Magnetic Resonance Water Proton Relaxation in Protein Solutions and Tissue: T$_{1p}$ Dispersion Characterization

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Abstract

**Background:** Image contrast in clinical MRI is often determined by differences in tissue water proton relaxation behavior. However, many aspects of water proton relaxation in complex biological media, such as protein solutions and tissue are not well understood, perhaps due to the limited empirical data.

**Principal Findings:** Water proton T$_1$, T$_2$, and T$_{1p}$ of protein solutions and tissue were measured systematically under multiple conditions. Crosslinking or aggregation of protein decreased T$_2$ and T$_{1p}$ but did not change high-field T$_1$. T$_{1p}$ dispersion profiles were similar for crosslinked protein solutions, myocardial tissue, and cartilage, and exhibited power law behavior with T$_{1p}(0)$ values that closely approximated T$_2$. The T$_{1p}$ dispersion of mobile protein solutions was flat above 5 kHz, but showed a steep curve below 5 kHz that was sensitive to changes in pH. The T$_{1p}$ dispersion of crosslinked BSA and cartilage in DMSO solvent closely resembled that of water solvent above 5 kHz but showed decreased dispersion below 5 kHz.

**Conclusions:** Proton exchange is a minor pathway for tissue T$_1$ and T$_{1p}$ relaxation above 5 kHz. Potential models for relaxation are discussed, however the same molecular mechanism appears to be responsible across 5 decades of frequencies from T$_{1p}$ to T$_1$.

Introduction

Image contrast in clinical MRI is often determined by differences in tissue water relaxation behavior. Although the observed properties of proton relaxation in homogeneous liquids such as pure water, ethanol, and glycerol have been successfully explained by the theory of Bloembergen, Purcell, and Pound (BPP) [1], the mechanism of water relaxation in more complex environments such as tissues is still highly speculative. In part to gain insight into tissue relaxation, many studies have evaluated the relaxation characteristics of protein solutions, since for most tissue, relaxation behavior is dominated by the water-macromolecule interaction [2]. However, few studies have attempted to systematically investigate the relationship between the physico-chemical properties of macromolecules and bulk water relaxation, and there are diverse hypotheses concerning the mechanism of water proton relaxation in protein systems—perhaps due to the limited empirical data.

Of particular interest has been the character of the magnetic field dependence (dispersion) of relaxation in these protein systems. Prior investigations have shown that solutions of immobile proteins have spin-lattice relaxation dispersion characteristics similar to that of various soft tissues [3,4]. Most of these studies measured T$_1$ at low field (<20 MHz) or T$_{1p}$ (spin-lattice relaxation time in the rotating frame, which is measured at B$_1$ field strength [5,6]) since it is known that water proton T$_1$ at high field is insensitive to significant protein structural changes such as the addition of crosslinks [7]. Rationale for the improved sensitivity of low-field dispersion to detect protein or tissue structural changes includes arguments concerning the long correlation times of motion ($\tau_c$) in systems containing large macromolecules [8]. For instance, T$_{1p}$ will presumably be sensitive to motion with $\tau_c$ on the order of tens of microseconds, depending on the achievable RF power and proton solvent linewidth, respectively. However, the determination of motional correlation times—whether single, multiple, or even a continuous distribution—requires assumptions about the characteristic shape of the spectral density function. More recently, several investigators have suggested that conventional, BPP-type relaxation theory is inadequate to explain the low field dispersion behavior of solutions of immobilized proteins or tissues, the implications obviously relating to the validity of previous analyses of molecular motion in these systems [6,9,10]. For example, Brown and Koenig proposed that the observed low-field dispersion of T$_1$ and T$_{1p}$ of tissue water protons is unrelated to a specific correlation time but rather is due to a field dependence of magnetization transfer between water protons and solid-state broadened protein protons [6]. In any case, further data relating specific structural and/or chemical properties of various tissues and protein solutions with properties of water relaxation will be...
essential to clarify the contributing processes that lead to tissue water relaxation.

In this study, the \( T_1 \), \( T_2 \), and \( T_1p \) dispersion of solvent protons in solutions of Bovine Serum Albumin (BSA) were evaluated in detail under conditions of varying crosslink density of proteins, pH, solvents, methylation of proteins, and \( B_0 \) field strength. The results were compared with a similar evaluation of myocardial tissue and cartilage. In addition, the \( T_1p \) dispersion profiles of both BSA solutions and tissue were analyzed for simple power law or BPP model characteristics. There were two aims: first, to provide data relating water relaxation in protein solutions and tissue to variations in macromolecular environment and structure, and second, to evaluate molecular models of tissue water relaxation using \( T_1p \) dispersion analysis.

**Materials and Methods**

**Experimental Preparation: BSA**

Relaxation characteristics were studied using fraction V albumin, which is a mixture of different molecular weight BSA (Sigma Chemical), chromatographically purified monomer BSA (98% pure, Sigma), and dimer BSA (95% pure, Sigma).

**Crosslinking.** Variations in BSA crosslink density were produced by reacting 10% (1.5 mM) or 20% (3.0 mM) solutions of BSA with different amounts of glutaraldehyde (from 10 to 200 mM GA). In order to control for changes in small solute (i.e. various forms of unreacted GA), a series of BSA samples were reacted with GA, at concentrations of 10 mM to 60 mM, and then dialyzed (3.5 kDa cutoff) in excess distilled and deionized H\(_2\)O. Grade I GA (50% aqueous solution of pure monomeric GA, stored at \( \pm 20^\circ\)C, Sigma) was used for all experiments at 4.7 Tesla and for experiments at 2 Tesla with BSA monomers and dialyzed samples. Grade II GA (25% aqueous solution of monomeric and small quantities of polymeric GA, 25\(^\circ\)C, Sigma) was used for fraction V BSA experiments at 2 Tesla. Care was taken to maintain consistent reaction times (\( \pm 8 \) hrs for all experiments) before NMR measurements were obtained.

