Measurement of the exchange rate of waters of hydration in elastin by 2D $T_2$–$T_2$ correlation nuclear magnetic resonance spectroscopy

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Abstract. We report on a direct measurement of the exchange rate of waters of hydration in elastin by $T_2$–$T_2$ exchange spectroscopy. The exchange rates in bovine nuchal ligament elastin and aortic elastin at temperatures near, below and at the physiological temperature are reported here. Using an inverse Laplace transform (ILT) algorithm, we are able to identify four components in the relaxation times. While three of the components are in good agreement with previous measurements that used multi-exponential fitting, the ILT algorithm distinguishes a fourth component having relaxation times close to that of free water and is identified as water between fibers. With the aid of scanning electron microscopy, a model is proposed that allows for the application of a two-site exchange analysis between any two components for the determination of exchange rates between reservoirs. The results of the measurements support a model (described by Urry and Parker 2002 J. Muscle Res. Cell Motil. 23 543–59) wherein the net entropy of waters of hydration should increase with increasing temperature in the inverse temperature transition.

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1. Introduction

A well-known characteristic of elastin, an insoluble protein that is responsible for the elasticity of vertebrate tissues, is that the complex microscopic solvent–protein relationship dictates its macroscopic behavior. For example, experimental measurements of Young’s modulus demonstrated a dependence of the stress–strain relationship on solvent polarity [2]–[8]. The development of the remarkable resilience of the protein is believed to be correlated with the entropy change of elastin upon extension [1]. Using molecular dynamics simulations of a poly(GVGVP) peptide, it was shown that hydrophobic hydration is an important source of entropy-based elasticity [9, 10]. Fundamental to the mechanical property of elastin is the inverse temperature transition—a process pointed out by DW Urry in numerous experimental studies of a poly(GVGVP) peptide [1], [11]–[13]. During the inverse temperature transition, there is an increase in order of the backbone upon raising the temperature—microscopically a hydrophobic association of $\beta$ turns occurs, resulting in a macroscopic volumetric contraction. During the phase transition, structured hydration becomes less ordered bulk water due to the hydrophobic association of elastin $\beta$ turns [1]. The goal of this paper is to quantify in detail the exchange of waters of hydration in elastin and shed light on the complex water–protein association.

In this work, we implement a relatively new experimental technique known as relaxation exchange spectroscopy [14, 15]. This method allows for observing the displacement of mobile molecules over time between interconnected pores. The experiment involves correlating the spin–spin relaxation ($T_2$) rates of mobile molecules over an experimentally variable mixing time. In the measurement, the spins of the ensemble are encoded and decoded by their $T_2$ relaxation rates. Between the encoding and decoding stages, the experimentally controlled mixing time allows us to track the displacement of molecules from one site to another. Central to the method is a two-dimensional (2D) inverse Laplace transform (ILT) algorithm allowing for multi-exponential fitting to the data [16]. The net result of the 2D algorithm is a map of relaxation times with cross peaks indicating exchange between sites distinguishable by their $T_2$ times.

Relaxation exchange spectroscopy has been implemented with success in a variety of porous systems. The method has been validated by considering a model porous medium consisting of mixtures of nonporous borosilicate and soda lime spheres in water [17]. In the measurement, the expected length scales between domains correlated well (within 25%) with that computed by the exchange time and molecular diffusion coefficient of water. A detailed analysis was presented recently on the 2D $T_2$–$T_2$ exchange experiment and applied successfully to white cement paste for extracting the exchange rates and an estimate of the pore sizes [18]. Recently, a novel propagator-resolved relaxation exchange experiment has been proposed by

**References**

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enabling spatial resolution together with $T_2 - T_2$ exchange [15]. One of the challenges in 2D relaxation exchange NMR is that the encoding times may be comparable to the exchange times. In order to analyze the resulting experimental data, in this limiting case, it is required that one performs simulations to determine quantitative information, such as the exchange rates [19]. Nevertheless, 2D relaxation exchange spectroscopy is quickly gaining popularity in applications where quantifying the details of exchange between reservoirs may prove to be significant in understanding connectivity and structure in a complex system.

The experimental data presented in this paper highlight the mutual exchange between various sites in bovine nuchal ligament elastin and aortic elastin at temperatures below, near and at the physiological temperature. In both of the elastin samples, at all temperatures, we measure four clearly distinguishable relaxation rates corresponding to four discernable domains. A model is proposed, supported by scanning electron microscopy (SEM), where the waters of hydration in close proximity to the protein can be further divided into four groups that exchange with the three other components independently. Using this model, it is argued that a two-site exchange analysis may be performed to determine the exchange rates between the various reservoirs.

