Changes in Proton Dynamics in Articular Cartilage Caused by Phosphate Salts and Fixation Solutions

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Abstract
The objective was to study the effect of phosphate salts and fixation solutions on the proton dynamics in articular cartilage in vitro. Microscopic magnetic resonance imaging (µMRI) T2 anisotropy and nuclear magnetic resonance (NMR) double quantum–filtered (DQF) spectroscopy were used to study the full-thickness articular cartilage from several canine humeral heads. The in-plane pixel size across the depth of the cartilage tissue was 13 µm. The acid phosphate salt was an effective exchange catalyst for proton exchange in the cartilage with an organized structure of collagen fibrils, while the alkaline phosphate salt was not. For cartilage tissue containing less organized collagen fibrils, both acid and alkaline phosphate salts have no significant effect on the T2 value at low concentration but decrease the T2 value at high concentration. The solutions of NaCl, KCl, CaCl2, and D-PBS were found to have no significant effect on T2 and DQF in cartilage. This study demonstrates the ability to modify the proton exchange in articular cartilage using the solutions of phosphate salts. The ability to modify the proton exchange in articular cartilage can be used to modulate the laminar appearance of articular cartilage in MRI.

Keywords
cartilage, collagen, pH, phosphate salt, T2 relaxation, microscopic MRI, double quantum–filtered spectroscopy (DQF)

Introduction
The depth-dependent structure of the collagen fibrils in articular cartilage conceptually divides the tissue from the articular surface to the cartilage/bone interface into 3 consecutive structural zones: the superficial zone (SZ), where the fibrils are parallel to the surface; the transitional zone (TZ), where the fibrils are mostly randomly oriented; and the radial zone (RZ), where the fibrils are oriented perpendicularly to the surface.1-4 This depth-dependent fibril structure results in structural anisotropy of articular cartilage as viewed by many imaging tools, such as the laminar appearance (the magic angle effect) in magnetic resonance imaging (MRI),4-6 the birefringence in polarized light microscopy,7,8 and the amide anisotropies in Fourier-transform infrared imaging.9,10 The disruption of this fibril network will inevitably change the tissue anisotropy, which has been used in MRI as a molecular marker to detect the early lesions in cartilage such as osteoarthritis.11,12

The experimental fact13 that the anisotropic characteristics of MRI T2 relaxation in articular cartilage quantitatively follow the geometric factor in the dipolar Hamiltonian (3cos²θ – 1) endorses the concept that T2 anisotropy in cartilage is mainly influenced by the dipolar interaction among the water protons whose motional dynamics are modulated by the structure of the collagen fibril network.13-17 Because the depth-dependent T2 anisotropy is the origin of the laminar appearance of articular cartilage in MRI,17 and because such an appearance can complicate the clinical interpretation of the cartilage images, several methods were proposed to reduce the laminar appearance by minimizing the dipolar interaction, including the uses of solid echo,18 spin lock,19 and ultrashort echo time.20,21 In one of the recent studies,22 high concentration phosphate buffered saline (PBS) solution was found to have a strong influence on the water dynamics in the tendon and cartilage, and the immersion of articular cartilage in high concentration PBS was found to result in a significant reduction in the laminar appearance of cartilage on MRI. These observations were explained as a consequence of the catalyzing effect of phosphate ions on the proton exchange among water molecules.23-29
hypothesis was supported by additional nuclear magnetic resonance (NMR) spectroscopy experiments\textsuperscript{22} using the proton double quantum–filtered (DQF) sequence,\textsuperscript{30,31} where the signal comes exclusively from the mobile protons experiencing anisotropic motion.

The purpose of this report is to investigate the influence of the individual chemical components in the PBS solution on the dynamics of water molecules in articular cartilage using quantitative MRI T\textsubscript{2} anisotropy and NMR DQF spectroscopy. In addition to the phosphate solutions, the effects of several commercial buffer solutions, with pH value ranging from 3 to 9, and 2 fixation solutions were also investigated.