**Validation.** Samples of BSA reacted with varying quantities of GA were analyzed with polyacrylamide gel electrophoresis (PAGE) to document progressive increases in BSA molecular weight with increases in [GA]. A standard SDS (Sodium Dodecylsulfate) reducing buffer (Fig. 1a) or a non-denaturing buffer without SDS (Fig. 1b and 1c) was used. Note in lane 1 of figure 1a that purified monomer BSA migrates to a single band near 70 kDa. Lane 2 of figure 1a shows that BSA dimers can exhibit multiple bands. Lanes 4, 5, and 7–10 clearly demonstrate that reactions with increasing quantities of GA resulted in the increase not only in the molecular weight of the largest species detected but also in the relative amount of larger to smaller species of BSA. Specifically, as [GA] increases from lane 4 to 10, the monomer band becomes fainter while higher molecular weight species (first BSA in the dimer range, then BSA between 250 and 300 kDa, then BSA polymers that cannot migrate past the 2% stacking gel) become stronger. Figure 1b demonstrates that non-crosslinked, fraction V BSA (lane 2) is composed of a mixture of albumin with different molecular weights unlike purified monomer BSA. In contrast, the strongest bands in the crosslinked, fraction V BSA samples were above 200 kDa. Lanes containing methylated BSA will be described below in Results.

![Figure 1. Analysis of bovine serum albumin (BSA) samples by polyacrylamide gel electrophoresis (PAGE).](https://www.plosone.org/doi/10.1371/journal.pone.0008565.g001)
pH dependence. Since pH can significantly alter proton chemical exchange rates [11], a subset of BSA relaxation measurements were performed at both pH 5.5 and 7.0. After hydrochloric acid (1 N) was added to the BSA solutions, pH was measured at room temperature using a Metrohm pH meter.

DMSO solvent. The importance of chemical exchange effects on relaxation may be studied by substituting dimethyl sulfoxide (DMSO) solvent for water since DMSO does not have exchangeable protons. A 10% solution of crosslinked BSA (60 mM GA) was dialyzed (3.5 kDa cutoff) twice against excess DMSO (ACS Reagent, Sigma) at room temperature for 24 hours each. Both the resultant dialysate as well as the original BSA in water solvent were analyzed. The presence of the methyl proton of DMSO and the absence of observable water resonance was confirmed on 1H NMR spectra of the dialysate.

Methylation. The methylation of BSA in this study refers to the methyl esterification of the carboxyl groups on the BSA molecule (CH3-O-BSA). The lyophilized form of methylated BSA (Sigma Chemicals) was dissolved in H2O (10% w/v) resulting in a clear solution with neutral pH. Deuterated methyl ester of BSA was synthesized by reacting fraction V BSA with deuterated methanol (Aldrich) (CD3OD, 99.8 atom % D) following the protocol of Fraenkel-Conrat [12]. As a control for the synthesis, unlabeled methyl ester of BSA (CH3-O-BSA) was synthesized in the same manner except unlabeled methanol (CH3OH) was used and then compared to the purchased form of methylated BSA.

Experimental Preparation: Tissues

Myocardium. A 3.5 kg New Zealand White rabbit and a 400 g Sprague-Dawley rat were anesthetized with intravenous sodium pentobarbital (0.5 mg/kg) or diethyl ether respectively. The hearts were rapidly excised and then arrested in cold (4°C) cardioplegic solution containing in mM: NaCl 110, NaHCO3 10, KCl 16, MgCl2 16, and CaCl2 1.2. The posterior papillary muscle of the rabbit left ventricle was then quickly excised keeping the majority of the covering intimal layer intact. It was then dabbed dry and placed in a parafilm-sealed glass tube. After the left ventricular free wall of the rat heart was equilibrated in excess saline (4°C), it was also dabbed dry and placed in a sealed glass tube.

Cartilage. Five cubes (4 mm) were cut from a disk of calf patella cartilage which was stored in saline at −20°C. The cubes were thawed, padded dry and equilibrated overnight at room temperature with an excess of one of three different solvents: normal saline (0.9% NaCl in H2O), phosphate buffered solutions (100 mM KH2PO4/KHPO4 at pH 9.2, 7.0 and 4.4), and DMSO. After equilibration, the cubes were padded dry and sealed in a glass tube for NMR measurements.

NMR Measurements

Relaxation measurements at 2 T or 4.7 T were obtained at room temperature with the sample inside a parafilm-sealed 5 mm diameter spherical glass vial using a 4-turn (6 mm diameter) solenoid RF coil. The size and shape of the samples and coils were designed to minimize the spectral linewidth, as well as the RF power required so that the largest range of B1 values could be studied. T1p was measured with solvent proton (water or DMSO) on resonance and linewidth less than 30 Hz for all protein samples. Solvent proton linewidth was less than 110 Hz for the tissue samples. The T1p pulse sequence consisted of a hard 90° pulse (13 to 50 µs), a 10 µs delay followed by a spinlock pulse on resonance with a 90° phase offset, and then a 100 µs delay and acquisition. The sequence was repeated with step changes in spinlock pulse duration (from 5 msec to approximately 4-fold T2).

T1 was measured using inversion recovery, and T2 was measured using single Hahn spin echoes. At least 11 step changes were used for all T2 and T1p measurements, and at least 21 step changes for all T1 measurements. As T2 values varied widely, the upper range of T1 also varied and was individually adjusted depending on the signal received (TE ranged from 2 msec to approximately 1–2 fold T2). For T1, T2, and T1p measurements, the repetition time (TR) was always at least 5 × T1.

