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Experimental Issues in the Measurement of Multi-component Relaxation Times in Articular Cartilage by Microscopic MRI

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Abstract

A number of experimental issues in the measurement of multi-component T_2 and T_1 relaxations in native and enzymatically digested articular cartilage were investigated by microscopic MRI (μ MRI). The issues included the bath solutions (physiological saline and phosphate buffered saline (PBS)), the imaging resolution (35 to 140 μ m), the specimen orientations (0° and 55°), and the strength of spin-lock frequencies (0.5 to 2 kHz) in the T_1 experiments. In addition to cartilage, the samples of agar gel and doped water solution were also used in the investigation. Two imaging sequences were used: CPMG-SE and MSME. All raw data were analyzed by the non-negative least square (NNLS) method. The MSME sequence was shown to result in the observation of multi-component T_2 , even in the gel and liquid samples, demonstrating the artificial uncleanness of this sequence in the multi-component measurements. The soaking of cartilage in PBS reduced the observable T_2 components to one at both 0° and 55° , suggesting the effect of phosphate ions on proton exchange between different pools of water molecules. The cartilage orientation with respect to the external magnetic field and the spin-lock strengths in the T_1 experiment both affected the quantification of the multi-component relaxation. The transitions between a mono-component and multi-components in cartilage under various experimental conditions call for the extra caution in interpreting the relaxation results.

Keywords

cartilage; T_2 relaxation; T_1 relaxation; multi-component; MRI; PBS; chemical exchange

INTRODUCTION

The anisotropic properties (experimental dependence of the measurement on the orientation of the specimen to the magnetic field B_0) of connective tissues, such as tendon, nasal cartilage, and articular cartilage, have been well studied by NMR spectroscopic and imaging techniques [1–9], where the phenomena are commonly attributed to the dipolar interaction between the water protons and the collagen fibril structure. The existence of multiple pools of water molecules (e.g., macromolecule-bound vs. free) can in principle result in the measurement of multi-component relaxation [10; 11]. For example, the tendon sample has consistently been confirmed to have multi-component T_2 relaxations [1; 12–14]. Recently,

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after the discovery of the anisotropy in nasal cartilage by μ MRI and polarized light microscopy [9], the residual fibril orientation in nasal cartilage was found to be able to modulate the number of the relaxation components in nasal cartilage: both T_2 and T_1 at the spin-lock strength of 500 Hz having two relaxation components when the fibril orientation was 0° , but reducing to one component for both T_2 and T_1 when the fibril orientation was 55° (the magic angle) [8].

Articular cartilage has a similar molecular composition in tendon and nasal cartilage, namely, the negatively charged proteoglycans, collagen fibrils, and water. However, articular cartilage has a more complex morphological structure [15; 16], where the thickness of the tissue is commonly sub-divided conceptually into three sub-structural zones, the superficial zone (SZ) at the top, the transition zone (TZ) in the middle, and the radial zone (RZ) that anchors the tissue to the underlining bone [17]. These structural zones have different thicknesses (both absolute and relative), depending upon the joint type and animal species. The orientation of collagen fibrils in these zones is depth-dependent, resulting in a laminar appearance in MRI of cartilage, when the axis of cartilage surface is parallel with B_0 [18]. The disappearing of the laminar appearance and higher signal intensity, due to the minimization of the dipolar interaction when the axis is about 55° to B_0 , is called the “magic-angle effect” in MRI of cartilage [3].

The discovery of the anisotropy of the multi-component relaxation in nasal cartilage clearly indicates the experimental complication of the multi-component relaxation in articular cartilage by any imaging method, because the size of the imaging voxel now becomes another critical factor in the measurement. The two MRI parameters of particular concern in this project are T_2 and T_1 , both having been shown to be sensitive to the interactions between water molecules and local macromolecular environments in connective tissues. The limited imaging reports on the topic so far contained inconsistent results. For example, Keinan-Adamsky et al noticed two T_2 components in a deep portion of porcine articular cartilage [19]. Reiter et al also showed two T_2 components in both fresh and trypsin degraded articular cartilage [20]. However, Zheng et al. observed only a single component of T_2 in articular cartilage, as long as the resolution was sufficiently high in μ MRI [14]. Since many experimental parameters can subtly affect the outcomes of the measurement, comparing the results among the published reports, each only dealing with a subset of the experimental parameters, is often not conclusive.

This μ MRI project aimed to systematically investigate the influences of several experimental factors on the multi-component T_2 and T_1 relaxation in articular cartilage. These experimental factors included the resolution of MRI experiments (35 μ m to 140 μ m), the effect of different bath solutions (saline and PBS), the influence of the specimen orientations (0° , 55°), the effect of the different pulse sequences (MSME and CPMG-SE), and the strengths of the spin-lock frequencies in T_1 experiments (0.5 kHz to 2 kHz). We aimed to provide a coherent baseline for the complex and convoluted factors that can subtly influence the measurement of multi-component relaxation in an organized biological tissue.

MATERIALS AND METHODS

Solutions of Saline and PBS

All solutions of saline and PBS used in the experiments were prepared in the laboratory. A saline solution refers simply to physiological saline (154 mM NaCl in deionized water). A PBS (phosphate buffered saline) solution was prepared as follows. First, 276 grams of sodium phosphate monobasic (monohydrate) (Sigma, Missouri) were added to deionized water. The volume of the phosphate buffer was then brought to one liter after adjusting the pH value to 7.3 by NaOH (Sigma, Missouri). Then nine grams of NaCl was added to 50 ml

of the phosphate buffer and diluted to one liter with deionized water. The final PBS solution contained a phosphate concentration of 100 mM, and had a pH corresponding to ~ 7.4 [21].

