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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) T_2 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 T_2 value at low concentration but decrease the T_2 value at high concentration. The solutions of NaCl, KCl, $CaCl_2$, and D-PBS were found to have no significant effect on T_2 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, T_2 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.[1-3] 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 fact[13] that the anisotropic characteristics of MRI T_2 relaxation in articular cartilage quantitatively follow the geometric factor in the dipolar Hamiltonian ($3\cos^2\theta - 1$) endorses the concept that T_2 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 T_2 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] This

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Table 1. The pH Values of the Phosphate Salt Solutions

Concentration (mM)	pH (KH_2PO_4)	pH (NaH_2PO_4)	pH (K_2HPO_4)	pH (Na_2HPO_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

hypothesis was supported by additional nuclear magnetic resonance (NMR) spectroscopy experiments[22] using the proton double quantum–filtered (DQF) sequence,[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_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 $CaCl_2$; (2) 1 mM, 4 mM, 7 mM, 10 mM, 50 mM, and 100 mM solutions of Na_2HPO_4, NaH_2PO_4, K_2HPO_4, and KH_2PO_4 (these chemical components are contained in the recipe of Dulbecco's PBS [D-PBS][32]); (3) the mixtures of KH_2PO_4/Na_2HPO_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_2PO_4/Na_2HPO_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.

Table 2. The pH Values of the KH_2PO_4/Na_2HPO_4 Solutions

Solutions	Concentration ratio				
KH_2PO_4/Na_2HPO_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

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 $\mathbf{B}_0$ (unless indicated otherwise).

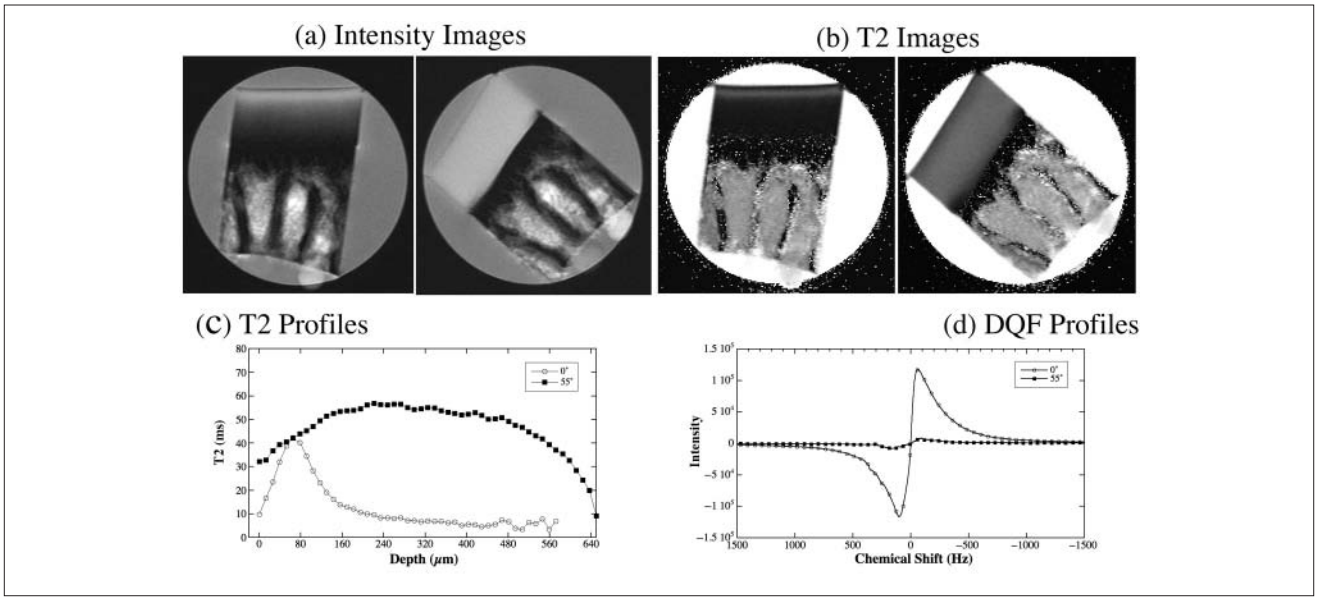

Figure 1. Microscopic magnetic resonance imaging (μMRI) intensity (**a**) and T_2 images (**b**) when the tissue was immersed in pure water. The T_2 profiles from the images are shown (**c**). The proton signals of nuclear magnetic resonance (NMR) double quantum–filtered (DQF) spectroscopy from the tissue blocks immersed in water (**d**). Note that μMRI and NMR measurements are orientational dependent. The 0° direction causes the cartilage to have the laminar appearance in MRI. The direction of the external magnetic field (B_0) was pointing upward. The depth of 0 μm is the articular surface of the cartilage; the depth of approximately 600 μm is the interface between articular cartilage and the underlining bone.