Data Analysis

T2 and T1p relaxation curves were first plotted in semi-log scale to determine the presence of non-single exponential behavior. Relaxation data of all samples except for rabbit papillary muscle appeared single exponential within the time resolution of the NMR experiment. T1, T2, and T1p relaxation times were then obtained by fitting magnitudes of spectral peaks to two-parameter single exponential functions. All relaxation time values were calculated from a one-time measurement. The T1p dispersion data of both BSA and tissue samples were analyzed for simple power law or BPP model characteristics using equations of the form:

\[ T_{1p} = a + bV_1^{c} \]  \hspace{1cm} \text{(1)}

\[ \frac{1}{T_{1p}} = a + bV_1^{c} \]  \hspace{1cm} \text{(2)}

\[ \frac{1}{T_{1p}} = \frac{1}{a} \left( 1 + \left( \frac{1}{bV_1} \right)^n \right) + \frac{1}{c} \]  \hspace{1cm} \text{(3)}

where \( V_1 = \gamma B_1/2\pi \), and a, b, and c are dispersion parameters whose values are determined by the fitting algorithm. The relaxation times and dispersion parameters were obtained via non-linear least squares fit of the data using the Marquardt-Levenberg algorithm (IDL, Research Systems, Inc.). Convergence ceased when the relative decrease in chi-square between iterations was smaller than 0.01%. Up to 100 iterations were performed before determining a failure to converge.

Results

Table 1 summarizes the relaxation times and T1p dispersion characteristics for all samples. The standard error of the estimate for the relaxation time curvfits were on average less than 1.0% of the calculated relaxation time values for all protein samples and less than 2.2% for tissue samples. Unless specifically reported, pH was not measured.

BSA Samples

Figure 2a demonstrates two distinct patterns of T1p dispersion for fraction V BSA samples at 2 Tesla. In the absence of crosslinking (0% GA), T1p sharply increased for γB1 from 1 to 5 kHz (21% and 34% increase in T1p values for acidic and neutral samples respectively) and then quickly plateaued beyond 10 kHz (>3% increase up to 60 kHz for both acidic and neutral samples). High concentrations of GA (≥80 mM), in contrast, led to smooth and monototonically increasing T1p values from 1 to 60 kHz. For example, T1p increased 25% from 1 to 5 kHz and 53% from 10 to 60 kHz for the sample reacted with 80 mM GA, and the dispersion curves no longer displayed an acute transition zone near 5 kHz. Since BSA reacted with 80 mM GA was a
homogeneous liquid and BSA reacted with 100 mM GA was a gel, as demonstrated in Fig. 2a. Gelation, however, had little effect on T1 dispersion from 1 to 60 kHz.

Figure 2b shows the T2/T1 ratio as a function of B1 for the same samples as in Fig. 2a. Note that this ratio approaches unity at 1 kHz as [GA] increases, indicating that highly crosslinked BSA has minimal dispersion (i.e. T1 changes little with B1) below 1 kHz. Uncrosslinked samples, on the other hand, had ratios significantly less than 1, indicating significant dispersion below 1 kHz. The maximum difference in T1p between the acidic and neutral uncrosslinked samples occurred at zero field (measured as T2) where the T2 of the acidic sample was 40% above that of the neutral sample.

Figure 3 shows T1p dispersion plots of purified monomers (or dimer) of BSA rather than fraction V BSA. T1p values for native (0% GA) purified BSA dimers nearly coincided with those of monomers reacted with a low concentration of GA (20 mM) and showed only subtle changes compared to uncrosslinked BSA monomers (see also Table 1). Similar to the dispersion plots of fraction V BSA, purified monomers of BSA treated with increasing [GA] up to 60 mM showed increasing T1p dispersion for B1 beyond 5 kHz. Increasing [GA] above 60 mM did not significantly change the dispersion characteristics of crosslinked monomers of BSA.

Figure 4 plots the T2/T1 ratio of BSA samples reacted with varying concentration of GA at both 2.0 T and 4.7 T and for dialyzed samples. Although T2/T1 ratios were found to generally decrease with increasing [GA], there was a transition zone between 40 and 60 mM GA where most of the changes in the T2/T1 ratios occurred. This range of [GA] was also the transition zone for the BSA in H2O (4.7T).

### Table 1. Solvent Relaxation Parameters in Various Protein Solutions and Tissues.

| Sample Group | Sample Description | T1 (ms) | T2 (ms) | T2/T1 | % change in T1p (1–30 kHz) |
|--------------|--------------------|---------|---------|-------|----------------------------|
| BSA (Fraction V) in H2O (2T) | 10% BSA, 0mM GA, pH 5.5 | 1650 | 473.2 | 0.287 | 83.29 | 6.72 | 9.99 |
| | 10% BSA, 0mM GA, pH 7.0 | 1222 | 338.0 | 0.277 | 85.78 | 6.06 | 8.16 |
| | 10% BSA, 20mM GA | 1535 | 392.0 | 0.255 | 69.70 | 8.95 | 21.36 |
| | 10% BSA, 40mM GA | 1632 | 347.0 | 0.213 | 52.94 | 10.80 | 36.25 |
| | 10% BSA, 80mM GA | 1520 | 167.6 | 0.110 | 17.23 | 22.91 | 59.86 |
| | 10% BSA, 100mM GA | 1557 | 136.5 | 0.088 | 25.49 | 21.22 | 53.29 |
| | 20% BSA, 200mM GA | 766 | 58.7 | 0.077 | 17.96 | 23.78 | 58.26 |
| BSA (Monomers) in H2O (2T) | 10% BSA, 0mM GA | 1873 | 256.8 | 0.137 | 64.32 | 13.11 | 22.57 |
| | 10% BSA, 20mM GA | 1793 | 249.0 | 0.139 | 81.74 | 10.10 | 8.16 |
| | 10% BSA, 40mM GA | 1746 | 229.0 | 0.131 | 44.65 | 17.20 | 38.16 |
| | 10% BSA, 60mM GA | 1656 | 206.7 | 0.125 | 14.54 | 18.02 | 67.44 |
| | 10% BSA, 80mM GA | 1559 | 155.4 | 0.100 | 13.32 | 22.53 | 64.15 |
| | 10% BSA, 100mM GA | 1617 | 136.8 | 0.085 | 20.07 | 22.02 | 57.90 |
| | 10% BSA dimer | 1674 | 275.5 | 0.165 | 74.05 | 16.99 | 8.96 |
| BSA (Fraction V) in H2O (4.7T) | 10% BSA, 0mM GA | 1767 | 233.1 | 0.132 | 63.63 | 21.89 | 14.48 |
| | 10% BSA, 20mM GA | 1715 | 257.0 | 0.150 | 56.84 | 18.78 | 24.38 |
| | 10% BSA, 40mM GA | 1695 | 204.0 | 0.120 | 25.93 | 18.47 | 55.60 |
| | 10% BSA, 60mM GA | 1703 | 168.0 | 0.099 | 21.49 | 18.78 | 59.73 |
| | 10% BSA, 80mM GA | 1833 | 109.3 | 0.060 | 21.44 | 20.89 | 57.67 |
| | 10% BSA, 200mM GA | 1852 | 82.6 | 0.045 | 20.55 | 19.03 | 60.42 |
| 10% BSA (Fraction V), 60mM GA (2T) | undialyzed | 1453 | 146.3 | 0.101 | 19.95 | 20.89 | 59.16 |
| | dialyzed in DMSO | 1121 | 54.0 | 0.048 | 12.42 | 21.43 | 66.15 |
| | dialyzed in H2O | 1542 | 52.4 | 0.034 | 23.43 | 22.65 | 53.92 |
| 10% BSA (methylated) (2T) | methylated BSA (Sigma) | 1999 | 190.3 | 0.095 | 42.38 | 17.59 | 40.04 |
| | methylated BSA (synthesized) | 2080 | 275.3 | 0.132 | 47.13 | 14.27 | 38.60 |
| | 4-H-methylated BSA (synthesized) | 2313 | 272.8 | 0.118 | 41.84 | 11.91 | 46.24 |
| 4.7T | rabbit myocardium | 1396 | 44.0 | 0.032 | 26.80 | 20.83 | 52.37 |
| 2T | rat myocardium | 1131 | 50.5 | 0.045 | 19.84 | 20.37 | 59.79 |
| Cartilage (2T) | In saline | 847 | 64.2 | 0.076 | 26.10 | 24.61 | 49.29 |
| | In DMSO | 351 | 6.4 | 0.018 | 11.19 | 20.56 | 68.25 |
| | In phosphate buffer pH 9.2 | 1047 | 27.7 | 0.026 | 18.48 | 21.98 | 59.45 |
| | In phosphate buffer pH 7.0 | 987 | 48.1 | 0.049 | 26.25 | 24.40 | 49.35 |
| | In phosphate buffer pH 4.35 | 953 | 40.3 | 0.042 | 23.33 | 26.11 | 50.56 |