Artificial Specimens of Agar Gel and Gd Solution

Two kinds of artificial samples were made in the lab. The first kind was several gel samples, made by dissolving two grams of agar powder (Flinn Scientific, Batavia, IL) in 100 grams of heated deionized water. The cooling of the agar solution was under two different conditions: (a) the liquid was stirred for five minutes at 100 °C and (b) the liquid was occasionally stirred during the process. The cold gels were sealed in glass tubes and kept at room temperature before the experiment. The second kind of artificial specimen was simply a small glass tube filled with 1 mM Gd(DTPA)²⁻ solution (Magnevist, Berlex, NJ).

Cartilage Specimens

Canine humeral heads were harvested shortly after the sacrifice of three mature (1 – 2 years old) and skeletally healthy dogs that were used for an unrelated biomedical research (ongoing for more than 10 years at a neighboring institution). For each of the three dogs, three adjacent blocks consisting of the full thickness of the articular cartilage ($1.8 \times 1.6 \times 6$ mm³) were obtained from the central part of a humeral head. Before the MRI measurement, three blocks were immersed in saline solution and another three blocks were soaked in PBS solution for stabilization. The final three blocks were soaked in 10 µg/ml trypsin (Sigma, Missouri) solution for more than eight hours to digest proteoglycans [22] and then soaked in saline to remove excess trypsin before MRI experiments. All specimens were never frozen.

NMR and Microscopic MRI (µMRI) Protocols

All experiments were conducted at room temperature on a Bruker AVANCE II NMR spectrometer equipped with a 7-Tesla/89-mm vertical-bore superconducting magnet and microimaging accessory (Billerica, MA). A homemade 5-mm solenoid coil was used for the experiments. The orientation of the cartilage specimens with respect to B_0 was set at 0° and 55° (the magic angle) for the MRI experiments, respectively. T_2 imaging experiments were performed using a commercial multi-slice, multi-echo (MSME) pulse sequence (only one slice was used in this project) from Bruker, as well as a Carr-Purcell-Meiboom-Gill (CPMG) magnetization-prepared spin-echo imaging sequence (termed as the CPMG-SE in this report) [6]. The echo spacing in the CPMG T_2 -weighting segment was 1 ms. The number of echo times was 50, resulting in 50 delays from 2 to 600 ms. The T_1 imaging sequence was nearly identical to the T_2 imaging sequence, except that it was led by a T_1 -weighting segment, which had a 90° radio-frequency (rf) pulse followed by a spin-lock pulse. The frequency of the spin-lock pulse varied from 0.5 to 2 kHz. The strength of the spin-lock frequency was calibrated by the strength of the 90° rf pulse, which had a typical length of 6.5 µs. The lengths of the spin-lock pulses were equal to the 50 echo times in the T_2 imaging experiments.

The 2D imaging parameters were consistent for both T_2 and T_1 experiments: the echo time and repetition time was 3.0 ms and 2 s respectively; the number of scans was 16; the number of the dummy scans was 2; and the field of view (FOV) was 4.5 mm×4.5 mm. T_2 experiments were acquired at three different transverse pixel resolutions (140 µm, 70 µm, and 35 µm). The slice thickness was 1 mm. The MSME experiments were also performed for each cartilage block and agar specimens using the identical imaging parameters as in the CPMG-SE experiments, except for the following: the number of images was 64; and the repetition time was 3 s.

NMR spectroscopy experiments for the two kinds of gels (homogeneous and inhomogeneous) were conducted on the same instrument. The T_2 relaxation time was

measured by the standard CPMG spectroscopy sequence, which is similar to the CPMG-SE imaging sequence except without the imaging pulses. Seventy data points were acquired with 1 ms echo spacing. The repetition time was 10 s; the number of scans was 8; the number of dummy scans was 4. Other details have been documented previously in the literature [23].

Multi-component T_2 and $T_{1\rho}$ relaxation analysis

The non-negative-least-squares (NNLS) method was implemented in the Matlab codes (Mathworks, Natick, MA) and used to calculate the distribution profiles of T_2 and T_1 relaxation times [24; 25]. In the MRI data analysis, a rectangular region-of-interest (ROI) with a width of 10 contiguous columns were averaged from the 2D images to improve the signal-to-noise ratio of the 1D profiles. The ROI was selected from the region of the tissue with no visible variation. In the NNLS analysis of the T_2 and T_1 spectrum, any T_2 or T_1 component with a value below or above two constant thresholds (1.5 ms and 250 ms) was ignored to eliminate the dependence of the calculation on the experimental noises [26–28]. The use of the NNLS method meant that the results in the experiments were calculated without *a priori* assumptions about the number of T_2 and T_1 components and any initial guesses of the solution.

RESULTS

T_2 Relaxations of Agar Gel and 1mM Gd Solution by NMR Spectroscopy and μ MRI

Fig 1 summarizes the multi-component T_2 relaxation results by NMR spectroscopy in two agar gel phantoms, one homogeneous sample (after sufficient stirring) and the other inhomogeneous sample (with occasional stirring, hence the specimen was visibly un-uniform and also contained many small air bubbles in the gel). A convenient way of visualizing the multi-component relaxation decay is to plot the image intensity in the natural log scale, as shown in Fig 1a. The relaxation profiles of the same data by the NNLS analysis procedure are shown in Fig 1b. Compared to just one T_2 component in the homogeneous gel, two distinct T_2 components were clearly observed in the inhomogeneous gel.