2. Materials and methods

Purified bovine nuchal ligament and aortic elastin were purchased from Elastin Products Company (Elastin Products Co., Owensville, MO). The elastins were purified by the neutral extraction method of Partridge [20]. Two samples were made: (i) nuchal ligament elastin (NLE) suspended in D$_2$O and (ii) aortic elastin (AE) suspended in D$_2$O. The samples were prepared in a similar fashion: the elastin was immersed in abundant 99% D$_2$O and was mixed with D$_2$O by using a sonicator for 30 min (the temperature during this process was maintained at less than 40 °C), and then left at room temperature for more than 76 h. The samples were then placed in standard 5 mm NMR tubes and sealed using ethylene-vinyl acetate. The loss of water in any of the samples using this seal was less than 1% over the entire course of the experiments. Each sample was approximately 1.5 cm in length. The estimated concentration of elastin in D$_2$O was 0.5 g ml$^{-1}$.

All of the experiments were carried out on a Varian Unity 200 MHz NMR spectrometer using a Varian liquids NMR probe. The 90° pulse time on our system was 35 μs. The effect of this pulse time on the signal is negligible as the timescale of the water dynamics studied was of the order of milliseconds. For the NLE sample, the experiments were conducted at 10, 25 and 37 °C. The AE sample was studied at only 25 °C. At each temperature, a $^2$H 2D $T_1 - T_2$ experiment and several $^2$H 2D $T_2 - T_2$ experiments with different mixing times were carried out.

The NMR pulse sequence for the $T_1 - T_2$ experiments is illustrated in figure 1 [16]. In the experiments, the magnetization is inverted by the first 180° pulse and then recovers toward thermal equilibrium with a relaxation time of $T_1$ during a delay $t_1$. The 90° pulse transforms the magnetization into the transverse plane and the NMR signal is detected by a Carr–Purcell–Meiboom–Gill (CPMG) pulse train [21]. Information about $T_1$ is encoded by varying the delay time $t_1$. Using a 2D ILT of the data, a $T_1 - T_2$ correlation map is obtained. The caption in figure 1 highlights the four-step phase cycling scheme that was implemented.

The pulse sequence employed in the $T_2 - T_2$ experiments is illustrated in figure 2 [15]. The sequence consists of two 180° pulse trains that allow for the encoding of $T_2$ information before and after the mixing period. During the mixing time, denoted by $t_m$, water molecules that migrate between different compartments experience different $T_2^{app}$ relaxation times before and after the...
Figure 1. RF pulse sequence used for 2D $T_1$–$T_2$ correlation experiments in this work. In the experiments, phase cycling was $\varphi_1 = x, -x$, $\varphi_2 = x, x, -x, -x$, $\varphi_3 = y$ and the receiver phase was $\varphi_{\text{receiver}} = x, x, -x, -x$. The experimental values for $t_1$ and $\tau$ are described in the text.

Figure 2. RF pulse sequence used for 2D $T_2$–$T_2$ correlation experiments in this work. The phase cycling used was $\varphi_1 = x, -x$, $\varphi_2 = y, -y$, $\varphi_3 = x, x, -x, -x$ and the receiver phase was $\varphi_{\text{receiver}} = x, -x$. The experimental values for $t_m$ and $\tau$ are described in the text.

mixing time. The $T_{2\text{app}}$ are the apparent $T_2$ times measured in the presence of exchange and are different from the $T_2$ measured in the absence of exchange. The exchange is manifested in the 2D ILT map as a cross peak. Water that does not exchange between compartments is observed as a diagonal peak in the 2D ILT $T_2$–$T_2$ map as it has only one $T_2$ throughout the entire experiment. A four-step phase cycle, shown in the caption in figure 2, was used to eliminate the undesired signal that is built up during the mixing time. By incrementing the mixing time, a series of $T_2$–$T_2$ maps were obtained and the exchange of water between different compartments in elastin was studied via the time dependence of the cross peak intensities.

In all of the experiments, a $\tau$ value of 0.3 ms in between two successive $180^\circ$ pulses was used. A free induction decay (FID) of 2.4 s was acquired so that a $T_2$ in the range of 1 ms–1 s was measured. In the $T_1$–$T_2$ experiments, $t_1$ was incremented from 1 ms to 10 s to enable an accurate measurement of $T_1$ values from 10 ms to 1 s. In the $T_2$–$T_2$ experiments, the mixing time $t_m$ was incremented from 0.5 to 200 ms. In all of the experiments, the number of accumulated scans was set to 16, a recycle delay of 3 s was used and the temperature was regulated to within 2 °C.