NMR Spectroscopy and Microscopic MRI (μMRI)

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.[33]

Proton DQF spectroscopic signals were acquired using the following pulse sequence[22,30,31]:

$$90^\circ_{\phi 1} - \frac{\tau}{2} - 180^\circ_{\phi 2} - \frac{\tau}{2} - 90^\circ_{\phi 1} - t_1 - 90^\circ_{\phi 3} - (acquisition), \quad (1)$$

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

Quantitative T_2 imaging experiments were performed using a CPMG magnetization-prepared T_2 imaging sequence,[34] which separates the T_2 weighting and spin echo imaging segments to obtain an accurate measurement of T_2 in the tissue. The echo spacing in the CPMG T_2-weighting segment was 1 ms. Five images with echo numbers of 2, 4, 10, 30, and 60, which corresponded to 5 T_2 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 T_2 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 μm; the spectral bandwidth was 50 kHz, corresponding to a 20 μs 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 T_2-weighted intensity images, the T_2 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 T_2 component in cartilage.[13,34]

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 $\mathbf{B_0}$), cartilage has a strong orientational-dependent laminar appearance in both its proton intensity image and quantitative T_2 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_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_2O, the immersion H_2O, 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_2O 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_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_2 data, which returned a *t* probability of 0.9782, confirming that no significant change in T_2 could be found. We also carried out identical experiments for cartilage specimens immersed in KCl and $CaCl_2$ solutions and obtained nearly identical results (not shown).

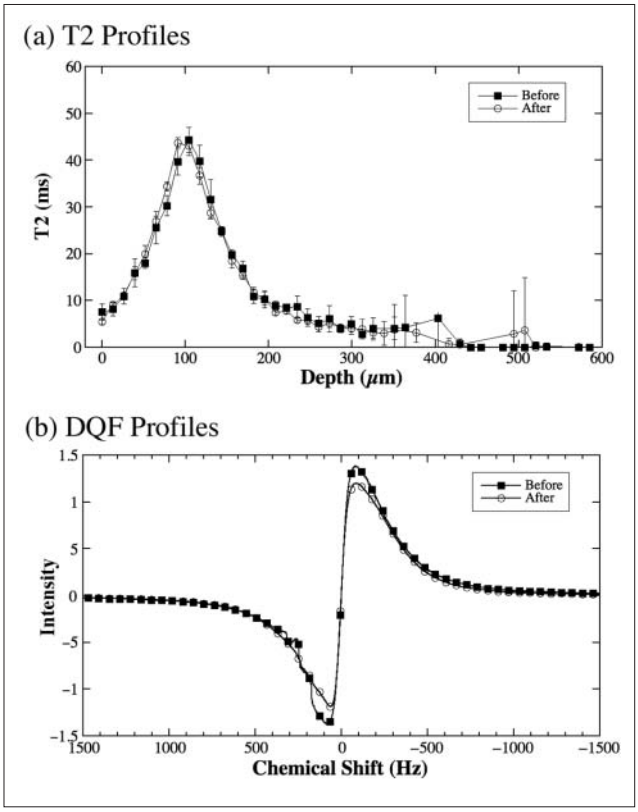

Figure 2. Microscopic magnetic resonance imaging (μMRI) T_2 anisotropy and nuclear magnetic resonance (NMR) double quantum–filtered (DQF) signals when the tissue was immersed in pure water (before) and in normal saline (after), when the tissue was oriented at 0°.

Cartilage in NaH_2PO_4, Na_2HPO_4, KH_2PO_4, and K_2HPO_4 Solutions

The proton μMRI T_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_2 and spectroscopic DQF results in articular cartilage after the tissue was immersed in various concentrations of (a) NaH_2PO_4 solutions (1 – 100 mM), (b) Na_2HPO_4 solutions (1 – 100 mM), and (c) the mixture of KH_2PO_4/Na_2HPO_4 solutions (0/10, 2.18/10, 4/10, 10/10, and 20/10 mM/mM). For tissue immersed in NaH_2PO_4 (**Fig. 3a**), the T_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_2 value in the superficial and deep-zone cartilage but decreasing the T_2 value in transition-zone cartilage. These sizeable changes of T_2 values were accompanied with a near disappearance of DQF signal in the spectroscopic experiments. For tissue immersed in Na_2HPO_4 (**Fig. 3b**), only the T_2 values in the transitional zone of the tissue changed. The T_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_2 profiles were accompanied by nearly consistent DQF signals for the Na_2HPO_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_2PO_4/Na_2HPO_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_2HPO_4 in the solution maintains T_2 anisotropy and DQF signal in the tissue, even in the presence of 4-mM KH_2PO_4 (cf. the NaH_2PO_4 cases in **Fig. 3a**). Another experiment