Fig 2a summarizes the multi-component T_2 relaxation results by μ MRI in the agar gel phantoms, which were imaged by both MSME and CPMG-SE sequences at three different resolutions (140 μ m, 70 μ m, 35 μ m). It is clear that the intensities of the CPMG-SE images decayed slower than those of the MSME images (at the same TE, slower the decay, brighter the image), which suggests a longer T_2 relaxation. The natural log plot of Fig 2b shows clearly the resolution dependency of the T_2 decays in the MSME experiments. Fig 2c is the results of the multi-component profiles of the gel samples, which were also quantified in Table 1.

While T_2 is a single component (centered at ~ 78 ms) in all CPMG-SE experiments of the agar gel at three different resolutions, two T_2 components are clearly visible in the MSME experiments of the gel. In addition, while the T_2 values in the CPMG-SE experiments are independent of the imaging resolutions, the values of the two T_2 components in the MSME experiments decrease as the resolution increased, where the values of the long component were only $\sim 1/2$ of the CPMG-SE values. Furthermore, the relative populations of the short T_2 components in MSME depend on the imaging resolution — the population of the short component becoming larger when the resolution becoming higher. Table 1 contains not only the summary of the gel data but also the summary of the T_2 data from a tube of 1 mM Gd(DTPA) $^{2-}$ solution, where the images and plots were similar to the gel samples (not shown). This set of gel and solution data demonstrates unmistakably the artificial uncleanness of the MSME sequence in the multi-component relaxation measurements.

T₂ of Native Cartilage in Saline and PBS

Fig 3 is representative of the intensity images of native articular cartilage when they were soaked in PBS and saline, and measured by CPMG-SE and MSME sequences at 0° and 55° respectively. Similar to the agar gel results, the intensity of the cartilage images depends strongly on the image resolution (140 μm , 70 μm , 35 μm) in MSME (the comparisons were not shown). In addition, in contrast to a clear laminar appearance of articular cartilage in saline, the laminar appearance of the tissue was less noticeable when the specimen was soaked in PBS (e.g., comparing the first and third images in the first row in Fig 3a).

Fig 4a – 4d show the T₂ relaxation results of cartilage when the tissue was soaked in saline, where the results were the bulk data, by averaging the entire thickness of the tissue including all structural zones. Several features could be noted from these bulk T₂ data. First, T₂ at 0° showed two components by MSME and three components by CPMG-SE. Second, T₂ at 55° showed two components by MSME and one component by CPMG-SE. Third, the T₂ values by CPMG-SE were independent of the image resolutions, while the T₂ values by MSME decreased when the imaging resolution increased, no matter whether the specimen was oriented at 0° or the magic angle. Finally, the relative population of the short T₂ component varied significantly as the specimen orientation – around 50% at 0° and 10–20% at the magic angle.

Fig 4e – 4h show the T₂ relaxation results of cartilage when the tissue was soaked in PBS. When the tissue was imaged by CPMG-SE, only a single T₂ component was observed at both 0° and 55° at all resolutions, with the T₂ values at 55° higher than at the T₂ values at 0°. When imaged by MSME, two T₂ components were observed at both 0° and 55° at all resolutions. Similar to the gel results and the cartilage saline results, higher resolutions in MSME resulted in shorter values of the T₂ components, as well as higher relative populations of the shorter components. All T₂ results of native cartilage were summarized in Table 2.

T₂ and T₁ ρ of Native and Degraded Cartilage

The dependence of the T₁ values on the spin-lock frequency (or field) is known as the T₁ dispersion in the MRI literature, which can also absorb the T₂ data as the T₁ data at the zero spin-lock frequency. This phenomenon in the context of the multi-component measurement was investigated in this project using both native and degraded cartilage. Fig 5 shows the representative images of both native and degraded cartilage, where all specimens were in saline and measured by the CPMG-SE sequence. At the 0° orientation, the laminar appearance of the tissue could be seen clearly in the T₂ images (two white arrows shown in the Fig 5a), but less clear in the T₁ images. At the magic angle, both T₂ and T₁ images appeared relatively uniform.

T₁ measurements at 1 kHz were performed at three different image resolutions (140 μm , 70 μm , 35 μm); additional T₁ experiments at 0.5 and 2 kHz were acquired at 140 μm image resolution (since the T₁ experiment is not sensitive to the image resolution). These images were analyzed for their bulk multi-component characteristics, which were summarized in Table 3, and illustrated in Fig 6 using the T₂ data and T₁ data when the spin-lock frequency was at 1 kHz. Several conclusions can be reached. (1) At the 0° orientation, a lower spin-lock frequency resulted in more relaxation components (Table 3). (2) The number of the relaxation components at any particular spin-lock field was identical and orientational dependent for both native and degraded tissues (Table 3). At the 0° orientation, the tissues had three components for T₂, two components at T₁ at 0.5 kHz, and a single component when the spin-lock frequency was 1 kHz or higher. At the magic angle by comparison, both T₂ and T₁ showed a single component. (3) The T₂ and T₁ values of the degraded tissue

were consistently higher than those of native tissue; and the T_1 values were consistently higher than the T_2 values regardless of native or degraded tissue. (4) T_1 value increased more significantly after the tissue was degraded (Fig 6c and 6d). (5) T_1 dispersion occurred in both native and degraded tissue, and at both 0° and 55° . (6) The number and the fractional concentration of the relaxation components were related to the spin-lock frequency, rather than the imaging resolution.