BSA = Bovine serum albumin, GA = Glutaraldehyde, DMSO = Dimethyl sulfoxide.
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for changes in the shape of $T_1^r$ dispersion seen in figures 2 and 3. $T_2/T_1$ ratios for crosslinked BSA samples dialyzed in excess H$_2$O to remove the possible effects of unreacted GA showed a similar dependence on [GA] as undialyzed samples although the transition zone was narrower. $T_2/T_1$ ratios and $T_1^r$ dispersion of BSA samples obtained at 4.7 T demonstrated a similar dependence on [GA] as those obtained at 2 T (see Table 1).

Methylation

Figure 5 compares the $T_1^r$ dispersion of methylated BSA with crosslinked (60 mM GA) and uncrosslinked BSA. Dispersion curves were normalized to the respective $T_1^r$ values at the maximum frequency studied (23 kHz) in order to allow direct comparison of dispersion shapes. Figure 5 shows that above 5 kHz $\gamma B_1$ methylated BSA had nearly the same $T_1^r$ dispersion as crosslinked BSA. Below 5 kHz, the dispersion of methylated BSA resembled uncrosslinked BSA. These results were consistent, independent of whether the methyl groups were protonated or 99% deuterated. Deuteron labeling of the methyl groups of methylated BSA led to a minor increase in $T_1$ (2313 vs. 2080 ms) and no change in $T_2$ values (273 vs. 275 ms) compared with $^1$H-methylated BSA (Table 1). The $T_1^r$ dispersion of methylated BSA synthesized in the same manner as $^2$H-methylated BSA (see Methods) is shown as a control. The similarity of $T_1^r$ dispersion of methylated BSA and crosslinked BSA should be interpreted in light of our results in Fig. 1c, which shows that methylated BSA tends to form large aggregates in aqueous solutions (non-denaturing PAGE analysis). Absence of covalent bonding in these aggregates is evidenced by the monomeric appearance of the methylated BSA in denaturing PAGE (Fig. 1a, lane 3).

Tissues

Figure 6a shows the $T_2/T_1^r$ ratio as a function of $\gamma B_1$ for cartilage samples as well as for rat myocardial tissue. Note the similarity of these curves with crosslinked BSA in Fig. 2b. Tissue samples uniformly showed smooth monotonically increasing $T_1^r$.
over the entire frequency range studied. For instance, of the total $T_1^r$ dispersion seen from 1 to 30 kHz for cartilage (pH 7.0, 2 T), 26% occurred between 1 and 5 kHz, 24% between 5 and 10 kHz, and 49% between 10 and 30 kHz. The frequency breakdown in $T_1^r$ dispersion for crosslinked BSA (10% solution, 100 mM GA) was remarkably similar (25%, 21%, and 53% respectively) as compared to uncrosslinked BSA where generally over 80% of the $T_1^r$ dispersion occurring from 1 to 30 kHz occurred below 5 kHz (see Table 1). Changes in solvent pH had minor effects on cartilage $T_1^r$ dispersion, although the $T_2/T_1^r$ ratio at 1 kHz $\gamma B_1$ for the acidic and basic samples were closer to unity than the neutral sample, indicating less dispersion below 1 kHz for these samples.

To compare the $T_1^r$ dispersion characteristics of cartilage in water solvent to cartilage in DMSO solvent, normalized dispersion curves are demonstrated in Fig. 6b. Below 5 kHz, cartilage in DMSO displayed minimal $T_1^r$ dispersion reaching nearly zero slope below 2 kHz. The dispersion curve of crosslinked BSA (10% solution, 60 mM GA) in DMSO nearly coincided with the dispersion curve of cartilage in DMSO from 1 to 33 kHz.