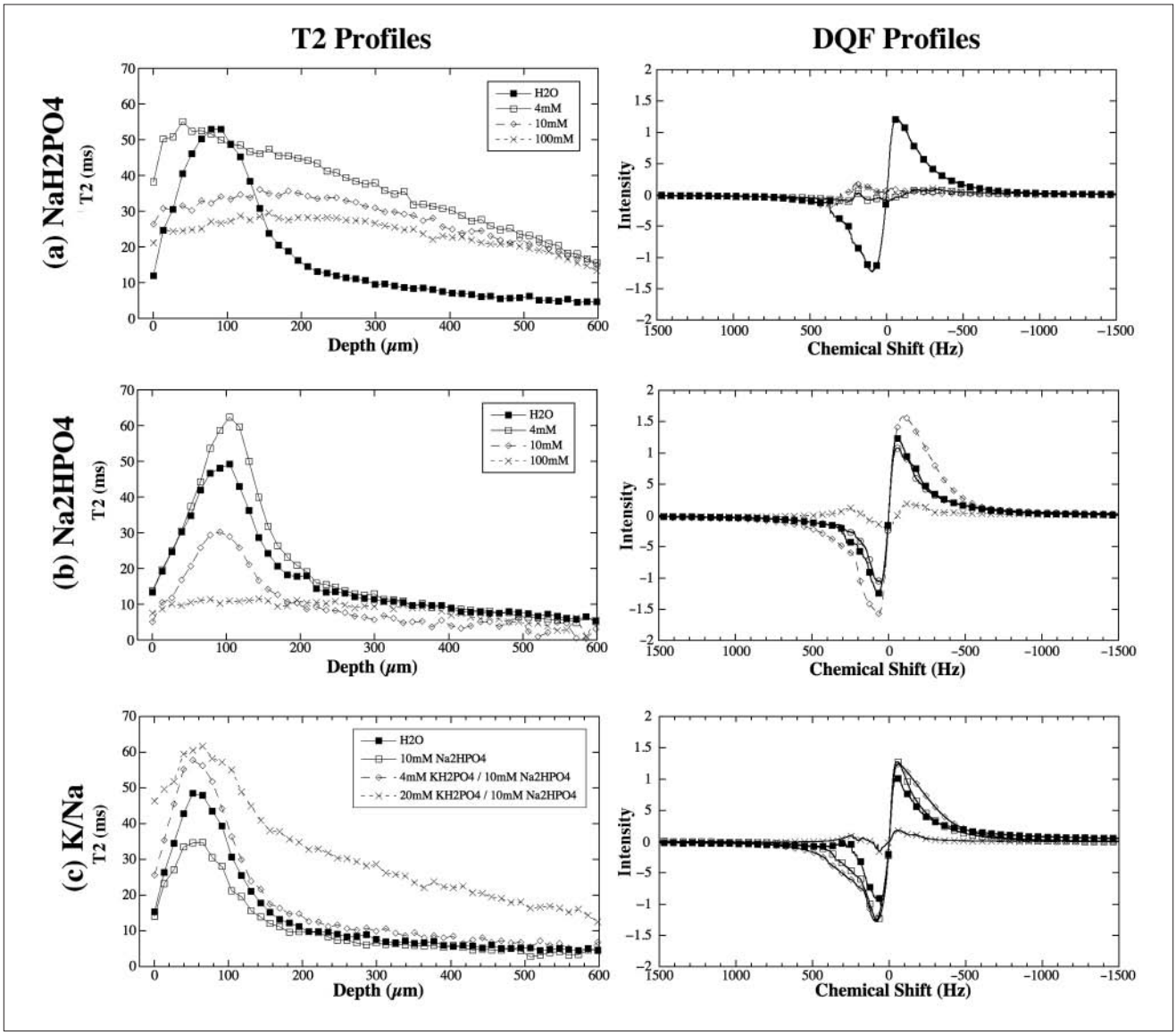

Figure 3. Microscopic magnetic resonance imaging (μMRI) T_2 anisotropy and nuclear magnetic resonance (NMR) double quantum–filtered (DQF) signals when the tissue was immersed in phosphate salt solutions.