**Methods and Materials**

**Solutions of Normal Saline and PBS**

The solutions used in this study were the following: (1) 154 mM solutions of NaCl, KCl, and CaCl\textsubscript{2}; (2) 1 mM, 4 mM, 7 mM, 10 mM, 50 mM, and 100 mM solutions of Na\textsubscript{2}HPO\textsubscript{4}, NaH\textsubscript{2}PO\textsubscript{4}, K\textsubscript{2}HPO\textsubscript{4}, and KH\textsubscript{2}PO\textsubscript{4} (these chemical components are contained in the recipe of Dulbecco’s PBS [D-PBS]\textsuperscript{32}); (3) the mixtures of KH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} solutions with a mole concentration ratio of 0/10, 2.18/10, 4/10, 10/10, and 20/10; (4) 2 fixation solutions (10% formalin and glutaraldehyde); and (5) 6 commercial buffer solutions at different pH values: (a) pH 3.00 buffer solution (Fisher Scientific SB97-500, Pittsburgh, PA) (potassium biphthalate-hydrochloric acid buffer, 0.05 molar in 500 mL water); (b) pH 4.00 buffer solution (VWR Scientific cat. no. 34170-127, West Chester, PA) (potassium hydrogen phthalate, 0.05 molar in 500 mL water); (c) pH 5.00 buffer solution (Fisher Scientific SB102-500) (potassium biphthalate-sodium hydroxide buffer, 0.05 molar in 500 mL water); (d) pH 7.00 buffer solution (Fisher Scientific SB 107-500 or SB 108-500) (potassium phosphate monobasic-sodium hydroxide buffer, 0.05 molar in 500 mL water); (e) pH 9.00 buffer solution (Fisher Scientific SB 114-500) (boric acid-potassium chloride-sodium hydroxide buffer, 0.1 molar in 500 mL water); and (f) 1 × D-PBS (ICN Biomedicals cat. no. 1860454, Solon, OH).

The pH values of these phosphate salt solutions were measured by a standard pH meter (Denver Instrument, Arvada, CO) at room temperature and are summarized in Table 1 and Table 2. It is clear that the pH value is a function of the concentration of a single phosphate solution. Higher phosphate concentrations lower the pH value of acid and raise the pH value of alkaline phosphate salts. Table 2 shows that the pH value of the mixture of KH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} solutions is a function of the relative concentration of acid and alkaline phosphate salts. These measurements were consistent with the accepted knowledge about the acid-base equilibrium.

**Cartilage Samples**

Several humeral heads from canine shoulder joints were harvested within 3 hours post mortem from dogs sacrificed for unrelated biomedical research. The animals were between 1 and 2 years old and musculoskeletally healthy. The humeral heads were cut into tissue slices (1.75 mm thick) using a diamond table saw. Three or 4 cartilage-bone blocks (about 1.75 × 2 × 10 mm) were obtained from the relatively flat part of each slice. Specimens were frozen in saline at -20 °C until imaging. Before MRI experiments, each specimen was removed from the freezer and soaked in deionized water for about 6 hours after thawing at room temperature. Samples were then sealed in a precision glass NMR tube with an internal diameter of 2.34 mm that was filled with deionized water. The MRI and DQF experiments were then carried out on these control specimens. After the control experiments, a series of MRI and DQF experiments were performed on the same specimen after the sample was soaked in a different solution for about 6 hours and sealed in the same NMR tube filled with the same solution. At least 3 specimens were used for each set of experiments. The cartilage block in the NMR tube was always oriented in such a way that the normal axis of the cartilage surface was parallel to the static magnetic field B\textsubscript{0} (unless indicated otherwise).

**Table 1. The pH Values of the Phosphate Salt Solutions**

| Concentration (mM) | pH (KH\textsubscript{2}PO\textsubscript{4}) | pH (Na\textsubscript{2}HPO\textsubscript{4}) | pH (K\textsubscript{2}HPO\textsubscript{4}) | pH (Na\textsubscript{2}HPO\textsubscript{4}) |
|-------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| 1.0               | 4.86                           | 4.92                           | 7.76                            | 7.76                            |
| 4.0               | 4.81                           | 4.87                           | 7.86                            | 7.87                            |
| 7.0               | 4.74                           | 4.83                           | 7.98                            | 7.98                            |
| 10.0              | 4.69                           | 4.78                           | 8.07                            | 8.10                            |
| 50.0              | 4.53                           | 4.61                           | 8.37                            | 8.33                            |
| 100.0             | 4.48                           | 4.54                           | 8.47                            | 8.44                            |