Dispersion Modeling

As a preliminary test for power law behavior, $T_1^r$ was plotted against $\gamma B_1$ for various BSA and tissue samples. Figure 7 demonstrates a near linear relationship between $T_1^r$ and $\gamma B_1$ for crosslinked BSA, which did not exist for uncrosslinked BSA. Near linear relationships were also observed for the water protons of rabbit myocardial tissue and the methyl protons of DMSO equilibrated in cartilage. Although some non-linear behavior was present for crosslinked BSA and tissues, especially at lower frequencies, perfect linearity was not expected since it would require the power law exponent to be exactly $\frac{1}{2}$ and the frequency independent component to be negligible.

Figure 8 demonstrates the fits of Eq. [1–3] to representative crosslinked BSA and tissue samples. Table 2 shows that the standard error of the estimate of the fit expressed as a percentage of the $T_1^r$ value (% SEE) for crosslinked BSA (GA=60 mM) and tissue samples was 2.2±1.1% for the relaxation-time power law Eq. [1] compared to 10.5±4.8% for the relaxation-rate power law Eq. [2] and 4.0±2.3% for the BPP model Eq. [3]. The improved fit of $T_1^r$ dispersion using the relaxation-time power law was found to be statistically significant ($P<0.005$ from analysis of variance with Bonferroni correction for both comparisons [13]). Table 2 lists the fitted values of the parameters ($a$, $b$, $c$) for all samples which were successfully fitted to Eq. [1].

Discussion

Low Field Dispersion Behavior

In this study we have obtained low field relaxation data of protein solutions and tissue under varying conditions. We show that the $T_1^r$ dispersion profiles of native BSA solutions are clearly distinct from that of crosslinked BSA. Above 5 kHz $\gamma B_1$, the $T_1^r$ dispersion of 10% native BSA was essentially flat. This result is similar to that of Zhou and Bryant [5] and Koenig and Brown [7] who found minimal $T_1^r$ dispersion of native BSA from 10 to 100 kHz. Below 5 kHz, we found a steep dispersion profile (i.e. $T_1^r$ changed rapidly with $B_1$). Our measured ratio of $T_2/T_1^r$ at 1 kHz for native BSA (Fig. 2b) also suggested continued dispersion below 1 kHz. This sharp dispersion from 0 to 5 kHz was sensitive
to changes in solution pH and likely is due to chemical exchange of the water protons with the ionizable protons of the protein. As far as we are aware, only Virta et al. [10] and Mäkelä et al. [14] have also measured the $T_{1p}$ of native BSA below 10 kHz. Although Virta et al. observed insignificant $T_{1p}$ dispersion, only two $T_{1p}$ data points were obtained below 5 kHz. In contrast, Mäkelä et al. demonstrated similar findings to the current study in that native BSA solutions showed significant $T_{1p}$ dispersion below 5 kHz and were strongly affected by pH.

For our experimental conditions, at least 60 mM GA was required to alter the BSA $T_{1p}$ dispersion to match the smooth monotonically increasing profile that was seen for tissues. Although 40 mM GA was sufficient to form $<300$ kDa BSA oligomers, 60 mM GA formed an additional species of BSA polymers that were unable to migrate through the pores of the 2% stacking gel (Fig. 1a) suggesting an order of magnitude increase in molecular weight for this band. The formation of these large BSA polymers was associated with significant $T_{1p}$ dispersion above 5 kHz as well as an abrupt change in the $T_2/T_1$ ratio (fig. 4). This result indicates that a high degree of immobilization is required for protein solutions to accurately model tissue. Not surprisingly, Gore and Brown [15] evaluating proteins with molecular weight range from 1.4 to 483 kDa, and Menon and Allen [16] assessing serum proteins from 69 to 725 kDa found these protein solutions to be poor models for tissue relaxation behavior. Increasing GA above 80 mM, which obviously led to macromolecular structural changes since gelation occurred between 80 and 100 mM GA, did not lead to further changes in dispersion profile (Fig. 2–3) or the $T_2/T_1$ ratio even with an increase in [BSA] to 20%. Apparently a plateau is reached whereby further increases in macromolecular crosslinking does not enhance relaxation. Our finding of a plateau for samples with GA/BSA mole ratios greater than 53 (80 mM GA, 10% BSA) is in contrast to the results of Zhou and Bryant [5]. They showed increases in $T_1$ relaxation dispersion with increasing concentrations of GA with no sign of plateau even at a mole ratio of 256 (8.25% BSA). Since BSA polymerization is known to be highly sensitive to concentrations of BSA as well as GA [17], their results may relate to poor production of sufficiently large BSA polymers even at high GA concentrations.
Table 2. T₁ρ Dispersion Analysis of Crosslinked BSA and Tissues.