3. Results and discussion

In the $T_1$–$T_2$ experiments, 40 values of $t_1$ ranging logarithmically from 1 ms to 10 s were used to achieve a 2D map. Among these 40 FIDs, three FIDs with $t_1 = 1$ ms, 110 ms and 10 s are presented in figure 3 as examples from the NLE sample at 25 °C. It is clear from the experimental data that the inversion of the longitudinal magnetization shows the information about $T_1$, and that $T_2$ is encoded in each FID. Using an ILT of the 2D $T_1$–$T_2$ data of the NLE sample at 25 °C, a 2D ILT map was obtained and the result is shown in figure 4. Four distinguishable peaks are discernable. Similar results were obtained at 10 and 37 °C, as well as on the AE sample. The $T_{1\text{app}}$ and $T_{2\text{app}}$ values measured from the ILT maps are tabulated in table 1; the waters of hydration in and around elastin may be separated into four groups as indicated in figure 4.
Figure 3. FIDs in the $T_1$–$T_2$ experiment on NLE at 25°C. In the experiments, $t_1$ was 1 ms, 110 ms and 10 s, respectively, as indicated in the figure. The figure illustrates an inversion recovery of magnetization along the $t_1$ dimension, as well as a $T_2$ decay in each FID.

Figure 4. A 2D ILT result of the $T_1$–$T_2$ experiment on NLE at 25°C. Four distinguishable peaks are observed, and they are denoted by $\alpha_1$, $\alpha_2$, $\beta$ and $\gamma$, respectively. The measured $T_{1\text{app}}$ and $T_{2\text{app}}$ values are tabulated in table 1. To guide the eye, the dashed lines in the figure represent the location of $T_1 = T_2$. The 2D map intensity is shown on a logarithmic scale.

labeled $\alpha_1$ in figure 4 has a $T_{1\text{app}} = 351$ ms and a $T_{2\text{app}} = 224$ ms, which is very close to that of free water and has therefore been assigned as bulk water outside the elastin fibers (in our system, we measured $T_1 = 450$ ms and $T_2 = 400$ ms for a sample of free D$_2$O). For water in a porous medium, it is known that the observed relaxation time $T_{2\text{app}}$ is proportional to the total volume to
Table 1. Measured apparent longitudinal \( (T_{1\text{app}}) \) and apparent transverse \( (T_{2\text{app}}) \) relaxation rates for D\(_2\)O in bovine NLE and bovine AE. The uncertainties are within 5% for all the data. The symbols \( \alpha_1 \), \( \alpha_2 \), \( \beta \) and \( \gamma \) refer to different compartments, as discussed in the text.

| Relaxation times (ms) | NLE (10 °C) | NLE (25 °C) | NLE (37 °C) | AE (25 °C) |
|-----------------------|-------------|-------------|-------------|------------|
| \( T_{1\text{app}}(\alpha_1) \) | 320 | 351 | 464 | 423 |
| \( T_{1\text{app}}(\alpha_2) \) | 126 | 152 | 201 | 242 |
| \( T_{1\text{app}}(\beta) \) | 50 | 66 | 87 | 139 |
| \( T_{1\text{app}}(\gamma) \) | 30 | 34 | 41 | 66 |
| \( T_{2\text{app}}(\alpha_1) \) | 230 | 224 | 287 | 299 |
| \( T_{2\text{app}}(\alpha_2) \) | 69 | 63 | 88 | 50 |
| \( T_{2\text{app}}(\beta) \) | 23.0 | 19.9 | 29.0 | 17.3 |
| \( T_{2\text{app}}(\gamma) \) | 6.1 | 5.3 | 7.2 | 6.6 |



surface ratio [22]. A slightly smaller \( T_{2\text{app}} \) of \( \alpha_2 \) than that of \( \alpha_1 \) suggests \( \alpha_2 \) has a smaller volume to surface ratio, thereby indicating that it may be located between fibers, yet quite mobile. This will be discussed and supported by a SEM image below. In a previous study of the relaxation times of the waters of hydration in elastin, it was argued that three distinguishable dynamical properties could be discerned by fitting a single FID to a tri-exponential decay model [23, 24]. The multi-exponential fitting routines employed in [22] produced results similar to the 2D ILT algorithm. However, the ILT algorithm is capable of distinguishing a fourth component similar to that of free water, which we have labeled \( \alpha_2 \). It is worth noting that in figure 4, the component denoted as \( \alpha_1 \) appears to be made up of four peaks that are separated but close to one another. We treated this conglomerate of peaks as one component and labeled it \( \alpha_1 \). We believe that this feature may be an artifact of the ILT algorithm as we found that it was dependent on the temporal resolution that we implemented.

Referring to table 1, the \( T_{1\text{app}} \) of all components increase as the temperature of NLE is increased. This indicates that there is an increase in the mobility of the water molecules upon raising the temperature. The \( T_{2\text{app}} \) of all components are observed to decrease from 10 to 25 °C. This is indicative of a reduction in the volume of the fibers during the inverse temperature transition of elastin and is consistent with the trend found in the exchange rate, and this will be discussed below. By comparing \( T_{2\text{app}} \) of all components between the two samples, it is found that the \( T_{2\text{app}} \) are similar; this demonstrates that the water in both elastins may have a similar structural environment as the \( T_{2\text{app}} \) is reflective of the volume to surface ratio and the exchange rates [22]. This finding will also be supported by the results from the \( T_2-T_2 \) experiments discussed later.