Zonal Differences of T_2 in Native and Degraded Cartilage

Since articular cartilage has a depth-dependent zonal structure, the high-resolution images of articular cartilage are commonly sub-divided into multiple zones [17]. For the high-resolution results in this project ($70\text{ }\mu\text{m}$, $35\text{ }\mu\text{m}$), the full-thickness cartilage contained sufficient number of pixels to be sub-divided into the three zones based on the established zonal division criteria [17]. For the $140\text{-}\mu\text{m}$ -resolution data, the entire tissue had approximately three reliable pixels (since the canine humeral cartilage is about $550\text{--}600\text{ }\mu\text{m}$ in the total thickness). Therefore these three pixels from the tissue surface to bone were named as the surface, middle, and deep zones, and analyzed individually in each zone by the NNLS method.

Table 4 summarized the T_2 zonal results at different image resolutions when the specimen was oriented at 0° . (To simplify the labels, the three zones were named as the surface, middle, and deep zones in the table, regardless of the imaging resolution.) Several distinct characteristics can be identified in these complex relaxation results. (1) T_2 relaxation at the deep zone always showed a single component, centered at $\sim 2.8\text{ ms}$ regardless of degradation or imaging resolution. (2) T_2 had multi-components at the surface zone and the middle zone when the resolution was lower ($70\text{ }\mu\text{m}$ and $140\text{ }\mu\text{m}$). In contrast, no multi T_2 component was noticed when the imaging resolution was increased to $35\text{ }\mu\text{m}$ (where the voxel size was $35\text{ }\mu\text{m} \times 35\text{ }\mu\text{m} \times 1\text{ mm} = 12.25 \times 10^{-4}\text{ mm}^3$). Hence the number of relaxation components depended upon the imaging resolution. (3) The value of the long T_2 components has the largest increase after the tissue degradation.

DISCUSSION

An accurate and faithful measurement of multi-component T_2 and T_1 relaxations in tissue by either NMR or MRI is not trivial. In addition to the complex relaxation mechanism in biological tissues itself, the measurement can be influenced subtly by many factors related to the specimen specifics, instrumentation, experimental details, and data-analyzing methods. Finally, for a structured tissue like articular cartilage, the imaging resolution becomes a critical factor in the multi-component measurement because of the volume averaging. In this NMR and μMRI project, we aimed to investigate the influences of the common adjustable parameters in any practical experiments related to the multi-component T_2 and T_1 experiments. These parameters included: (1) the influence of the different pulse sequence (MSME, CPMG-SE) for T_2 measurements; (2) the influence of bath solution (saline, PBS); (3) the influence of the image resolution in μMRI T_2 experiments (from $35\text{ }\mu\text{m}$ to $140\text{ }\mu\text{m}$); (4) the influence of the specimen orientation in the magnetic field (0° , 55°) on both T_2 and T_1 relaxation; and (5) the influence of the spin-lock frequencies on the T_1 relaxation (from 0.5 kHz to 2 kHz).

Multi-component Relaxation by Different Pulse Sequences and Sample Inhomogeneity

To disassociate the complex influences of both the cartilage structure and imaging method on the multi-component relaxation measurement, the samples of agar gel and simple liquid solution were made in this investigation. Agar is a gelatinous substance with agarose as its predominant component, which has been studied in a number of NMR and MRI projects

[29; 30]. The gel is commonly considered to have a two-system pool consisting of a liquid pool and a small semisolid pool with a T_2 of 13 μ s [30]. The echo spacing used in this imaging project was 1 ms, which prevented the identification of the rapidly relaxing component in the ranges of tens or hundreds of μ s. Therefore, the only T_2 component that could be measured under our experimental conditions represents the liquid pool in the gel sample [31; 32]. It's also worth noting that the inhomogeneity of the gel sample and the existence of the tiny air bubbles in the gel could cause the artificial measurement of the multi T_2 components, which is shown clearly in Fig 1.

The relaxation data of both solution and gel in Table 1 demonstrates unmistakably that a common imaging sequence MSME can cause the artificial T_2 components in a homogeneous gel sample or even the liquid solutions. In addition, the values of the T_2 components by MSME are resolution-dependent and about 40–70% shorter than the T_2 values by the other imaging sequence CPMG-SE. Furthermore, the relative populations of the T_2 components by MSME are also resolution dependent – the short T_2 component would increase its population when the resolution is finer (i.e., the use of larger imaging gradients). These relaxation artifacts by MSME are probably not entirely surprising since its T_2 -weighting pulses are entangled with the spatial mapping pulses. In contrast, the CPMG-SE sequence [6] uses the magnetization-prepared approach [3], which completely separates the T_2 -weighting segment and the spatial mapping segment – hence the T_2 relaxation is being weighted without the presence of any gradient. Consequently, the CPMG-SE sequence provides a much more faithful measurement of multiple T_2 components.

Multi-component Relaxation by Different Tissue-soaking Solutions

In addition to the experimental factors, the T_2 and T_1 relaxations measurement of biological tissues also depends on the solution in which the tissue specimen is immersed. This is because biological samples commonly contain water molecules and macromolecules; the motional interactions between the bound water molecules and the macromolecules consequently play a significant role in the complex relaxation processes. As early as the 1960s, Berendsen and Migchelsen [33; 34] noticed that the addition of certain salts that contained proton-donating or proton-accepting ions could influence the proton NMR spectra of hydrated collagen in the same way as raising the temperature or increasing the water content. Salts were also found to be able to stabilize native conformations and to possess hydrogen-bonding properties that could increase the exchange rate between water molecules. Phosphate and ammonium ions were found to be the most effective, and to a lesser extent sulfate ions, but not some other salts such as NaCl (as in common saline). Several research groups [35; 36] successfully used the mechanism of proton exchange in water to explain the water dynamics in tendon and cartilage.