**Table 2. The pH Values of the KH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} Solutions**

| Solutions | Concentration ratio |
|-----------|-------------------|
| KH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} (mM/mM) | 0/10 | 2.18/10 | 4/10 | 10/10 | 20/10 |
| pH value  | 8.10 | 7.59 | 7.36 | 6.97 | 6.64 |
The NMR spectroscopic and microscopic MRI (µMRI) experiments were performed at room temperature on a Bruker AVANCE II 300 NMR spectrometer (Billerica, MA) equipped with a 7-T/89-mm-wide vertical-bore superconducting magnet and microimaging accessory (Billerica, MA). A homemade 4-mm solenoid coil was used for the experiments, in which the orientation of the cartilage block with respect to the static magnetic field can be adjusted. Proton DQF spectroscopic signals were acquired using the following pulse sequence:

\[
\begin{align*}
\tau & \quad \varphi_1 - 90^\circ - \varphi_2 - 180^\circ - \frac{\tau}{2} - 90^\circ - t_1 - 90^\circ - (\text{acquisition})
\end{align*}
\]

where a \(\tau\) value of 200 \(\mu\)s (creation time), a \(t_1\) value of 4 \(\mu\)s (evolution time), a 90° RF excitation pulse of about 5 \(\mu\)s, and a repetition time (TR) of 5 s were used.

Quantitative T2 imaging experiments were performed using a CPMG magnetization-prepared T2 imaging sequence, which separates the T2 weighting and spin echo imaging segments to obtain an accurate measurement of T2 in the tissue. The echo spacing in the CPMG T2-weighting segment was 1 ms. Five images with echo numbers of 2, 4, 10, 30, and 60, which corresponded to 5 T2 delays of 2, 4, 10, 30, and 60 ms, respectively, were acquired for cartilage in water (the echo number was varied according to the actual T2 value in tissue when the tissue was immersed in a different solution). The imaging parameters in the imaging segment were as follows: the echo time was 7.2 ms; the field of view was 3.2 mm × 3.2 mm; the imaging matrix size was 128 × 256 (256 was in the readout direction); the in-plane pixel size, which was across the depth of the cartilage tissue, was 13 \(\mu\)m; the spectral bandwidth was 50 kHz, corresponding to a 20 \(\mu\)s of readout sampling dwell time; 0.8-ms and 0.507-ms hermite shape pulses were used as excitation and refocusing pulse, respectively; and the TR of the imaging experiment was 2 s. From the T2-weighted intensity images, the T2 relaxation in the cartilage was calculated by a single exponential fitting of the data on a pixel-by-pixel basis, which assumes that there is only one T2 component in cartilage.

Results

Cartilage in \(H_2O\), \(NaCl\), \(KCl\), and \(CaCl_2\) Solutions

When articular cartilage is immersed in \(H_2O\) and placed in the magnet at 0° (the angle between the normal direction of the tissue surface and the direction of \(B_0\)), cartilage has a strong orientational-dependent laminar appearance in both its proton intensity image and quantitative T2 image, as shown in Figures 1a and b. This laminar appearance largely disappears when the tissue is oriented toward 55°, the magic
angle, due to the minimization of the dipolar interaction among the water in the tissue. The 1-dimensional (1-D) profiles of T\textsubscript{2} relaxation in cartilage from the central part of the tissue in these images are shown in Figure 1c.

The corresponding results from the proton NMR DQF spectroscopy are shown in Figure 1d. When the tissue block is immersed in H\textsubscript{2}O, the immersion H\textsubscript{2}O, as well as any free water inside the tissue, makes no contribution to the DQF signal because of their isotropically averaged nature. The DQF signal in the experiments comes exclusively from the residual dipolar coupling, originating from the H\textsubscript{2}O and/or macromolecules inside the tissue that are experiencing anisotropic motion. A strong and distinct proton DQF spectrum with a peak-peak splitting of about 150 Hz can typically be observed when the tissue is placed at 0° (Fig. 1d). In comparison, the DQF signal at 55° is nearly completely eliminated due to the minimization of the dipolar coupling at the magic angle (Fig. 1d). The small residual DQF signal at 55° might come from the fact that there are always some small parts of the tissue (e.g., the superficial zone) where dipolar interaction is not completely eliminated due to the local collagen fibril orientation. Because the influence of the dipolar coupling for protons in articular cartilage is minimal when the tissue is at 55°, only the results of the tissue at 0° are presented and discussed in the rest of this report.