| Sample Groups                      | Samples                     | Number of values | Minimum | Maximum | Relaxation-Time Power Law Eq. [1] | Relaxation-Rate Power Law Eq. [2] | BPP Model Eq. [3] | ‘a’ (ms) | ‘b’ (10⁻⁶ sec⁻¹·C) | ‘c’ (exponent) |
|------------------------------------|-----------------------------|------------------|---------|---------|-----------------------------------|-----------------------------------|------------------|----------|-----------------|-----------------|
| BSA (Fraction V) in H₂O (2T)       | 10% BSA, 80mM GA            | 13               | 0.97    | 60.0    | 2.4                               | 6.8                               | 2.9              | 138      | 764             | 0.515           |
|                                   | 10% BSA, 100mM GA           | 13               | 1.01    | 59.2    | 2.1                               | 5.3                               | 5.0              | 93.8     | 2247            | 0.425           |
|                                   | 20% BSA, 200mM GA           | 22               | 0.14    | 33.1    | 2.3                               | 12                                | 3.3              | 45.7     | 31.1            | 0.731           |
| BSA (Monomers) in H₂O (2T)        | 10% BSA, 60mM GA            | 13               | 1.50    | 33.1    | 0.4                               | 9.7                               | 0.9              | 293      | 3.10            | 0.993           |
|                                   | 10% BSA, 80mM GA            | 13               | 1.50    | 33.1    | 0.8                               | 17                                | 1.5              | 187      | 21.7            | 0.833           |
|                                   | 10% BSA, 100mM GA           | 13               | 1.50    | 33.1    | 0.9                               | 4.0                               | 1.9              | 133      | 298             | 0.596           |
| BSA (Fraction V) in H₂O (4.7T)    | 10% BSA, 60mM GA            | 23               | 1.05    | 154     | 1.2                               | 10                                | 7.3              | 155      | 334             | 0.591           |
|                                   | 10% BSA, 80mM GA            | 19               | 1.50    | 100     | 2.0                               | 4.7                               | 4.5              | 81.8     | 2464            | 0.411           |
|                                   | 10% BSA, 200mM GA           | 23               | 1.00    | 154     | 3.0                               | 9.4                               | 9.5              | 64.2     | 785             | 0.501           |
|                                   | undialyzed                  | 23               | 0.10    | 33.1    | 1.2                               | 8.2                               | 1.9              | 156      | 42.7            | 0.757           |
|                                   | dialyzed in excess DMSO     | 23               | 0.10    | 33.1    | 2.7                               | 18                                | 1.8              | 53.2     | 3.98            | 0.964           |
|                                   | dialyzed in H₂O             | 15               | 0.79    | 22.8    | 1.4                               | 48                                | 28               | 91.9     | 466             | 0.577           |
| 4.7T                               | rabbit myocardial tissue    | 19               | 0.59    | 47.6    | 2.0                               | 7.2                               | 8.4              | 74.5     | 398             | 0.569           |
| 2T                                 | rat myocardial tissue       | 22               | 0.10    | 28.5    | 1.2                               | 12                                | 3.4              | 68.4     | 269             | 0.786           |
| Cartilage (2T)                     | in saline                   | 23               | 0.21    | 46.0    | 1.7                               | 6.0                               | 2.9              | 87.4     | 509             | 0.484           |
|                                   | in DMSO                     | 24               | 0.21    | 56.0    | 3.6                               | 17                                | 3.9              | 9.06     | 5.87            | 0.756           |
|                                   | in phosphate buffer pH 9.2  | 21               | 0.20    | 33.1    | 3.9                               | 17                                | 4.2              | 26.9     | 25.8            | 0.769           |
|                                   | in phosphate buffer pH 7.0  | 23               | 0.10    | 33.1    | 3.3                               | 13                                | 4.1              | 51.0     | 224             | 0.591           |
|                                   | in phosphate buffer pH 4.35 | 23               | 0.10    | 33.1    | 4.1                               | 16                                | 4.7              | 39.6     | 133             | 0.635           |

BSA = Bovine serum albumin, GA = Glutaraldehyde, DMSO = Dimethyl sulfoxide.
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It should be noted that the discussion, so far, assumes only intermolecular crosslinking of BSA is important in the observed changes in water relaxation. It is known that BSA is a rather rigid globular protein in its native state and that the addition of intramolecular crosslinks can warp or stiffen it only slightly [10]. Thus, intramolecular crosslinking of BSA is unlikely to affect protein motion significantly or lead to dipolar interactions that will substantially enhance relaxation.

Surprisingly, methylated BSA showed essentially the same $T_1^r$ dispersion as that of crosslinked BSA above 5 kHz (Fig. 5). The mechanism of this low field relaxation, however, is clearly independent of dipole-dipole interactions of the methyl protons either directly by spin exchange with tightly bound solvent protons or indirectly by spin diffusion with the protein protons. Significant reduction in both the intra- and intermolecular dipolar interactions of methyl protons by the substitution of deuterons for protons had essentially no effect on the $T_1^r$ dispersion profile. Rather, the similarity of $T_1^r$ dispersion of methylated BSA and crosslinked BSA can be explained by Fig. 1c, which shows that methylated BSA tends to form large aggregates in aqueous solutions (non-denaturing PAGE analysis). Presumably, the addition of methyl ester side groups to BSA allows nonspecific intermolecular binding of BSA monomers which, similar to crosslinking BSA, slows macromolecular tumbling below a critical threshold. The absence of covalent bonding in these aggregates is evidenced by the single monomer band of methylated BSA in denaturing PAGE (Fig. 1a).

Since the methylated BSA solutions contain mixtures of different sized aggregates (Fig. 1c), the sharp dispersion below 5 kHz can be accounted for by the presence of methylated BSA monomers and small aggregates that behave similar to native BSA, and the continued dispersion above 5 kHz is due to large aggregates which are functionally “immobilized” and behave similarly to crosslinked BSA. Thus, the methylated BSA data provide further evidence that differences in the relaxation properties of native and crosslinked BSA are a consequence of the increase in the polymerization of BSA, rather than other effects of GA, such as its attachment as a chemical side group to BSA. Although motion of proton containing side groups, such as methyl groups have been suggested to provide significant relaxation sinks for large proteins [19,20], their high mobility [19,21] implies that no significant enhancement of relaxation can be expected below 100 MHz much less in the kHz regime of $T_1^r$ [20].

Modeling

For tissue and solutions of sufficiently crosslinked BSA, significant $T_1^r$ dispersion was seen for the entire range of $\gamma B_1$ studied (up to 150 kHz for some samples, Table 2). Our plots of $T_1^r$ versus $\gamma B_1$ were remarkably similar to the plots of $T_1$ versus $\gamma B_0$ shown by Bottomley et al. [22] for many tissues. Specifically, both $T_1^r$ and $T_1$ dispersion profiles showed a weak field dependence, which was distinct from the $T_1 \propto \tau^6$ relationship expected for magnetic dipolar interactions in simple homogeneous systems (BPP model, see Eq. [3]). Although, the BPP equation can present a concave-down frequency relationship over a local range (Fig. 8), the same relationship cannot occur over an extended range from kHz to hundreds of MHz—5 decades of frequencies from $T_1^r$ to $T_1$—unless multiple or continuously distributed correlation times are assumed [5,23,24].

