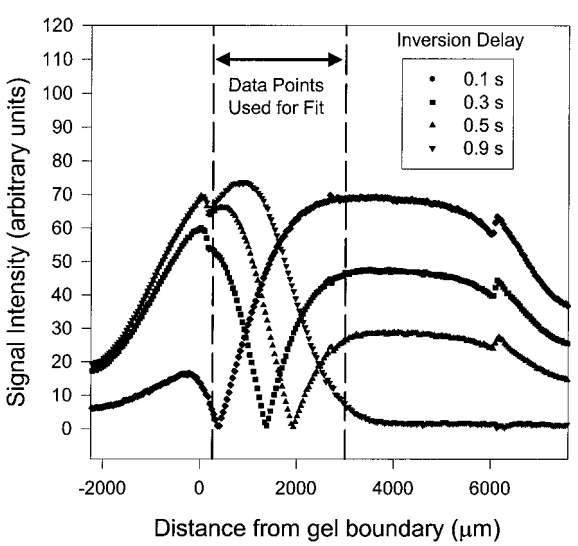

FIG. 4. Complete depth profiles at four inversion delays in 1% agarose. The stirred bath is located at negative distance values, while the gel is located at positive distance values between 0 and approximately 6000 μM. Only data points in the region between the vertical dashed lines were used for curve-fitting estimation of diffusivity and relaxivity. These plots were acquired during the following time intervals after placing $CuSO_4$ in the bathing solution: for TI = 0.1 s, 1510–1550 s; for TI = 0.3 s, 1590–1630 s; for TI = 0.5 s, 1670–1710 s; and for TI = 0.9s, 1750–1790 s.

The results of three experiments each for 1 and 3% concentrations of agarose gel are presented in Table 2. The diffusion constant of $CuSO_4$ in 1 and 3% agarose determined in these experiments is 98 and 86% of the diffusion constant of $CuSO_4$ in solution (6.5×10^{-6} cm^2/s) (*10*). These percentages agree well with a summary of investigations of diffusion in gels (*19*), which revealed that, in all cases where ions or small molecules like water were diffusing through agar or gelatin with a solid volume fraction of 1%, the D/D_0 ratios were between 90 and 99%. The diffusivity also reveals the expected trend with increasing agarose concentration. An estimate of the relaxivity b can be obtained from the parameter $P1$ by assuming a partition coefficient of 1.0 for $CuSO_4$ in the agarose gel. For the 1% experiment, $b = 9.81/(15\ mM \cdot 1.0) = 0.65$ $(mM\ s)^{-1}$), and for the 3% experiment, $b = 10.4/(15\ mM \cdot 1.0) = 0.69$ $(mM \cdot s)^{-1}$. These values compare well with a separately determined relaxivity of $CuSO_4$ in agarose of 0.64 ± 0.04 $(mM \cdot s)^{-1}$ obtained by equilibrating concentrations of $CuSO_4$ ranging from 0 to 20 mM in 1% agarose.

Cartilage

Graphs of a typical Gd–BSA diffusion experiment in trypsinized cartilage are shown in Fig. 5. Each of the panels depicts the magnitude of the signal intensity. The valley, or inverted peak, corresponds to the situation when a zero-crossing, or null point, would occur in a typical inversion-recovery experiment. The x-position of this null point corresponds to the x-position of a specific concentration of paramagnetic compound (*16*). Note the change in the null point location versus time in Figs. 5B, 5C, and 5D. The TI used for Figs. 5B, 5C, and