Figure 2 shows the T\textsubscript{2} and DQF profiles of articular cartilage before and after the tissue block was immersed in the 154-mM NaCl solution (normal physiological saline). The Student t test was performed on this set of T\textsubscript{2} data, which returned a t probability of 0.9782, confirming that no significant change in T\textsubscript{2} could be found. We also carried out identical experiments for cartilage specimens immersed in KCl and CaCl\textsubscript{2} solutions and obtained nearly identical results (not shown).

**Cartilage in NaH\textsubscript{2}PO\textsubscript{4}, Na\textsubscript{2}HPO\textsubscript{4}, KH\textsubscript{2}PO\textsubscript{4}, and K\textsubscript{2}HPO\textsubscript{4} Solutions**

The proton µMRI T\textsubscript{2} anisotropy and NMR spectroscopic DQF experiments were carried out for cartilage specimens immersed in several sodium phosphate solutions and potassium phosphate solutions. Figure 3 shows a selected set of 1-D profiles of µMRI T\textsubscript{2} and spectroscopic DQF results in articular cartilage after the tissue was immersed in various concentrations of (a) NaH\textsubscript{2}PO\textsubscript{4} solutions (1 – 100 mM), (b) Na\textsubscript{2}HPO\textsubscript{4} solutions (1 – 100 mM), and (c) the mixture of KH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} solutions (0/10, 2.18/10, 4/10, 10/10, and 20/10 mM/mM). For tissue immersed in NaH\textsubscript{2}PO\textsubscript{4} (Fig. 3a), the T\textsubscript{2} profiles changed considerably, starting from concentrations as low as 1 mM. It is interesting to note in Figure 3a that at low concentrations, ions had little effect on the transition-zone cartilage when compared with its considerable effect on the superficial and deep cartilage. At high concentrations, the same ions caused considerable changes over all the 3 zones of cartilage: increasing the T\textsubscript{2} value in the superficial and deep-zone cartilage but decreasing the T\textsubscript{2} value in transition-zone cartilage. These sizeable changes of T\textsubscript{2} values were accompanied with a near disappearance of DQF signal in the spectroscopic experiments. For tissue immersed in Na\textsubscript{2}HPO\textsubscript{4} (Fig. 3b), only the T\textsubscript{2} values in the transitional zone of the tissue changed. The T\textsubscript{2} values in most of the deep tissue, corresponding to the radial zone of articular cartilage, did not change much within the 1 to 100 mM concentration range. These features of the proton T\textsubscript{2} profiles were accompanied by nearly consistent DQF signals for the Na\textsubscript{2}HPO\textsubscript{4} concentrations ranging between 1 and 50 mM. The experimental results for the cartilage specimens immersed in the potassium phosphate solutions were similar to those in sodium phosphate solutions (data not shown).

Several cartilage specimens were also immersed in various mixtures of KH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} solutions (0/10, 2.18/10, 4/10, 10/10, and 20/10 mM/mM). The results are shown in Figure 3c. It is interesting to note that the presence of Na\textsubscript{2}HPO\textsubscript{4} in the solution maintains T\textsubscript{2} anisotropy and DQF signal in the tissue, even in the presence of 4-mM KH\textsubscript{2}PO\textsubscript{4} (cf. the NaH\textsubscript{2}PO\textsubscript{4} cases in Fig. 3a). Another experiment
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revealed that the immersion of tissue in 2.18/10 mM/mM KH$_2$PO$_4$/Na$_2$HPO$_4$ solution (the concentration ratio of these 2 phosphate salts in D-PBS solution) recovers the T2 value.