The native cartilage results summarized in Table 2 demonstrate the important role of the tissue soaking solution in the measurement of multi-component relaxation. The fact that the use of PBS can reduce the number of the T_2 components from three to one (CPMG-SE sequence, 0° specimen orientation) provides solid evidence for the role of some ions in the relaxation mechanism. The reduction of the laminar appearance in MRI of articular cartilage when the tissue is soaked in PBS (Fig 3) is another example of the fast exchange between the bound water and free water, which was also shown previously in a high concentration (100 mM) of PBS solution [21]. The reduction of T_2 components to one in the PBS experiments by CPMG-SE at both 0° and 55° implies that the fast exchanges between free and bound water occurs not only in the same zone, but also among different zones (SZ, TZ, RZ) in the presence of phosphate salt.

Averaged Multi-component T_2 Relaxation of Cartilage

Having understood the complex roles of the experimental details and specimen-soaking solutions in the multi-component measurement, let's turn our attention to the specific issues of multi-component T_2 in articular cartilage, which have at least one additional complexity – the depth-dependent magic angle effect of T_2 relaxation [4]. Because of the differences in the collagen orientation in each sub-tissue zone in the tissue, the T_2 of articular cartilage in each zone naturally has different values as well as different orientational dependences [37; 38]. Consequently, the ability to resolve multi-components in cartilage could depend critically on the imaging resolution, i.e., the molecular and structural heterogeneity of the tissue within each imaging voxel. Furthermore, because of the multiple orientations in a single piece of the cartilage specimen, the various degrees of the dipolar interaction co-exist simultaneously in the specimen, which cannot be completely eliminated by simply setting the specimen at a particular orientation. The ability to resolve multi-components in a large voxel, therefore, depends on an even larger array of complex experimental and sample factors.

How many T_2 components do we expect to see from a large voxel of articular cartilage that contains all possible cartilage structures? The T_2 data in Table 2 are averaged from the entire tissue and provide the experimental verification to the possible results in the multi-component T_2 measurement in low-resolution MRI or bulk spectroscopy. Provided that there is no cause of experimental artifacts, and all relaxation components can be resolved in the data analysis (i.e., an extremely short minimum echo time, a sufficient relaxation resolution, no overlaps in the relaxation values), the possible answers are listed below.

Case 1: One averaged component, when the chemical exchanges are sufficiently high among all pools of water and all zones of tissue structures;

Case 2: Two components (bound and free), when the chemical exchanges are not sufficiently high between two pools of water in the tissue that has no zonal structure;

Case 3: Three components (one for each structural zone), when the chemical exchanges are high enough among all pools of water but not sufficient for all zones of tissue structures, and when the dipolar interaction is not minimized (e.g., at 0°);

Case 4: Six components, two (bound and free) components for each structural zone, provided there is no overlap in the relaxation values. (We could also have four or five components, in the situation when one or two components overlap in value.)

The Case 1 situation occurs in this study when the CPMG-SE sequence is used to measure the tissue that is soaked in PBS, where the chemical exchanges are sufficiently high among all pools of water and all zones of tissue structures. The Case 2 situation did not occur in this articular cartilage study, but can happen in the studies of nasal cartilage and tendon, both have no complex zonal structures in their morphology [23; 39]. The Case 3 situation occurs in this study when the tissue is soaked in saline, where each of the three components represents each of the three structural zones. Compared to the lack of a laminar appearance when the tissue was in PBS [21], the clear laminar appearance when cartilage soaked in saline suggests the insufficient chemical exchange among different zones. The multi-components are, hence, due to the different relaxation times at different zones. When the dipolar interaction is minimized at the magic angle, the same experimental condition measures only one component regardless of the resolution.

When the MSME sequence is used, an additional short component could be resolved (see Table 1 results), if one has a sufficient relaxation resolution. Comparing the results in PBS between MSME and CPMG-SE, for example, the short T_2 component with the fraction around 10% – 15% (depending upon the resolution) can be attributed to the artifacts caused

by the MSME sequence. Since the T_2 values in MSME are always shorter than the corresponding T_2 values in CPMG-SE at the same imaging resolution, the saline results by MSME could have four components, the three components that can be measured by CPMG-SE, plus the artificial component. However, the ability to resolve the shortest T_2 components depends critically on the minimum echo time in the experimental sequence. In our experiments, any component shorter than ~ 1.5 ms is probably not resolvable. Hence the MSME experiments can only measure two longer components in the T_2 distribution.

Zonal Differentiated T_2 relaxation of articular cartilage at 0°

Three T_2 relaxation components (Table 2) in the full-thickness cartilage come from the multiple molecular architectures in its composition. Table 4 illustrates the trends and issues in the zonal differentiated T_2 components in articular cartilage, which depends highly on the imaging resolution. When the resolution is low ($70\ \mu\text{m}$, $140\ \mu\text{m}$ in this study), T_2 relaxation also shows two components in the surface part and middle part of the tissue, but only one component in the deep part of the tissue. The reason is the following.

Based on the work of high-resolution optical microscopy, we know in histology that each of the superficial and transitional zones occupies a small portion of the total tissue thickness [17]. In the low-resolution MRI, each of the surface and middle zones contains more than one type of the molecular architectures – the surface part contains the superficial and transitional zones, while the middle part contains the transitional and upper radial zone. Hence, in low-resolution MRI, each localized region still has more than one T_2 component, due to the multiple architectures in its voxel composition. By comparison, the radial zone is known to be the thickest in the tissue ($\sim 75\%$ of the total tissue) – hence the deep region only contains one architecture – the radial zone, where only one component can be measured. When the imaging resolution was high ($35\ \mu\text{m}$), the zonal results in this study were not equally divided – each contained only one type of the tissue architecture (based on the optical results [17]). Hence, in high-resolution MRI, only one T_2 component is resolved in each zone. This conclusion of only one T_2 component in cartilage is very important to the quantitative relaxation measurements in MRI, and also shows the significant potential difference between μMRI and clinical MRI.