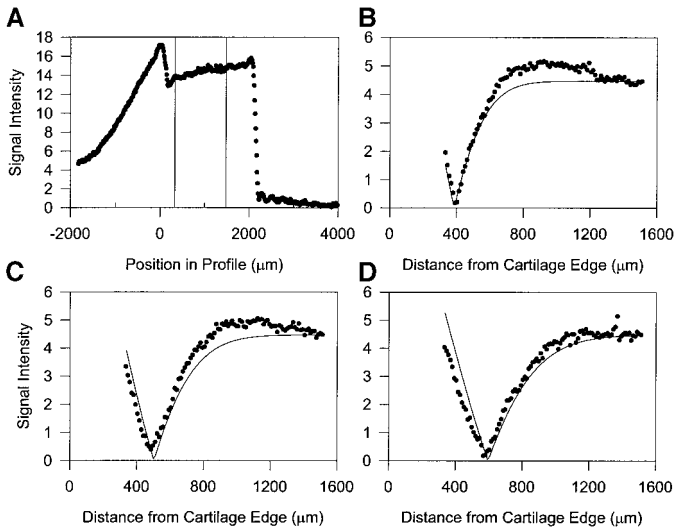

FIG. 5. Magnitude profiles from a Gd–DTPA–BSA diffusion experiment in trypsinized cartilage. (A) The entire profile of the signal from the NMR field of view. The cartilage sample itself resides between 0 and approximately 2200 μm. Negative positions correspond to the bathing solution. This profile was obtained before initiating Gd–DTPA–BSA diffusion and used a TI of 5.0 s. In order to speed curve-fitting and to avoid magnetic susceptibility artifacts at the cartilage–bath boundary, only the region between the two vertical lines (between 300 and 1500 μm) was used for data analysis. Only this region is shown in the other subplots. (B), (C), and (D) contain data (circles) obtained during the diffusion experiment and subsequent fits (lines). These plots were acquired with a TI of 0.5 s during the following time intervals after placing Gd–DTPA–BSA in the bathing solution: (B) 670–710 s, (C) 1140–1180 s, and (D) 1620–1660 s. The midpoint of each acquisition duration is used as the time value for curve-fitting.

5D was just 1 of 10 used for the entire 40-min experiment, with five cycles completed for each TI. One of the 10 TI values used was 5 s, which is long enough so that changes in T_1 from its original (no paramagnetic) value of around 1 s to shorter values will not affect signal intensity. However, if a shortening of the T_2 values due to the presence of Gd leads to a loss of signal intensity, this long TI value would reflect that. A small reduction of signal intensity at the fluid–cartilage boundary of less than 5% was occasionally observed for this 5-s TI value, with most occurrences of signal loss occurring for the Gd–BSA diffusion probe.

Table 3 lists the diffusion coefficients in healthy articular cartilage for each of the Gd-labeled compounds, along with that of H_20 examined in previous work. The table also lists the estimated size of the molecule, the diffusion coefficient of the same compounds in free solution (D_0), and the ratio of cartilage diffusivity to free solution diffusivity (D/D_0). In addition to the expected decrease in diffusion coefficient with increasing size, one can see that the diffusion coefficients of the larger molecules are a smaller fraction of the free solution diffusivity. The data reveal a steep decline in D/D_0 between the sizes of Gd–lysozyme and Gd–trypsinogen. Gd–BSA only entered the surface layers of each cartilage sample. After approximately 30 min, no further movement of Gd–BSA into the cartilage was observed. The average fixed charge density for all healthy cartilage samples was -240 ± 70 mM.

Table 4 is equivalent to Table 3, except that it provides diffusion information for cartilage samples that have been equilibrated in trypsin for 24 h. As expected, all solutes experienced enhanced diffusion in the trypsinized cartilage. A sharp transition in D/D_0 ratios for trypsinized cartilage can be seen but at larger molecular sizes than observed for healthy cartilage, namely between Gd–trypsinogen and Gd–ovalbumin. The diffusion of Gd–BSA was easily quantifiable within a 2-h experiment. The average fixed charge density for all trypsinized cartilage samples was -40 ± 44 mM.

DISCUSSION

A novel NMR technique for quantifying diffusion of paramagnetic macromolecules in gels and cartilage has been presented. One problem with other similar NMR techniques is the