(not shown) revealed that the immersion of tissue in 2.18/10 mM/mM KH_2PO_4/Na_2HPO_4 solution (the concentration ratio of these 2 phosphate salts in D-PBS solution[32]) recovers the T_2 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 T_2 values in the superficial and deep zones but changed little in the transition zone. Alkaline buffers (pH, 9) decreased the T_2 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 T_2 relaxation profiles and the spectroscopic DQF signal in cartilage after immersion in 10%

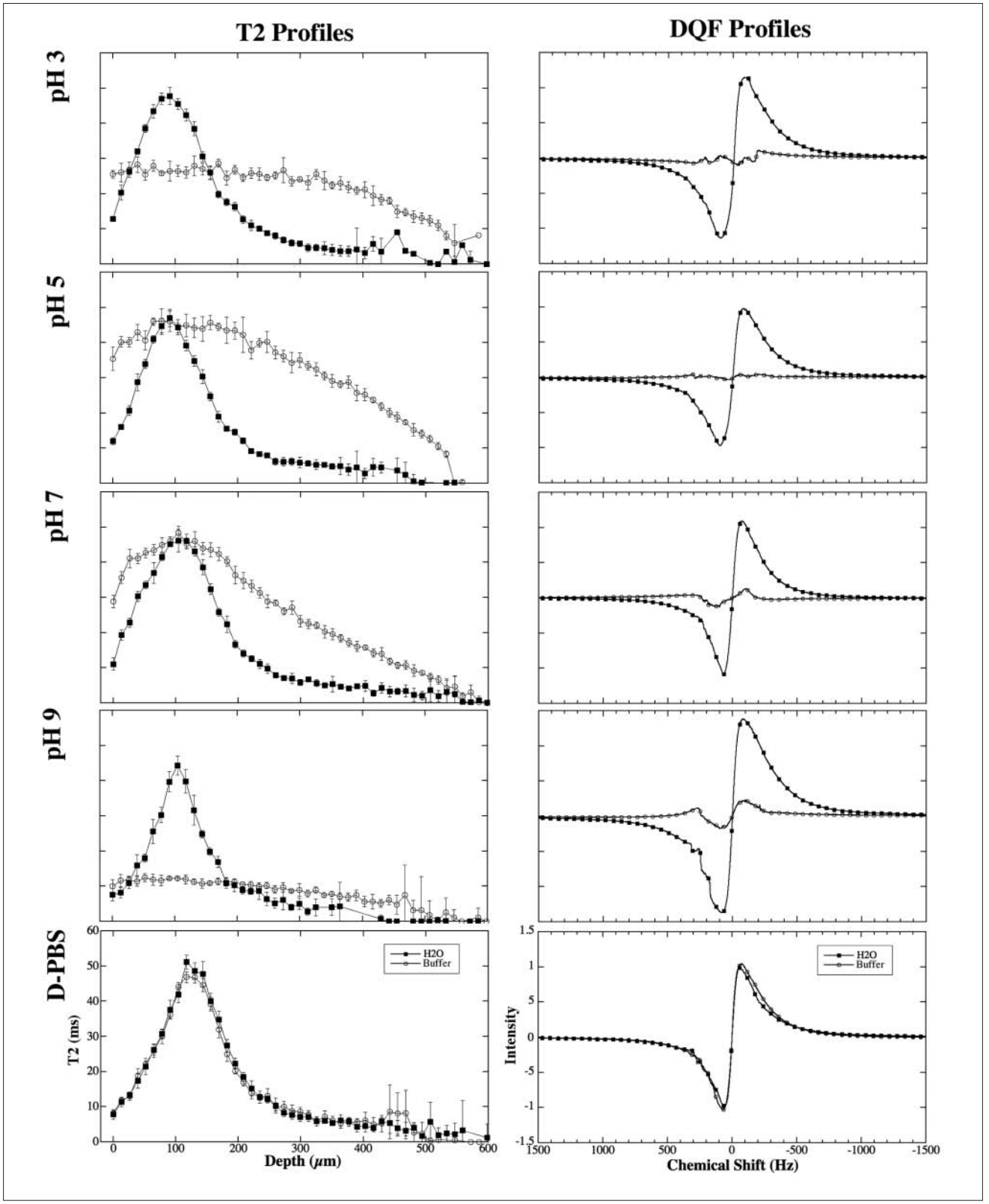

Figure 4. Microscopic magnetic resonance imaging (μMRI) T_2 anisotropy and nuclear magnetic resonance (NMR) double quantum–filtered (DQF) signals when the tissue was immersed in several commercial buffers with different pH values.