Cartilage in Commercial Buffer Solutions with Different pH Values

The cartilage tissues were immersed in several commercial buffer solutions with different pH values (Fig. 4). Except for the D-PBS solution, all other buffer solutions had considerable effects on the proton dynamics in articular cartilage, but the extent of these effects was different for different buffers. Both weak acidic (pH, 5) and neutral buffers (pH, 7) increased T2 values in the superficial and deep zones but changed little in the transition zone. Alkaline buffers (pH, 9) decreased the T2 value in the transition zone. The DQF signals in these buffer solutions (not D-PBS) also nearly vanished, qualitatively consistent with the experimental results in sodium and potassium phosphate solutions.

Cartilage in Fixation Solutions (10% Formalin and Glutaraldehyde)

Figure 5 shows 1-D T2 relaxation profiles and the spectroscopic DQF signal in cartilage after immersion in 10%
Figure 4. Microscopic magnetic resonance imaging (μMRI) $T_2$ anisotropy and nuclear magnetic resonance (NMR) double quantum-filtered (DQF) signals when the tissue was immersed in several commercial buffers with different pH values.
formalin and glutaraldehyde solutions, respectively. It is clear that both formalin and glutaraldehyde fixation solutions cause the proton dynamics in cartilage to change. Exposure to fixation solutions yielded similar results to those of high concentration NaH$_2$PO$_4$: increased T2 value in the superficial and deep zones but decreased T2 value in the transitional zone with a disappearance of the DQF signal.

**Discussion**

The proton dynamics in articular cartilage was measured by 2 different techniques in this project: MRI T2 anisotropy (an imaging approach) and NMR DQF spectroscopy (a bulk measurement). The strong anisotropy of T2 relaxation in articular cartilage is known to arise from the nonzero averaging of the dipolar interaction among the ordered molecules in the tissues.$^{17}$ This anisotropic T2 relaxation is depth dependent because the collagen fibril orientation in articular cartilage is organized into 3 structural zones. At the magic angle, the minimization of the dipolar interaction in the radial zone of cartilage restores the T2 relaxation to approximately isotropic, which eliminates the laminar appearance of articular cartilage in MRI.$^5$ The proton DQF signal in cartilage has a similar physical origin to that of T2 anisotropy, also arising from the residual dipolar interaction associated with the anisotropic averaging of the molecular motions. The unique feature of the proton DQF signal is its exclusive sensitivity to the anisotropic motion; the DQF signal comes entirely from the molecules experiencing anisotropic motion and contains no contribution from the large pool of molecules experiencing isotropic motion (hence, the meaning of “filtering” in the name of the pulse sequence, DQF).

**Effect of Phosphate Salts on Proton Dynamics in Cartilage**

As early as the 1960s, Berendsen and Migchelsen$^{23,24}$ noticed that the addition of certain salts containing protons that donate or accept ions to hydrated collagen could influence the proton NMR spectra in the same way as raising the temperature or increasing the water content. This phenomenon can be explained by the fact that these salts act as exchange catalysts to accelerate the proton exchange among different pools of protons.$^{35,36}$ Because the proton exchange rate of water is known to be sensitive to pH value$^{37,38}$ and buffer concentration,$^{35,39}$ the effect of these salts on NMR spectra must also be sensitive to pH and buffer concentration. While some salts such as NaCl were found to have little effect,$^{23,24}$ inorganic phosphate was found to be the most effective exchange catalyst.$^{25-29}$ These conclusions are...
supported by Liepins et al.,26 who found that proton exchange rate was pH and temperature dependent. They also found that although phosphate, carbonate, carboxyl groups, and amino groups can work as exchange catalysts to accelerate the proton exchange, inorganic phosphate is the most effective. More recently, pH-sensitive T2 relaxation time (and chemical shift) was used to track the change in pH in the food industry, to study skeletal muscle after exercising, and to detect cancer and other pathologies.30

A previous μMRI study14 had found only one T2 component in bovine nasal cartilage (BNC, which has a relatively random fibril structure) and canine articular cartilage (in a resolution of 26 μm over the entire tissue from the superficial zone to the radial zone). These observations suggest that the proton exchange between the bound water and free water in cartilage must be faster than the spin relaxation,16 resulting in our inability to observe the 2 individual populations. The results from the current project reveal that the effects of phosphate salt as an exchange catalyst in articular cartilage are not only pH and concentration dependent but also tissue structure dependent.