TABLE 3
Parameters to Curve-Fit of the Diffusion Experiment in Healthy Cartilage

	N	MW	Radius (nm)	a (s^{-1})	$P1$ (s^{-1})	D (10^{-6} cm^2/s)	D_0 (10^{-6} cm^2/s)	D/D_0
H_2O	NA	18	0.19[a]	NA	NA	9.4 ± 0.9[b]	23.4[b]	0.40 ± 0.04
Gd–DTPA	4	530	0.61[c]	1.3 ± 0.2	10 ± 4	1.4 ± 0.3	4.0[c]	0.34 ± 0.07
Gd–lysozyme	3	14,300	2.8[c]	1.5 ± 0.2	5.6 ± 0.6	0.25 ± 0.09	0.87[c]	0.3 ± 0.1
Gd–trypsinogen	4	24,000	3.5[c]	1.34 ± 0.08	2.4 ± 1.6	0.05 ± 0.03	0.70[c]	0.08 ± 0.04
Gd–ovalbumin	3	45,000	4.7[c]	1.4 ± 0.3	2.7 ± 0.6	0.04 ± 0.02	0.52[c]	0.07 ± 0.04
Gd–BSA	4	67,000	5.8[c]	NA	NA	NA	0.62[d]	~0

Note. NA, not applicable.
[a] Hydrodynamic radius.
[b] Burstein *et al.* (*7*).
[c] Radius was computed from the MW via $R = 0.0332 \cdot MW^{0.463}$ (*21*). D_0 was computed from the radius via the Stokes–Einstein relation.
[d] Measured value (*22*).

TABLE 4
Parameters to Curve-Fit of the Diffusion Experiment in Trypsinized Cartilage

	N	MW	Radius (nm)	*a* (s^{-1})	*P*1 (s^{-1})	*D* (10^{-6} cm^2/s)	D_0 (10^{-6} cm^2/s)	D/D_0
H_2O	NA	18	0.19[a]	NA	NA	11.0 ± 0.9[b]	23.4[b]	0.47 ± 0.04
Gd–DTPA	3	530	0.61[c]	1.1 ± 0.1	10.2 ± 0.4	2.4 ± 0.1	4.0[c]	0.60 ± 0.03
Gd–lysozyme	3	14,300	2.8[c]	0.88 ± 0.02	2.26 ± 0.05	0.35 ± 0.07	0.87[c]	0.40 ± 0.08
Gd–trypsinogen	4	24,000	3.5[c]	1.2 ± 0.2	2.6 ± 0.4	0.29 ± 0.06	0.70[c]	0.42 ± 0.09
Gd–ovalbumin	3	45,000	4.7[c]	1.22 ± 0.06	8.1 ± 2.0	0.09 ± 0.07	0.52[c]	0.16 ± 0.14
Gd–BSA	5	67,000	5.8[c]	1.2 ± 0.2	8.6 ± 5.4	0.07 ± 0.03	0.62[d]	0.11 ± 0.05

Note. NA, not applicable.
[a] Hydrodynamic radius.
[b] Burstein *et al.* (*7*).
[c] Radius was computed from the MW via $R = 0.0332 \cdot MW^{0.463}$ (*21*). D_0 was computed from the radius via the Stokes–Einstein relation.
[d] Measured value (*22*).

necessity of assuming that the relaxivity of the paramagnetic compound is unchanged when the paramagnetic compound moves from the bathing solution to the tissue. The technique presented here overcomes this problem by combining a well-defined geometry for monitoring diffusion into a sample with T_1 measurements during the diffusion process. Using this approach, $CuSO_4$ relaxivity and diffusion coefficients in agarose gels have been determined. When using Gd-labeled proteins, the D/D_0 ratio for healthy cartilage decreased as the molecular radius increased, with a steep drop off between the sizes of Gd–lysozyme and Gd–trypsinogen. When applied to cartilage that had been severely degraded by 24-h exposure to trypsin, the D/D_0 ratio went up in every case when compared to healthy cartilage. The decrease in D/D_0 ratio with increasing molecular size was still present, but the most rapid decline was now apparent at a slightly larger molecular size—namely between the sizes of Gd–trypsinogen and Gd–ovalbumin.

Regarding the resolution of the NMR profiles, 512 data points within a 6-mm field of view correspond to a slice 11.7 μm wide. For a pulse sequence of 0.5-s length on average, the diffusion of water molecules, $D = 9.4 \times 10^{-6}$ cm^2/s (*7*), would be expected to travel a mean distance of 31 μm. Thus a typical water molecule will travel near paramagnetic molecules from three adjacent slices, producing at most this level of imprecision in the location of the resulting signal. Averaging effects (a T_1 based on the average relaxivity of three adjacent slices is going to be very close to the T_1 produced by the center slice) will somewhat minimize the imprecision. Also, the data analysis, as graphically depicted in Figs. 2, 4, and 5, does not depend so much on precise locations as it does on monitoring the average signal trend over time from 50–100 experiments.