Employing the pulse sequence shown in figure 2, 28 \( T_2-T_2 \) 2D maps were obtained at experimentally controllable mixing times ranging from 0.5 to 200 ms. Figure 5 shows the results of D\(_2\)O-hydrated AE at 25 °C with mixing times of 1, 2, 10, 50, 60 and 200 ms. From the figure, it is clear that there are four distinguishable components along the diagonal, which arise from the four different environments that are also observed in the \( T_1-T_2 \) experiments shown in figure 4.

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Figure 5. 2D ILT results of the $T_2-T_2$ experiments on AE at 25°C. The figures show the results from when the mixing time was set to $t_m = 1, 2, 10, 50, 60$ and 200 ms, respectively. The figure demonstrates the exchange of water between different compartments in elastin. It is observed that the exchange rates between $\alpha_1 \leftrightarrow \gamma$, $\alpha_2 \leftrightarrow \gamma$, $\beta \leftrightarrow \gamma$ and $\alpha_1 \leftrightarrow \alpha_2$ are larger than that between $\alpha_1 \leftrightarrow \beta$. To guide the eye, the dashed lines in the figure represent the location of where $T_2$ in one dimension is equal to $T_2$ of the second dimension. The 2D map intensity is shown on a logarithmic scale.

The notation we have adopted in the remainder of the paper is indicated as well in table 1 for the AE sample. The measured relaxation times are consistent with the values obtained in the $T_1-T_2$ experiments.

At $t_m = 1$ ms in figure 5, a cross peak having apparent $T_2$ relaxation times of 299 and 6.6 ms is evident. This reveals exchange between compartments $\alpha_1$ and $\gamma$ during the mixing time of 1 ms. Comparing results from different mixing times in figure 5, two observations can be made. (i) During short mixing times (e.g. 1, 2 and 10 ms) four cross peaks (i.e. $\alpha_1 \leftrightarrow \gamma$, $\alpha_2 \leftrightarrow \gamma$, $\beta \leftrightarrow \gamma$ and $\alpha_1 \leftrightarrow \alpha_2$) are observed, whereas a cross peak between $\alpha_1$ and $\beta$ appears later in time (e.g. 60 ms). The physics of this observation is that the exchange of water between the former four pairs of compartments is approximately 5 ms, and is much faster than the exchange rate between $\alpha_1$ and $\beta$, which is estimated to be longer than 100 ms. (ii) All of the diagonal peaks except peak $\gamma$ are always observed, even under mixing times as long as 200 ms. The disappearance of $\gamma$ at the mixing time of 200 ms is due to its $T_1$ being approximately 66 ms. Given a fast exchange time of 5 ms between $\alpha_1 \leftrightarrow \gamma$, $\alpha_2 \leftrightarrow \gamma$ or $\beta \leftrightarrow \gamma$, the existence of the diagonal peaks $\alpha_1$, $\alpha_2$ and $\beta$ in long timescales suggests that there are always water molecules within each reservoir that do not exchange with other reservoirs. The observed cross peaks arise from the water that can exchange with other compartments during the mixing time. Comparing the measured $T_2$
results from an independent measurement that we performed of free D$_2$O (again, free D$_2$O has an approximate $T_2$ of 400 ms at 25 °C), it is clear that compartment $\alpha_1$ is water that is outside the elastin fiber. The observed data in the $T_2$–$T_1$ data for this component are consistent with the $T_2$ measured in the $T_1$–$T_2$ data set. Furthermore, the samples were prepared so that there was always some excess free water surrounding the elastin fibers. Therefore, it is expected that some water in compartment $\alpha_1$ does not exchange with other compartments given the timescale of our experiments and the molecular diffusion coefficient of water. It should be noted that the positions of some of the cross peaks are slightly shifted and asymmetric on the $T_2$–$T_2$ maps. We believe that these effects are due to noise and baseline offset, as well as multisite exchange, as demonstrated in simulation [19]. In addition, we observed a larger variation in the $T_{app}^2$ in the first dimension of any given cross peak (the x-axis) over the second dimension (the y-axis). This is due to the fact that we only sampled 100 points in the first dimension, while we sampled 8000 points in the second dimension.