Our $T_1^r$ dispersion profiles for crosslinked BSA and tissue from 1–100 kHz displayed continuous, monotonic increases with frequency that did not suggest obvious inflections. Likewise, $T_1$ tissue dispersion curves from 1–100 MHz summarized by Bottomley et al. [22] and crosslinked BSA dispersion curves from 10 kHz to $\sim$100 MHz shown by Koenig and Brown [4] (notwithstanding small, local changes in dispersion due to $^{14}N\cdot^1H$ quadrupole dips) do not show obvious inflections. Similar to Bottomley et al. [22] who found an excellent fit to $T_1$ dispersion using the relationship $T_1 = A\tau^6$, the $T_1^r$ dispersion data fit well to the simple relaxation-time power law, $T_1^r = a \tau^b$ [Eq. (1)], where parameter “$a$” was added to account for the zero-field offset, $T_1^r(0) = T_2$. Not only did this equation present a significantly improved fit to the data compared to the relaxation-rate power law [Eq. (2)] and the BPP model [Eq. (3)], it was able to provide a calculated $T_1^r(0)$ value that closely approximated $T_2$. Specifically, the $T_1^r(0)/T_2$ ratio was near unity (1.10 $\pm$ 0.32) for the relaxation-time power law, whereas it was significantly higher ($P < 0.001$) for the BPP model (1.40 $\pm$ 0.40).

The relaxation-rate power law [Eq. (2)] is similar to the Escanye et al. [23] expression $1/T_1 = A\Delta^{1/2} + B$. This expression was found to adequately fit $T_1$ dispersion of mouse muscle from 7–90 MHz and has the advantage that it can be easily interpreted mechanistically as a fast-exchange two-state model. However, this expression cannot account for properties of relaxation at or near zero field where it predicts $T_1(0)$ to be zero. The relaxation-rate power law demonstrated a poor fit to our $T_1^r$ dispersion data.

The exponent “$c$” in the relaxation-time power law was calculated to be 0.66 $\pm$ 0.20, 0.68 $\pm$ 0.15, and 0.66 $\pm$ 0.12 for crosslinked BSA (GA=60 mM), myocardial tissue, and cartilage, respectively. Neglecting the effects of the $T_1^r(0)$ offset, these values are higher than the exponent reported by Bottomley et al. [22] for water proton $T_1$ dispersion of skeletal muscle (0.42) and heart muscle (0.36). These values, however, are near the exponent reported by Kimmich et al. [20] for $^1H$ $T_1$ dispersion of either lyophilized or minimally D$_2$O-hydrated (16% by weight) proteins and polypeptides (0.74 $\pm$ 0.06). Potential relaxation models that account for simple power law field dependence are discussed below.

Relaxation Mechanisms

**Protein-associated water.** Nearly all models of water relaxation in macromolecular systems consider one or more new groups of protein-associated water with altered motion that contributes to bulk water relaxation. For example, “hydration layers” at the macromolecular interface have been proposed with increased correlation times in order to explain the dispersion data [23]. In the case of $T_1^r$ relaxation data, invariably an additional correlation time is added to the model to account for the low-field regime [8]. However, observations obtained by high-resolution NMR spectroscopy of proteins [25], relaxation dispersion of water $^{17}O$ [26], and paramagnetic spin labeling [27], strongly suggest that surface hydration water is highly mobile with sub-nanosecond residence times. Thus, it is unlikely that models based on distributions of surface water with restricted motional characteristics or the “exchange diffusion” of water molecules to and from a bound hydration layer can explain the relaxation dispersion of protein solutions or tissue.

Later models have focused on a small number of water molecules buried inside proteins, which are clearly distinguished from surface hydration water by their longer residence times [25]. Denisov and Halle [26] report that the internal water molecules of the globular protein, bovine pancreatic Trypsin inhibitor (BPTI), have residence times ($t_{RES}$) on the order of $10^{-8}$ to $10^{-6}$ seconds, whereas the water molecules on the surface of the protein have an average reorientational correlation time of approximately 20 picoseconds. By studying the relaxation behavior of water $^{17}O$ nuclei, the complicating effects of cross-relaxation and hydrogen exchange were avoided, and they postulate that the origin of the water $^{17}O$ relaxation dispersion of BPTI solutions can be
explained by a small number of interior water molecules exchanging with bulk water on the submicrosecond time-scale. Although a consensus view is still lacking, our experimental data will be examined considering this model of protein hydration.

**Dilute globular protein solutions.** Similar to our $T_{1p}$ data in native and crosslinked BSA solutions, several investigators have shown that the $T_1$ dispersion profiles of dilute globular protein solutions are clearly distinct from those of immobilized protein solutions [5,7]. At least for mobile protein solutions, the dispersion relation is generally Lorentzian, and the dispersion inflection frequency of water $^1H$, $^2H$, and $^17O$ nuclei has been shown to correspond to $\tau_R$, the rotational correlation time of the protein molecule [26,28,29]. Thus, the conventional BPP model along with the condition of motional narrowing ($\omega_0\tau_c<<1$) is apparently applicable in these protein solutions as the effective correlation time of motion, $\tau_c$, is easily identified with $\tau_R$. As suggested by Venu et al [28], interior water molecules with residence times greater than $\tau_R$ ($\sim$6 ns for BPTI) can sense the Brownian motion of the protein molecule, exchange with bulk water, and thereby contribute to the observed relaxation dispersion. The intrinsic relaxation rate of these buried relaxation sinks was explained quantitatively by intramolecular dipole couplings ($\sim$70%) and many intermolecular dipole couplings with BPTI protons ($\sim$30%). Labile protein protons were also thought to make a significant contribution to the observed water relaxation rate. Contributions from direct nuclear Overhauser effect (NOE) cross-relaxation between protein protons and interior or surface water protons were found to be negligible, which is not surprising given the motional narrowing condition [28].

Irrespective of the actual mechanism, the relation $\omega_0\tau_c<<1$ for the spinlock experiment predicts an essentially flat $^1H$ $T_{1p}$ dispersion below $\sim$1 MHz. As suggested by Hills [30], proton exchange then becomes the remaining relaxation mechanism that is operative in the low-field regime. Our results (Fig. 2–3) show that this is indeed the case. Native BSA solutions exhibited a sharp dispersion profile below 5 kHz that was sensitive to changes in pH and showed a flat dispersion above 5 kHz. Furthermore, the active dispersion range was consistent with the intrinsic proton exchange rates ($700$–$10,000$ s$^{-1}$) measured by Liepinsh and Otting from OH and NH groups of several amino acid side chains under physiologic conditions [31]. Our results, therefore, are consistent with the theoretical $T_{1p}$ dispersion of dilute globular protein solutions proposed by Hills [30].