The orientational dependent dispersion of $T_{1\rho}$ relaxation in native and degraded cartilage

T_1 is known to be sensitive to the slow motional interactions between water and macromolecules (proteins) in biological tissues and has been used in clinical MRI as a diagnostic parameter for the cartilage quality [8; 40–42]. Compared to the T_2 relaxation, the characteristic of T_1 relaxation has one additional variable, the dispersion of its values that depends upon the strength of the spin-lock field, in which T_2 can be considered as T_1 at the zero spin-lock field. The data in Table 3 shows clearly that the value and population of T_1 components depend strongly on the strength of the spin-lock field. In addition, the values of T_1 and T_2 are both sensible to the cartilage degradation by trypsin. In this project, the significant increase of T_1 values after the trypsin degradation, especially at higher spin-lock strength (increased by 43 ms or 62% at a 2 kHz spin-lock field), confirms the superior sensitivity of T_1 than T_2 (maximum $\sim 30\%$ at the magic angle) towards the tissue degradation regardless of the specimen orientation.

It is interesting to see that at the low spin-lock fields (e.g., 500Hz), the bulk T_2 and T_1 both have multiple relaxation components, which can be attributed to the combination of multiple zones and, hence, the various degrees of dipolar interaction (results in lower T_1 relaxation time at RZ than the values of SZ and TZ). When the spin-lock field is sufficiently high (1000 Hz or higher), T_1 relaxation has only one component in MRI measurements. Finally, T_1 at 55° always shows one component, regardless of the strength of the spin-lock field.

This orientation-dependent transition among multiple components and a single component implies that there are sufficient exchange processes in the tissue system that are able to mask the difference between different pools of water molecules when the dipolar interaction is minimized (at the magic angle).

When the tissue is degraded by trypsin, most of the GAG in cartilage is digested, with the remaining tissue containing a collagen matrix filled with a larger quantity of water. Although the zone-dependency of T_2 relaxation values changed after trypsin degradation, the population changes in the T_2 fractions at different zones were not significant before and after the trypsin treatment (~ 35% for SZ, ~ 40% for TZ, and ~ 25% for RZ). The GAG depletion would increase the free space inside the tissue matrix and the influx of water [20], but the low GAG fraction in cartilage means a stronger dipolar interaction at 0° [18]. Consequently, the sufficient high chemical exchange rate at each zone could reduce the change of the fractional distribution after the trypsin degradation. At the magic angle, due to the minimization of the dipolar interaction, only one component was found in both native and degraded tissue, which demonstrated that T_2 has an excellent sensitivity toward the cartilage degradation (change from ~ 54 ms to ~ 70 ms) [8; 43].

Other Experimental Factors that can Influence the Multi-component Measurements

In addition to the experimental and sample factors that have been discussed so far in this report, several other factors could also influence the accurate measurement of multi-component relaxations in biological tissues. For example, a previous report revealed the generation of the artificial T_2 components in the multi-component relaxation measurement by several other instrumental factors [6], including the use of a sample longer than the homogeneous length of the rf coil, the mis-match of the magnetic susceptibility between the sample and the solution, the reduction of accuracy of 90° and 180° rf pulses, and the bad shimming. Furthermore, one must have a sufficiently high resolution in *the relaxation time scale* to ensure the ability to resolve the possible components of multi-component relaxation times. Moreover, the effect of the frozen storage was shown to be able to introduce inaccurate measurement of relaxation components, due to the disruption of cartilage microstructure by the formation of the ice crystals [44]. Sufficient care must therefore be taken at *all* steps of the experiment to ensure a faithful measurement of multi-component relaxation.

In conclusion, the anisotropic T_2 and T_1 in articular cartilage are likely the indications of dipolar interaction-induced depth-dependency of relaxation times at different structural zones in the collagen matrix of the tissue. The transition between a mono-component and multi-component in articular cartilage demands the caution in interpreting the multi-component relaxation results, where the measurable values and numbers of the relaxation times could be subject to several experimental factors. For example, (a) Specimen orientation: there are more relaxation components at 0° than at the magic angle; (b) Pulse sequence: CPMG-SE sequence is robust and reliable, while MSME could cause artificial T_2 components; (c) Bath solution: the ions in the bath solution can influence the measurable number of relaxation components; (d) Spin-lock strength: the components of T_1 become less when the spin-lock strength is increased; (e) Imaging resolution: for articular cartilage or any other tissue/sample that has structural inhomogeneity, the measureable number of the relaxation components is imaging-resolution dependent. Finally, the changes in the T_2 and T_1 relaxation times due to the depletion of the tissue's proteoglycans could be used in future clinical MRI study of connective tissues.

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Research Highlights

Articular cartilage has multiple T_2 and T_1 relaxation components by μ MRI
Multi-slice multi-echo imaging sequence results in artificial relaxation component
Cartilage soaking in PBS reduces the number of observable relaxation components
Cartilage orientation affects the quantification of the multi-component relaxation
 T_1 spin-lock strength affects the quantification of the multi-component relaxation
The imaging resolution affects the quantification of the multi-component relaxation.

Fig 1.

(a) The normalized signals in NMR spectroscopy experiment of the two different kinds of the gel specimens in the natural log scale. (b) The T_2 distribution profiles of the gel samples by the NNLS calculat.