A simulation was performed, using MATLAB, with mixing times mimicking a condition wherein all of the water molecules undergo an exchange process. While this is not the condition realized in the experiment, the simulations presented below will make clear that the expected results had all of the water molecules exchanged. In the simulation, an exchanging four-site spin system was considered. The Bloch equation that governs the relaxation of spins is modified by the exchange rates and is given by the following expression,

$$
\frac{d}{dt} \bigg( \vec{M}_{1,2} - \vec{M}_{1,2}^{eq} \bigg) = (\vec{R}_{1,2} + \vec{K}) \vec{M}_{1,2}.
$$

(1)

In equation (1), the subscripts 1 and 2 denote longitudinal and transverse magnetization and associated relaxation terms, respectively. The magnetization of the spins, $\vec{M}_{1,2}$, is given by

$$
\vec{M}_{1,2} = \begin{pmatrix}
M_{1,2}^{\alpha_1} \\
M_{1,2}^{\alpha_2} \\
M_{1,2}^\beta \\
M_{1,2}^\gamma
\end{pmatrix}
$$

(2)

and the equilibrium magnetization $\vec{M}_{1,2}^{eq}$ is given by

$$
\vec{M}_{1,2}^{eq} = \begin{pmatrix}
M_{1,2}^{\alpha_1,eq} \\
M_{1,2}^{\alpha_2,eq} \\
M_{1,2}^{\beta,eq} \\
M_{1,2}^{\gamma,eq}
\end{pmatrix}.
$$

(3)

In equations (2) and (3), the superscripts $\alpha_1$, $\alpha_2$, $\beta$ and $\gamma$ all denote the component of the magnetization. The relaxation matrix is written as

$$
\vec{R}_{1,2} = \begin{pmatrix}
1/T_{1,2}^{\alpha_1} & 0 & 0 & 0 \\
0 & 1/T_{1,2}^{\alpha_2} & 0 & 0 \\
0 & 0 & 1/T_{1,2}^\beta & 0 \\
0 & 0 & 0 & 1/T_{1,2}^\gamma
\end{pmatrix}.
$$

(4)
Figure 6. A simulation result using the experimental values of the relaxation times and exchange rates from the $T_1-T_2$ and $T_2-T_2$ experiments. The mixing time was $t_m = 1, 60$ and $200$ ms, respectively. In the simulation, a four-site spin system with exchange was assumed. The $T_1^{\text{app}}$ and $T_2^{\text{app}}$ values were used from those tabulated in table 1. The nine independent elements in the $4 \times 4$ $\overline{K}$ matrix were $k_{\alpha_1\beta} = 0$, $k_{\alpha_1\gamma} = \frac{1}{5}$ ms$^{-1}$, $k_{\alpha_2\alpha_1} = \frac{1}{10}$ ms$^{-1}$, $k_{\alpha_2\beta} = 0$, $k_{\alpha_2\gamma} = \frac{1}{2}$ ms$^{-1}$, $k_{\beta\alpha_1} = \frac{1}{100}$ ms$^{-1}$, $k_{\beta\gamma} = \frac{1}{5}$ ms$^{-1}$, $k_{\gamma\alpha_2} = 0$ and $k_{\gamma\beta} = 0$. The disagreement with the experimental results in figure 5 shows that not all water molecules in the experiments were exchanging, as discussed in detail in the text. To guide the eye, the dashed lines in the figure represent the location of where $T_2$ in one dimension is equal to $T_2$ of the second dimension. The 2D map intensity is shown on a logarithmic scale.

\[ \overline{K} = \begin{pmatrix} k_{\alpha_1\alpha_1} & -k_{\alpha_1\alpha_2} & -k_{\alpha_1\beta} & -k_{\alpha_1\gamma} \\ -k_{\alpha_2\alpha_1} & k_{\alpha_2\alpha_2} & -k_{\alpha_2\beta} & -k_{\alpha_2\gamma} \\ -k_{\beta\alpha_1} & -k_{\beta\alpha_2} & k_{\beta\beta} & -k_{\beta\gamma} \\ -k_{\gamma\alpha_1} & -k_{\gamma\alpha_2} & -k_{\gamma\beta} & k_{\gamma\gamma} \end{pmatrix} \] (5)

Although $\overline{K}$ is a $4 \times 4$ matrix, only nine out of the 16 elements are independent. These elements are correlated according to the principle of detailed mass balance,

\[ \overline{K} \overline{M}^{\text{eq}} = 0. \] (6)