Ogston's theory for diffusion of large molecules through randomly oriented straight fibers accounts for limited pore sizes and can be used to estimate the root-mean-square radius of spherical spaces R_{rms} within the fiber network (*19*). The relevant equation which can be derived is

$$R_{rms} = \frac{r \cdot (1 - \sqrt{\varphi})}{-\ln \frac{D}{D_0} - \sqrt{\varphi}}, \quad [5]$$

where r is the radius of the diffusing macromolecule and φ is the solid volume fraction of matrix. Using estimates of solid volume fraction for healthy cartilage of 0.12 and trypsinized cartilage of 0.096 (*7*), application of this theory to the D/D_0 ratios given in Tables 3 and 4 produces the estimates for R_{rms} given in Table 5. In all cases, R_{rms} increased with trypsinization as expected. Also, the near impermeability of healthy cartilage to Gd–BSA is not surprising given this range of pore sizes. However, several pore size estimates don't appear to follow the trends in the table—namely the low pore size estimate for Gd–DTPA in healthy cartilage and the elevated pore size estimates for Gd–lysozyme and Gd–trypsinogen in degraded cartilage. These anomalies may indicate that factors other than steric hindrance may affect diffusion within the gel, such as nonrandom orientation of fibers (i.e., channels within the tissue) or charge interactions.

Since the curve-fit parameter $P1$ is equal to $b \cdot C_0 \cdot k$, one can obtain an estimate for the relaxivity b of these chelates in the cartilage tissue if one has knowledge of the partition coefficient k. Estimates of $P1$ for protein diffusion experiments

TABLE 5
Calculation of R_{rms} using Ogston's Theory

	R_{rms}, healthy cartilage (nm)	R_{rms}, trypsinized cartilage (nm)
Gd–DTPA	0.54	2.09
Gd–lysozyme	2.05	3.18
Gd–trypsinogen	1.05	4.33
Gd–ovalbumin	1.32	2.13
Gd–BSA	NA	2.10

in cartilage were 5.6 s^{-1} for Gd–lysozyme in healthy cartilage (Table 3) and 8.7 s^{-1} for Gd–BSA in trypsinized cartilage (Table 4). If one conservatively assumes that the partition coefficient k for these proteins is around 0.1 (*9*), then the product of $b \cdot C_0$ is 56 s^{-1} for Gd–lysozyme and 87 s^{-1} for Gd–BSA. The value of $b \cdot C_0$ in saline solution was measured just prior to each experiment and was found to be 9.7 ± 1.4 s^{-1} for Gd–lysozyme and 4.2 ± 0.5 s^{-1} for Gd–BSA. Since C_0 is fixed, these results indicate that these large paramagnetic molecules experienced a greatly enhanced relaxivity b in the confined spaces of articular cartilage. Since the proton relaxation enhancement due to a paramagnetic species is a function of the molecular-scale interactions between the paramagnetic species and its local environment (*17, 20*), the study of the relaxivity of paramagnetically labeled macromolecules may provide an additional tool for understanding the motion of large molecules in cartilage tissue.

CONCLUSIONS

The technique described here has been shown to accurately estimate the diffusion coefficient both in mathematical simulations and in a diffusion experiment of $CuSO_4$ in agarose gel. The advantage of the technique is that separate measurements of relaxivity and partition coefficient are not necessary to determine the diffusion coefficient. Also, the diffusion coefficient of paramagnetically labeled proteins in healthy and degraded cartilage was measured. The ratio D/D_0 was found to decline with increasing molecular size, in contrast to an earlier study using a range of smaller molecular sizes (*7*). These estimates of macromolecular diffusion coefficients can be used to quantify the time needed for a particular compound to fully penetrate the avascular cartilage, such as when one wishes to treat cartilage or chondrocytes with a drug. Estimates of pore sizes from these experiments indicate that simple steric hindrance may be insufficient to explain diffusion of large molecules in cartilage. Also, preliminary evidence indicates that the relaxivity of these paramagnetically labeled proteins may be enhanced over their values in solution.

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