Fig 2.

(a) Selected proton-intensity images of agar gel at different resolutions by the MSME (left) and CPMG-SE (right) sequences, where each specimen had 64 and 50 T_2 weighting images (each at a different echo time) respectively. All intensity images were displayed using the same maximum (32767) and minimum (0) values on the usual gray scale. The direction of the external magnetic field B_0 was vertically up. (b) The normalized signals of the gel imaging data in the natural log scale. (c) The T_2 distribution profiles of the gel samples by the NNLS calculation. The central region of 5 pixels by 5 pixels in (a) was averaged to improve the signal-to-noise ratio

Fig 3. Selected proton-intensity images of the native cartilage soaked in PBS (left) and saline (right) solutions by both MSME and CPMG-SE sequences at 0° and 55° . All images were displayed using the same minimum and maximum values.

Fig 4. The T_2 relaxation distributions in the bulk cartilage samples (combined SZ, TZ, and RZ together) at both 0° and 55° by NNLS analysis, when the specimens were in saline (a – d) and PBS (e – h).

Fig 5.

Selected proton-intensity images of the native tissues (left) and degraded tissue (right) where each specimen was imaged 50 times, each at a different T_2 or T_1 (1 kHz) weighting. All intensity images were displayed using the same maximum and minimum values on the usual gray scale. All the images in this figure were at the 70 μm resolution. (The similar images at two different resolutions (35 μm , 140 μm) were not shown.) The laminar appearance of T_2 images at 0° for both native and degraded tissue is marked by two white arrows.

Fig 6.

The T_2 and $T_{1\rho}$ at 1 kHz results of the native and degraded tissues at 0° and 55°. (The T_2 plots of the native cartilage (shown as the dash lines without the individual data points) were duplicated from the plots of Fig 4, for the ease of comparison.)

Table 1

T₂ imaging results of gel and 1mM Gd(DTPA)²⁻ solution at different imaging resolutions

Sample	Pulse Sequence	Imaging Resolution					
		140 μ m		70 μ m		35 μ m	
		T ₂ (ms)	%	T ₂ (ms)	%	T ₂ (ms)	%
Gel	MSME	6.8 $\pm$ 1.3	7.9 $\pm$ 3.1	4.5 $\pm$ 0.9	8.4 $\pm$ 4.1	3.9 $\pm$ 0.6	13.2 $\pm$ 4.6
		39.4 $\pm$ 2.8	92.1 $\pm$ 3.4	31.3 $\pm$ 1.8	91.6 $\pm$ 5.3	21.5 $\pm$ 3.0	86.8 $\pm$ 5.8
1mM Gd	CPMG-SE	78.4 $\pm$ 4.0	100	78.0 $\pm$ 3.7	100	77.9 $\pm$ 4.6	100
	MSME	7.1 $\pm$ 1.9	5.0 $\pm$ 2.3	5.5 $\pm$ 2.1	7.6 $\pm$ 3.7	4.0 $\pm$ 1.2	13.9 $\pm$ 3.1
		52.3 $\pm$ 3.3	95.0 $\pm$ 5.1	39.8 $\pm$ 3.4	92.4 $\pm$ 5.8	22.8 $\pm$ 3.6	86.1 $\pm$ 5.2
	CPMG-SE	173.3 $\pm$ 7.4	100	172.6 $\pm$ 7.8	100	169.4 $\pm$ 6.9	100

Table 2T₂ imaging results of native cartilage at different soaking solutions

Solution	Pulse Sequence	Angle	Imaging Resolution					
			140 μ m		70 μ m		35 μ m	
			T ₂ (ms)	%	T ₂ (ms)	%	T ₂ (ms)	%
Saline	MSME	0°	6.8 $\pm$ 1.4	45.9 $\pm$ 5.6	5.4 $\pm$ 1.2	53.0 $\pm$ 4.6	3.9 $\pm$ 1.0	51.2 $\pm$ 4.1
			29.8 $\pm$ 3.4	54.1 $\pm$ 4.3	23.3 $\pm$ 2.8	47.0 $\pm$ 4.2	17.2 $\pm$ 3.3	48.8 $\pm$ 4.9
		55°	18.5 $\pm$ 2.2	12.5 $\pm$ 4.6	12.7 $\pm$ 1.6	12.0 $\pm$ 3.8	11.5 $\pm$ 2.0	23.4 $\pm$ 5.2
			40.7 $\pm$ 3.5	87.5 $\pm$ 4.1	36.8 $\pm$ 3.0	88.0 $\pm$ 5.0	27.1 $\pm$ 2.9	76.6 $\pm$ 5.0
	CPMG-SE	0°	2.7 $\pm$ 0.9	24.1 $\pm$ 3.9	2.8 $\pm$ 1.1	24.4 $\pm$ 4.1	2.9 $\pm$ 1.3	23.8 $\pm$ 4.3
			7.8 $\pm$ 1.6	35.8 $\pm$ 4.2	7.7 $\pm$ 1.4	35.6 $\pm$ 5.3	7.7 $\pm$ 1.4	35.5 $\pm$ 5.5
		55°	33.0 $\pm$ 2.6	40.1 $\pm$ 5.2	32.1 $\pm$ 3.5	40.0 $\pm$ 4.7	32.6 $\pm$ 3.9	40.7 $\pm$ 5.1
			54.0 $\pm$ 3.9	100	54.8 $\pm$ 3.6	100	54.3 $\pm$ 3.6	100
PBS	MSME	0°	6.7 $\pm$ 1.3	8.9 $\pm$ 2.5	4.8 $\pm$ 1.2	9.6 $\pm$ 2.6	4.1 $\pm$ 1.0	15.2 $\pm$ 4.1
			23.8 $\pm$ 2.4	91.1 $\pm$ 4.3	22.9 $\pm$ 2.7	90.4 $\pm$ 4.2	19.2 $\pm$ 3.2	84.8 $\pm$ 4.7
		55°	7.6 $\pm$ 2.8	4.3 $\pm$ 3.6	6.0 $\pm$ 1.8	9.8 $\pm$ 3.3	5.5 $\pm$ 1.4	20.4 $\pm$ 3.5
			27.5 $\pm$ 3.6	95.7 $\pm$ 3.8	25.0 $\pm$ 3.1	90.2 $\pm$ 5.1	19.8 $\pm$ 2.9	79.6 $\pm$ 4.4
	CPMG-SE	0°	29.2 $\pm$ 3.2	100	28.8 $\pm$ 3.1	100	28.5 $\pm$ 3.3	100
		55°	41.8 $\pm$ 3.9	100	41.2 $\pm$ 3.8	100	41.9 $\pm$ 3.3	100