In articular cartilage, where the collagen fibrils are well organized and hence under a strong influence of dipolar interaction (e.g., the tissue in the superficial and radial zones of articular cartilage at 0°), the T2 value is short when the tissue is immersed in pure water or saline solution. This short T2 is a result of the strong intramolecular proton dipolar interaction that dominates the relaxation scheme. When immersed in acid potassium or sodium phosphate solutions (e.g., Fig. 3a), the ions accelerate the proton exchange not only between the bound water and free water but also among each proton population, resulting in an increased T2 value. The nearly completely vanishing of the DQF signal at a concentration as low as 1 mM supports the concept that acid phosphate salt is a very effective catalyst for the proton exchange for this part of tissue that otherwise has a strong dipolar influence. However, the alkaline phosphate salt is not an effective catalyst for the proton exchange in the organized tissue (e.g., Fig. 3b). This implies that the dipolar interaction remains as the dominant mechanism for proton dynamics. The near consistency of a strong DQF signal (Fig. 3b) for the 1 to 50 mM solutions of Na2HPO4 supports this conclusion.

In areas where the collagen fibrils are relatively randomly organized (e.g., in the transitional zone of articular cartilage [e.g., depth of 80-120 μm in Fig. 2a] and in bovine nasal cartilage), the T2 relaxation is long when the tissue is immersed in pure water or saline solution. This long T2 is the consequence of the relative random fibril organization, which results in relative random motion of protons and weak dipolar interaction in the tissue. When this relative random tissue is immersed in phosphate solutions (either acid phosphate salt solution or alkaline phosphate salt solution), the T2 of the tissue becomes a function of the solution’s pH (or salt concentration). This concentration-dependent feature might be ascribed to the fact that, for randomly structured tissues having a weak dipolar interaction, any increase in ion content, whether acidic or alkaline, could result in decreased water concentration in the tissue. As a result, the ion equilibrium between the tissue and the immersed solution was altered (which reduces T2 value).41,42

**Effect of Fixation Solution on Proton Dynamics in Cartilage**

Recently, Fishbein et al. found that T2 value in bovine nasal cartilage decreased by 59.4% upon fixation in 10% formalin.43 This was attributed to the “noncovalent binding or chemical exchange of free water with polyoxymethylene oligomers formed when formaldehyde is dissolved in water.”43,44 In our experiments, the T2 value in the transition zone of articular cartilage was also reduced by about 50% after being immersed in the fixation solutions (10% formalin or glutaraldehyde). In addition, we also found that the T2 value in the superficial and radial zones of articular cartilage increased upon fixation. This phenomenon could be explained by the fixation-induced cross-linking in the tissue’s macromolecules, which disrupts the otherwise ordered collagen organization in the superficial and radial zones of articular cartilage. This was supported by the marked reduction of the DQF intensity, indicating a reduced dipolar interaction in a less ordered fibril structure.

In conclusion, this in vitro microscopic imaging study reports acid phosphate salts to be an effective catalyst that accelerates the proton exchange among water molecules in areas where the collagen matrix is organized and the dipolar interaction is dominant. However, alkaline phosphate salt was not an effective catalyst for the proton exchange in the organized tissue. Where collagen fibril structure is less ordered, both acid and alkaline phosphate salts have no significant effect on the T2 value at low concentrations. At high concentrations, however, these salts decrease the T2 value as water concentration in the tissue is reduced.