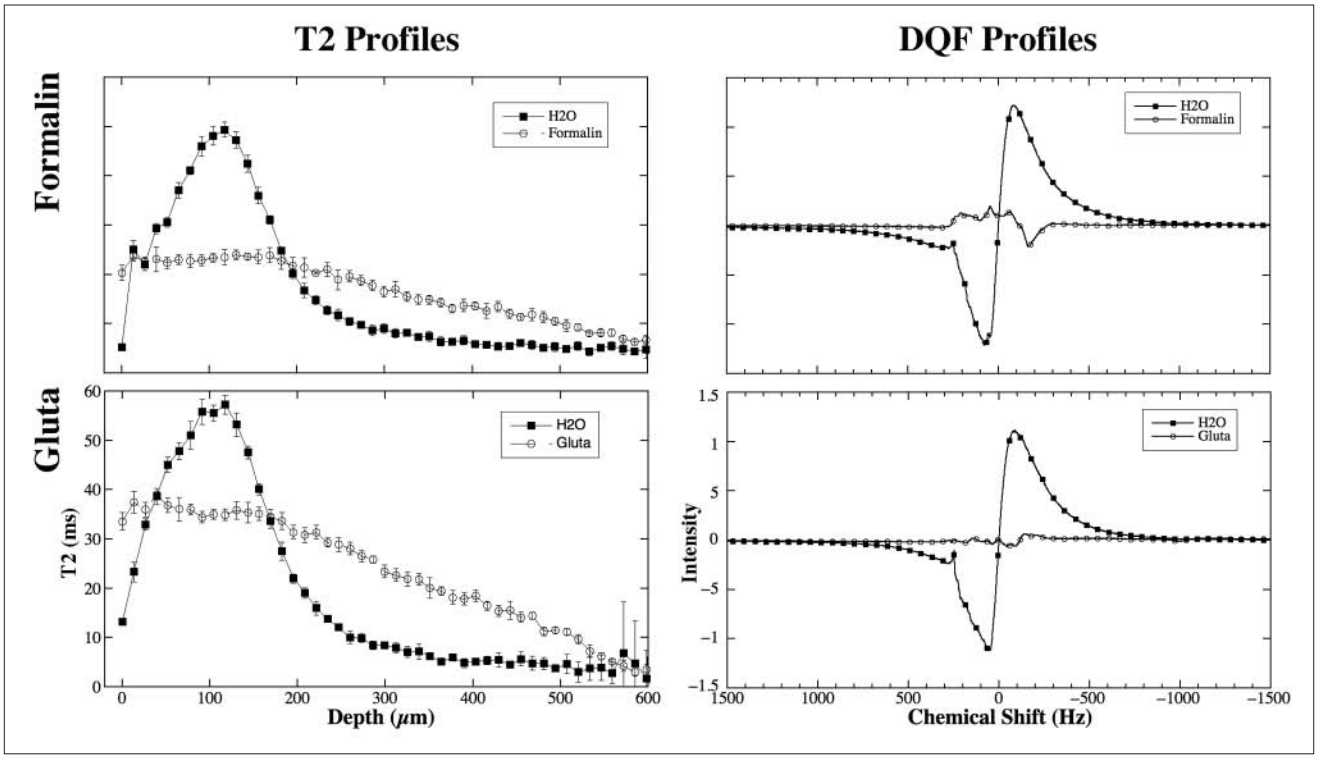

Figure 5. Microscopic magnetic resonance imaging (μMRI) T_2 anisotropy and nuclear magnetic resonance (NMR) double quantum–filtered (DQF) signals when the tissue was immersed in 2 fixation solutions.

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_2PO_4: increased T_2 value in the superficial and deep zones but decreased T_2 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 T_2 anisotropy (an imaging approach) and NMR DQF spectroscopy (a bulk measurement). The strong anisotropy of T_2 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 T_2 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 T_2 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 T_2 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 Liepinsh 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 T_2 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.[40]

A previous μMRI study[34] had found only one T_2 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 T_2 value is short when the tissue is immersed in pure water or saline solution. This short T_2 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 T_2 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 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 Na_2HPO_4 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 T_2 relaxation is long when the tissue is immersed in pure water or saline solution. This long T_2 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 T_2 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 T_2 value).[41,42]

Effect of Fixation Solution on Proton Dynamics in Cartilage

Recently, Fishbein et al. found that T_2 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 T_2 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 T_2 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 T_2 value at low concentrations. At high concentrations, however, these salts decrease the T_2 value as water concentration in the tissue is reduced.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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