**Immobilized proteins and tissues.** Rotational immobilization of solute protein can be achieved by a chemical [3] or thermal crosslinking reaction [10] or by non-covalent interactions at high ($>50\%$ w/w) protein concentrations [9]. For such solutions and biological tissues, it is assumed that the dispersion inflection frequency no longer reflects protein rotation but instead the residence times of long-lived water molecules that are associated with the protein [32]. However, the dispersion curves are not simply scaled, Lorentzian profiles with shifted inflection frequencies, but are distinctly non-Lorentzian [9]. In addition, immobilized protein solutions also exhibit broader inflection frequencies, but are distinctly non-Lorentzian [9]. In immobilized protein solutions and tissues, calculation of mechanistic parameters using BPP-type models—with their inherent assumptions about the nature of the local interactions causing relaxation and the shape of the spectral density function—is likely erroneous.

Furthermore, unlike for mobile protein solutions, abundant evidence exists for direct NOE cross-relaxation between immobilized protein and solvent protons [37]. Bryant et al. [3] have suggested that the longitudinal relaxation of water protons in solutions of immobilized proteins and tissue is due to magnetic coupling of macromolecular protons with water protons and that the magnetic field dependence of the solid component could be transferred at least partially to the liquid component. The simple power law dispersion profiles found for solid protein protons has been explained by intrinsic motions characteristic of protein backbones by Kimmich and Winter [38]. Independent of the mechanism by which protein protons acquire their relaxation field dependence, Zhou and Bryant [5] have proposed that cross-relaxation could then allow “water spins to report a scaled replica” of the relaxation behavior of the solid system. Efficient coupling is required to allow cross-relaxation, and long-lived water molecules buried inside macromolecules ($\tau_{RES}$ up to 200 ns for BPTI [39]) could be an important pathway for the magnetization transfer. Long-lived hydration water in junction zones formed by protein crosslinking have also been postulated [40]. In considering this cross-relaxation model, we note that the similarity of our $T_{1p}$ dispersion profiles to that of published $T_1$ profiles for immobilized proteins [20] and tissue [22] indicate a common relaxation mechanism is dominant across 5 decades of modulating field strength. The importance of this model is not settled, however, other relaxation models will need to consider dynamic processes that span this large range of frequencies.

An additional mechanism by which magnetization can be transferred from the solid phase to the solvent phase has been suggested by Hills [30]. A three-site model whereby spin diffusion in the solid phase allows spin exchange between non-exchangeable and exchangeable protein protons followed by proton exchange between water and exchangeable proton protons could provide a quantitative interpretation of the relaxation data without the need to invoke special hydration water. Our $T_{1p}$ dispersion profiles of the methyl protons of DMSO for cartilage and crosslinked BSA, however, show strong field dependence throughout the studied range (Fig. 6b). In fact, normalized dispersion profiles for the DMSO solvent samples were nearly identical to the dispersion profiles of the corresponding samples with water solvent above 5 kHz. Since the methyl protons of DMSO are not exchangeable, proton exchange is obviously not necessary for the strong field dependence of DMSO protons in immobilized protein solutions or tissue. In addition, the minor effect of solvent pH on water $T_{1p}$ dispersion of cartilage as compared to native BSA solutions suggests that protein immobilization attenuates the contribution of proton exchange to water relaxation (Fig. 2b & 6a). Thus, proton exchange appears to have a minor role on $T_{1p}$ dispersion in immobilized protein solutions and tissues above 5 kHz. Interestingly, Mäkelä et al. [14] using some similar sample preparations,
drew nearly the opposite conclusion. Namely, they conclude that there is "...a crucial role of proton exchange on R1p and R1p dispersion in immobilized protein solution mimicking tissue relaxation properties." However, we note that Makela et al. assessed T1p over a much narrower range of \( \gamma B_2 \) (1–11 kHz), did not analyze solvent samples, and did not study any tissues. The latter is particularly important, since Makela et al. specifically focus on their results from heat-denatured rather than glutaraldehyde cross-linked BSA, postulating that glutaraldehyde treated BSA is a poor model for tissue, albeit without tissue data to support this supposition.

We also note that our T1p dispersion profiles of BSA and tissue samples showed no significant \( \phi_b \) dependence between 86 and 200 MHz (2 T and 4.7 T). Therefore, exchange models that produce \( \phi_b \) dependence because of a resonance offset, \( \delta \), between water and labile protein protons or between long-lived protein associated water and bulk water, cannot account for the T1p dispersions measured in this study.

The similarity of our DMSO and water solvent T1p dispersions implies similar molecular mechanisms for relaxation. Long-lived DMSO molecules, if present, should also have comparable residence times to that of water molecules. It is consistent then that Denisov and Halle [26] indicate that buried water molecules have long residence times due to the free energy cost of local protein unfolding rather than due to a full complement of strong hydrogen bonds. In addition, we note that DMSO solvent in crosslinked BSA and cartilage show nearly identical 1H-T1 hydrogen bonds. In addition, we note that DMSO solvent in crosslinked BSA and cartilage show nearly identical 1H-T1 hydrogen bonds. In addition, we note that DMSO solvent in crosslinked BSA and cartilage show nearly identical 1H-T1 hydrogen bonds. In addition, we note that DMSO solvent in crosslinked BSA and cartilage show nearly identical 1H-T1 hydrogen bonds. In addition, we note that DMSO solvent in crosslinked BSA and cartilage show nearly identical 1H-T1 hydrogen bonds.

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Author Contributions

Conceived and designed the experiments: EIC RJK. Performed the experiments: EIC. Analyzed the data: EIC RJK. Contributed reagents/materials/analysis tools: EIC. Wrote the paper: EIC RJK.
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