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**References**

1. Maroudas A, Venn M. Chemical composition and swelling of normal and osteoarthritic femoral head cartilage. II: swelling. Ann Rheum Dis. 1977;36(5):399-406.
2. Jeffery AK, Blunn GW, Archer CW, Bentley G. Three-dimensional collagen architecture in bovine articular cartilage. J Bone Joint Surg. 1991;73B(5):795-801.
3. Buckwalter JA, Mankin HJ. Articular cartilage. Part I: tissue design and chondrocyte-matrix interactions. J Bone and Joint Surgery. 1997;79A:600-11.
4. Hayes CW, Parelleda JA. The magic angle effect in musculoskeletal MR imaging. Topics Magn Reson Imaging. 1996;8(1):51-6.
5. Xia Y. Magic angle effect in MRI of articular cartilage: a review. Invest Radiol. 2000;35(10):602-21.
6. Mosher TJ, Smith H, Dardzinski BJ, Schmithorst VJ, Smith MB. MR imaging and T2 mapping of femoral cartilage: in vivo determination of the magic angle effect. Am J Roentgen. 2001;177:665-9.
7. Arokoski JP, Hytinen MM, Lapveteläinen T, Takacs P, Kosztaczky B, Modis L, et al. Decreased birefringence of the superficial zone collagen network in the canine knee (stifle) articular cartilage after long distance running training, detected by quantitative polarized light microscopy. Ann Rheum Dis. 1996;55(4):253-64.
8. Xia Y, Moody J, Burton-Wurster N, Lust G. Quantitative in situ correlation between microscopic MRI and polarized light microscopy studies of articular cartilage. Osteoarthritis Cartilage. 2001;9(5):393-406.
9. Xia Y, Ramakrishnan N, Bidhanapally A. The depth-dependent anisotropy of articular cartilage by Fourier-transform infrared imaging (FTIRI). Osteoarthritis Cartilage. 2007;15(7):780-8.
10. Ramakrishnan N, Xia Y, Bidhanapally A. Polarized IR microscopic imaging of articular cartilage. Phys Med Biol. 2007;52(15):4601-14.
11. Bredella MA, Tirman PF, Peterfy CG, Zarlingo M, Feller JF, Bost FW, et al. Accuracy of T2-weighted fast spin-echo MR imaging with fat saturation in detecting cartilage defects in the knee: comparison with arthroscopy in 130 patients. Am J Roentgenol. 1999;172(4):1073-80.
12. Alhadjagh H, Xia Y, Moody JB, Matyas J. Detecting structural changes in early experimental osteoarthritits of tibial cartilage by microscopic MRI and polarized light microscopy. Ann Rheum Dis. 2004;63(6):709-17.
13. Xia Y. Relaxation anisotropy in cartilage by NMR microscopy (μMRI) at 14 μm resolution. Magn Reson Med. 1998;39(6):941-9.
14. Fullerton GD, Cameron IL, Ord VA. Orientation of tendons in the magnetic field and its effect on T2 relaxation times. Radiology. 1985;155:433-5.
15. Peto S, Gillis P, Henri VP. Structure and dynamics of water in tendon from NMR relaxation measurements. Biophys J. 1990;57(1):71-84.
16. Henkelman RM, Stanisz GJ, Kim JK, Bronskill MJ. Anisotropy of NMR properties of tissues. Magn Reson Med. 1994;32:592-601.
17. Xia Y, Farquhar T, Burton-Wurster N, Lust G. Origin of cartilage laminae in MRI. J Magn Reson Imaging. 1997;7(5):887-94.
18. Shinar H, Eliav U, Keinan-Adamsky K, Navon G. The effect of refocusing the dipolar interaction on the measured T2 of articular cartilage. Proc Intl Soc Magn Reson Med. 2002;64. Available from: http://cds.ismrm.org/ismrm-2002/PDF1/0064.PDF
19. Akella SV, Regatte RR, Wheaton AJ, Borthakur A, Reddy R. Reduction of residual dipolar interaction in cartilage by spinlock technique. Magn Reson Med. 2004;52(5):1103-9.
20. Young IR, Bydder GM. Magnetic resonance: new approaches to imaging of the musculoskeletal system. Physiol Meas. 2003;24(4):R1-23.
21. Gatehouse PD, Thomas RW, Robson MD, Hamilton G, Herlihy AH, Bydder GM. Magnetic resonance imaging of the knee with ultrashort TE pulse sequences. Magn Reson Imaging. 2004;22(8):1061-7.
22. Zheng S, Xia Y. Effect of phosphate electrolyte buffer on the dynamics of water in tendon and cartilage. NMR Biomed. 2009;22(2):158-64.
23. Berendsen HJC, Miegelensen C. Hydration structure of fibrous macromolecules. Annals N Y Acad Sci. 1965;125:365-79.
24. Berendsen HJ, Miegelensen C. Hydration structure of collagen and influence of salts. Fed Proc. 1966;25(3):998-1002.
25. Carpenter KA, Wilkes BC, Schiller PW. Influence of sample pH on the conformational backbone dynamics of a pseudopeptide (H-Tyr-Tic psi [CH2-NH]Phe-OH) incorporating a reduced peptide bond: an NMR investigation. Biopolymers. 1995;36(6):735-49.
26. Liepinsh E, Otting G. Proton exchange rates from amino acid side chains: implications for image contrast. Magn Reson Med. 1996;35(1):30-42.
27. Mori S, Eleff SM, Pilatus U, Mori N, van Zijl PC. Proton NMR spectroscopy of solvent-saturable resonances: a new approach to study pH effects in situ. Magn Reson Med. 1998;40(1):36-42.
28. Denisov VP, Halle B. Hydrogen exchange rates in proteins from water (1)H transverse magnetic relaxation. J Am Chem Soc. 2002;124(35):10264-5.
29. Zhou J, Payen JF, Wilson DA, Traystman RJ, van Zijl PC. Using the amide proton signals of intracellular proteins and peptides to detect pH effects in MRI. Nat Med. 2003;9(8):1085-90.
30. Tsoref L, Shinar H, Navon G. Observation of a 1H double quantum filtered signal of water in biological tissues. Magn Reson Med. 1998;39(1):11-7.
31. Seo Y, Ikoma K, Takamiya H, Kusaka Y, Tsoref L, Eliav U, et al. 1H double-quantum-filtered MR imaging as a new tool
for assessment of healing of the ruptured Achilles tendon.  
Magn Reson Med. 1999;42(5):884-9.