In the simulation, the values of $\overline{K}$ and $\overline{M}$ were set to those measured in the $T_1-T_2$ and $T_2-T_2$ experiments with a 0.5 ms mixing time. The nine independent exchange matrix elements were $k_{\alpha_1\beta} = 0$, $k_{\alpha_1\gamma} = \frac{1}{5}$ ms$^{-1}$, $k_{\alpha_2\alpha_1} = \frac{1}{10}$ ms$^{-1}$, $k_{\alpha_2\beta} = 0$, $k_{\alpha_2\gamma} = \frac{1}{2}$ ms$^{-1}$, $k_{\beta\alpha_1} = \frac{1}{100}$ ms$^{-1}$, $k_{\beta\gamma} = \frac{1}{5}$ ms$^{-1}$, $k_{\gamma\alpha_2} = 0$ and $k_{\gamma\beta} = 0$. These numbers are approximately equal to the observed build-up rates in the cross peaks of the $T_2-T_2$ experimental data. The simulated results are shown in figure 6 with three mixing times: 1, 60 and 200 ms. It is clear that only two diagonal peaks are observed in the simulation and their $T_2^{\text{app}}$ are measured to be 38 and 14 ms, which are an average of the four $T_2^{\text{app}}$ that are measured in the experiments. The results of this simulation demonstrate that if all of the spins exchange with each other over the timescale set by $t_m$, the resulting 2D map will show only averaged diagonal peaks at a mixing time that is comparable with or much longer than the exchange time. This is true because the exchange of spins is fast and the
Figure 7. A cartoon representing the model proposed for the water/elastin system studied in this work. In the model, four types of water are distinguishable: $\alpha_1$ and $\alpha_2$ are outside and between elastin fibers, respectively; $\beta$ is the water that is buried in the fibers; $\gamma$ is the water that is in closest proximity to the protein backbone. In this model, $\gamma$ can access all other water types—it exists both on the surface of the fiber and within the fiber. The component $\beta$ needs to diffuse outside the fiber via a tortuous channel before it can exchange with $\alpha_1$ or $\alpha_2$. Exchange of water from different reservoirs is indicated by arrows.

Different $T_2^{\text{app}}$ are averaged. However, our experimental data in figure 5 show that even with a fast exchange rate between $\gamma$ and $\alpha_1$, $\alpha_2$, or $\beta$, the diagonal peaks are still distinguishable and have the same $T_2^{\text{app}}$ values when the mixing time is long compared to the exchange time. This can only be accounted for by the aforementioned interpretation, that is, some of the water in each compartment must not be exchanging. It should be pointed out that within each compartment (e.g. $\alpha_1$), the measured apparent $T_2$ time is an average over the dynamical properties of all water in a similar structural environment. The measured value of any reservoir represents an average over the properties of molecules that exchange with other compartments, and those that do not exchange. Moreover, within each compartment, all molecules also undergo fast exchange with one another via molecular diffusion, resulting in the averaging effect—only a single $T_2^{\text{app}}$ is measured for each compartment. A model is described below that provides insight into the exchange between the four components measured in our experimental data.

A cartoon representing our model is shown in figure 7. In the cartoon, four different compartments for water are labeled according to the observations of the experimental data in figure 5. In figure 7, $\alpha_1$ is free water that is spatially located outside the fiber. The component denoted $\alpha_2$ is also free water, but it is located in the interspace of elastin fibers and undergoes anisotropic motion, given its confined space compared with that of free water. A SEM image of NLE is shown in figure 8, as a supplementary justification of the fact that spaces indeed exist between fibers in our samples. The component $\beta$ is water within fibers, and it moves along tortuous channels formed by the complex inner fiber morphology. Finally, component $\gamma$ is water that is in closest proximity to the protein. Based on this model, $\gamma$ is both inside and on

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Figure 8. A SEM image of NLE. The average diameter of elastin fibers was determined to be 3–5 µm. In the image, it is clear that there are spaces between fibers, which accommodate the component α₂ proposed in figure 7.

The component γ resides within and on the surface of the elastin fiber is true due to the fact that we observe a fast exchange rate between it and all other components α₁ ↔ γ, α₂ ↔ γ as well as β ↔ γ. The conclusion that β rests within the fiber morphology is discussed below.

The exchange between α₁ ↔ β shows up later in time, and the exchange time is estimated to be longer than 100 ms in the growth of the cross peak in the $T_2$–$T_2$ experimental data. The root mean square displacement, $\Delta r_{\text{rms}}$, of water due to diffusion is given by

$$\Delta r_{\text{rms}} = \sqrt{6D\tau}.$$

The time-dependent diffusion coefficient of water in elastin fibers was measured previously by our group and is $D = 8 \times 10^{-6}$ cm$^2$ s$^{-1}$ for 1 ms and $D = 8 \times 10^{-7}$ cm$^2$ s$^{-1}$ for 60 ms at 10 °C [25]. Therefore, for a time of 1 ms, the displacement is approximately 2.1 µm, and it is 5.4 µm when $\tau = 60$ ms, ignoring the tortuosity of the inner fiber morphology. The average diameter of elastin fibers, referring to figure 8, was measured to be 3–5 µm. Thus, according to the computation, it takes more than 60 ms for water inside fibers to diffusively exchange with water outside fibers, and vice versa. This prediction is consistent with the experimental observation of a slow exchange rate between α₁ ↔ β, which again was greater than 100 ms.