Table 3

T₁ dispersion results of native and degraded cartilage at different spin-lock fields (the specimens were soaked in saline and imaged by CPMG-SE)

Specimen Angle	Spin-lock Field (Hz)	Tissue	Imaging Resolution					
			140 μ m		70 μ m		35 μ m	
			T ₁ (ms)	%	T ₁ (ms)	%	T ₁ (ms)	%
0°	0	Native	2.7±0.9	24.1±3.9	2.8±1.1	24.4±4.1	2.9±1.3	23.8±4.3
			7.8±1.6	35.8±4.2	7.7±1.4	35.6±5.3	7.7±1.4	35.5±5.5
			33.0±2.6	40.1±5.2	32.1±3.5	40.0±4.7	32.6±3.9	40.7±5.1
		Degraded	2.8±0.8	24.0±3.7	2.9±1.0	23.3±3.9	2.9±1.7	23.6±3.4
			8.1±1.9	34.4±4.4	10.1±2.4	35.2±4.2	9.4±3.2	34.1±4.1
			40.6±2.8	41.6±5.3	41.9±4.4	41.5±5.0	42.3±4.9	42.3±5.2
	500	Native	9.2±2.1	35.6±3.7				
			46.5±3.9	64.4±5.3				
			11.1±2.3	36.5±3.9				
		Degraded	64.0±3.5	63.5±5.5				
			53.6±2.7	100	53.4±2.9	100	53.2±2.5	100
			88.5±4.9	100	88.4±4.5	100	88.2±4.8	100
55°	0	Native	69.1±3.8	100				
			112.0±5.9	100				
		Degraded	54.0±3.9	100	54.8±3.6	100	54.3±3.6	100
			70.1±5.2	100	70.0±4.8	100	70.6±5.0	100
	1000	Native	70.2±3.8	100	69.3±4.1	100	70.6±4.3	100
			100.6±5.1	100	100.5±5.2	100	100.0±4.8	100
		Degraded						

* For the ease of comparison, the native tissue (in saline by CPMG-SE) results in Table 2 were duplicated here as the T₁ results at the zero spin-lock field.

Table 4

T₂ imaging results of cartilage with the zone divisions (in saline, at 0°)

Tissue	Zone	Imaging Resolution					
		140 μ m		70 μ m		35 μ m	
		T ₂ (ms)	%	T ₂ (ms)	%	T ₂ (ms)	%
Native	SZ	9.1 $\pm$ 2.9	15.5 $\pm$ 3.8	9.0 $\pm$ 2.1	58.2 $\pm$ 4.9	12.0 $\pm$ 3.5	100
		34.9 $\pm$ 3.8	84.5 $\pm$ 5.9	38.8 $\pm$ 4.4	41.8 $\pm$ 4.2		
	MZ	7.7 $\pm$ 2.3	55.0 $\pm$ 4.9	9.3 $\pm$ 3.2	24.1 $\pm$ 4.9	36.9 $\pm$ 4.4	100
		32.2 $\pm$ 3.8	45.0 $\pm$ 4.6	36.4 $\pm$ 5.8	75.9 $\pm$ 6.2		
Degraded	DZ	3.0 $\pm$ 1.4	100	2.9 $\pm$ 1.5	100	2.8 $\pm$ 1.1	100
		10.6 $\pm$ 3.3	35.0 $\pm$ 4.8	10.4 $\pm$ 3.1	44.2 $\pm$ 4.9		
	SZ	50.1 $\pm$ 5.8	65.0 $\pm$ 5.6	45.1 $\pm$ 5.4	55.8 $\pm$ 5.2	11.4 $\pm$ 3.8	100
		11.8 $\pm$ 2.5	25.0 $\pm$ 3.9	11.1 $\pm$ 3.1	10.5 $\pm$ 4.8		
	MZ	47.9 $\pm$ 4.8	75.0 $\pm$ 4.2	47.2 $\pm$ 5.9	89.5 $\pm$ 6.2	50.0 $\pm$ 4.7	100
		2.9 $\pm$ 1.2	100	2.9 $\pm$ 1.7	100		
	DZ					2.9 $\pm$ 1.3	100

* For the 140 μ m results, SZ, MZ and DZ represent the three reliable pixels inside the entire thickness of articular cartilage (surface, middle deep). For the 35 μ m results, SZ, MZ and DZ represent the relevant zonal pixels that are located accurately inside the individual zones, which are based on the correlation μ MRI study with the higher resolution optical microscopy (see the reference 17). For the 70 μ m results, the zonal locations of the pixels are less accurate.