32. Dulbecco R, Vogt M. Plaque formation and isolation of pure  
lines with poliomyelitis viruses. J Exp Med. 1954;99(2):  
167-82.

33. Xia Y, Moody J, Alhadlaq H. Orientational dependence of T2  
relaxation in articular cartilage: a microscopic MRI (μMRI)  
study. Magn Reson Med. 2002;48(3):460-9.

34. Zheng S, Xia Y. Multi-components of T2 relaxation in ex vivo  
cartilage and tendon. J Magn Reson. 2009;198(2):188-96.

35. Dehl RE, Hoeve CA. Broad-line NMR study of H2O and D2O  
in collagen fibers. J Chem Phys. 1969;50(8):3245-51.

36. Mïchelsen C, Berendsen HJC. Proton exchange and molecular  
orientation of water in hydrated collagen fibers: an NMR study  
of H2O and D2O. J Chem Phys. 1973;59(1):296-305.

37. Meiboom S. Nuclear magnetic resonance study of the proton  
transfer in water. J Chem Phys. 1961;34(2):375-88.

38. Berger A, Loewenstein A, Meiboom S. Nuclear magnetic  
resonance study of the protolysis and ionization of N-methyl-  
acetamide. J Am Chem Soc. 1959;81:62-7.

39. Luz Z, Meiboom S. Nuclear magnetic resonance study of the  
protolysis of trimethylammonium ion in aqueous solution:  
order of the reaction with respect to solvent. J Chem Phys.  
1963;39:366-70.

40. Gillies RJ, Raghunand N, Garcia-Martin ML, Gatenby RA. pH  
imaging: a review of pH measurement methods and applica-  
tions in cancers. IEEE Eng Med Biol Mag. 2004;23(5):57-64.

41. Lüsse S, Knauss R, Werner A, Gründer W, Arnold K. Action  
of compression and cations on the proton and deuterium  
relaxation in cartilage. Magn Reson Med. 1995;33:483-9.

42. Donnan FG. The theory of membrane equilibria. Chem Rev.  
1924;1:73-90.

43. Fishbein KW, Gluzband YA, Kaku M, Ambia-Sobhan H,  
Shapses SA, Yamauchi M, et al. Effects of formalin fixation  
and collagen cross-linking on T2 and magnetization transfer in  
bovine nasal cartilage. Magn Reson Med. 2007;57(6):1000-11.

44. Thelwall PE, Shepherd TM, Stanisz GJ, Blackband SJ. Effects  
of temperature and aldehyde fixation on tissue water diffusion  
properties, studied in an erythrocyte ghost tissue model. Magn  
Reson Med. 2006;56(2):282-9.