The fast exchange rates for α₁ ↔ γ, α₂ ↔ γ and β ↔ γ observed in the experiments can also be explained by the model wherein γ on the surface is in close proximity to α₁ and α₂ and is also buried within the fiber and fast exchanges with β.

Given that a component of γ resides within the complex folding of the protein backbone and fast exchanges with α₁, α₂ or β, it follows that the exchange of γ with other components can be considered separately. The component α₁ fast exchanges with the γ molecules that are on the surface of the elastin fibers. The component α₂, which is trapped within the spaces between fibers, fast exchanges with γ on the surface of elastin fibers as well. The component β fast exchanges with γ inside the fiber morphology. Therefore, the complex, four-site ($\alpha_1$, $\alpha_2$, $\beta$, and $\gamma$) system may be simplified to several two-site (e.g. $\alpha_1 \leftrightarrow \beta$, $\alpha_2 \leftrightarrow \gamma$) systems. Thus, for
each subsystem, an equation that involves only two sites may be implemented as follows,

\[
\frac{d}{dt} \begin{pmatrix} M_i - M_i^{eq} \\ M_j - M_j^{eq} \end{pmatrix} = \begin{pmatrix} -R_i - k_i & k_j \\ k_i & -R_j - k_j \end{pmatrix} \begin{pmatrix} M_i - M_i^{eq} \\ M_j - M_j^{eq} \end{pmatrix}.
\]

(8)

In the above expression, the subscripts \(i\) and \(j\) denote two distinguishable sites, based on their \(T_2\) times. The apparent relaxation times, accounting for exchange (i.e. \(K \neq 0\)), are given by

\[
S_{1,2}^{\text{app,+/−}} = - \frac{1}{2} \left( R_{i,j}^{1,2} + k_i + R_{j,i}^{1,2} + k_j \right)
\]

\[
\pm \frac{1}{2} \left[ (R_{i,j}^{1,2} + k_i + R_{j,i}^{1,2} + k_j)^2 - 4 (R_{i,j}^{1,2} R_{j,i}^{1,2} + R_{i,j}^{1,2} k_j + R_{j,i}^{1,2} k_j) \right]^{1/2},
\]

(9)

where \(S_{1,2}^{\text{app,+/−}}\) denotes the long (+) and short (−) \(T_1^{\text{app}}\) and \(T_2^{\text{app}}\) relaxation rates [18]. The intensity of the cross peak is a function of \(S_{1,2}^{\text{app,+/−}}\) and \(K\) and may be written as

\[
P = \exp(T_{1,2}^{\text{app,+/−}} \tau_m) \left( (A_{1}^{+} + F_{2}) \left[ A_{1}^{-} (A_{2}^{-} M_{i}^{eq} - G_{2}^{-} M_{j}^{eq}) - G_{1}^{-} (B_{2}^{-} M_{j}^{eq} - F_{2}^{-} M_{i}^{eq}) \right] + (B_{2}^{+} + G_{2}) \left[ B_{1}^{+} (B_{2}^{-} M_{j}^{eq} - F_{2}^{-} M_{i}^{eq}) - F_{1}^{-} (A_{2}^{-} M_{i}^{eq} - G_{2}^{-} M_{j}^{eq}) \right] \right) + \exp(T_{1,2}^{\text{app,−/−}} \tau_m) \left( (A_{1}^{+} + F_{2}) \left[ A_{1}^{+} (A_{2}^{-} M_{i}^{eq} - G_{2}^{-} M_{j}^{eq}) - G_{1}^{-} (B_{2}^{-} M_{j}^{eq} - F_{2}^{-} M_{i}^{eq}) \right] + (B_{2}^{+} + G_{2}) \left[ B_{1}^{+} (B_{2}^{-} M_{j}^{eq} - F_{2}^{-} M_{i}^{eq}) - F_{1}^{-} (A_{2}^{-} M_{i}^{eq} - G_{2}^{-} M_{j}^{eq}) \right] \right),
\]

(10)

where

\[
A_{1,2}^{+/−} = \frac{S_{1}^{\text{app,+/−}} + k_i + R_{i,j}^{1,2} + k_j}{S_{1}^{\text{app,+/−}} - T \cdot S_{1}^{\text{app,−/−}}},
\]

(11)

\[
B_{1,2}^{+/−} = \frac{S_{1}^{\text{app,+/−}} + k_j + R_{j,i}^{1,2} + k_i}{S_{1}^{\text{app,+/−}} - T \cdot S_{1}^{\text{app,−/−}}},
\]

(12)

\[
F_{1,2} = \frac{k_i}{S_{1}^{\text{app,−/−}} - S_{1}^{\text{app,+/−}}},
\]

(13)

\[
G_{1,2} = \frac{k_j}{S_{1}^{\text{app,−/−}} - S_{1}^{\text{app,+/−}}},
\]

(14)

Comparing the measured \(T_1\) values with the estimated exchange rates, it is evident that \(1/T_1 \ll k\) for all compartments. Under this condition, equation (9) simplifies to

\[
S_{1}^{\text{app,+/−}} = 0
\]

(15)

and

\[
S_{1}^{\text{app,−/−}} = k_i + k_j.
\]

(16)

Equation (10) may be written as

\[
P = C_{1} - C_{2} \exp(-t_m/\tau_{ij}^{\text{ex}}),
\]

(17)

where

\[
C_{1} = (A_{1}^{+} + F_{2}) \left[ A_{1}^{-} (A_{2}^{-} M_{i}^{eq} - G_{2}^{-} M_{j}^{eq}) - G_{1}^{-} (B_{2}^{-} M_{j}^{eq} - F_{2}^{-} M_{i}^{eq}) \right] + (B_{2}^{+} + G_{2}) \left[ B_{1}^{+} (B_{2}^{-} M_{j}^{eq} - F_{2}^{-} M_{i}^{eq}) - F_{1}^{-} (A_{2}^{-} M_{i}^{eq} - G_{2}^{-} M_{j}^{eq}) \right]
\]

(18)
Two typical cross peak growth curves determined by the mixing time dependence in the $T_2-T_2$ experiments. The open points are the experimental data from NLE at 25 °C and the solid lines are the fitted curves to equation (17). The error bars of the experimental data are within 5% of the number shown.

In equation (17), $\tau_{ij}^{ex} = -(k_i + k_j)^{-1}$ is the exchange time for waters exchanging between sites $i$ and $j$. This result is similar to that implemented by Washburn and Callaghan [15]. Equation (17) is fitted to the experimentally measured build-up of cross peak intensity as a function of mixing time, where an exchange time is the best-fit parameter for $\tau_{ij}^{ex}$. To be clear, we fitted the parameters $C_1$, $C_2$ and $\tau_{ij}^{ex}$ to the experimental data. Two examples of cross peak growth curves between $\alpha_1 \leftrightarrow \gamma$ and $\alpha_2 \leftrightarrow \gamma$ on NLE at 25 °C are shown in figure 9. In the figure, the open points are experimental data, and the solid lines are the best-fit curves to equation (17). The only difference between the two curves is that the constant $C_1$ is similar to $C_2$ in equation (17) for the open circles, whereas $C_1$ is larger than $C_2$ for the open squares in the fitting. The exchange time extracted for the open circles is $\tau_{\alpha_1 \leftrightarrow \gamma}^{ex} = 4.8$ ms and $\tau_{\alpha_2 \leftrightarrow \gamma}^{ex} = 3.5$ ms for the open squares. This measurement is consistent with the estimate on exchange times made earlier. It should be noted that the curves ultimately decay at longer mixing times due to $T_1$ relaxation and are not shown in figure 9. By fitting equation (17) to all of the cross peak intensities, the exchange times between different compartments were determined and are tabulated in table 2. It is evident from the table that the $T_1^{app}$, $T_2^{app}$ and exchange times are similar for water in both the NLE and the AE; this observation indicates that water molecules experience similar dynamics in both samples.

\begin{equation}
C_2 = -(A_2^+ + F_2) \left[ A_1^+ (A_2^- M_i^{eq} - G_2 M_j^{eq}) - G_1 (B_2^- M_j^{eq} - F_2 M_i^{eq}) \right] \\
- (B_2^+ + G_2) \left[ B_1^+ (B_2^- M_j^{eq} - F_2 M_i^{eq}) - F_1 (A_2^- M_i^{eq} - G_2 M_j^{eq}) \right],
\end{equation}
and $C_1$ and $C_2$ are constants for a certain $K$ and $R_2$. New Journal of Physics 13 (2011) 025026 (http://www.njp.org/)
Table 2. Measured exchange times ($\tau_{ij}^{ex}$) (the inverse of the exchange rate) of D$_2$O in bovine NLE and bovine AE, determined by fitting equation (17) to the cross peak build-up curves in the $T_2-T_2$ exchange experiments. The uncertainties are within 5% for all the data.

| Exchange times (ms) | NLE (10°C) | NLE (25°C) | NLE (37°C) | AE (25°C) |
|---------------------|------------|------------|------------|----------|
| $\tau_{\alpha_1 \leftrightarrow \gamma}^{ex}$ | 6.0        | 4.8        | 4.9        | 4.2      |
| $\tau_{\alpha_2 \leftrightarrow \gamma}^{ex}$ | 6.6        | 3.5        | 5.1        | 2.7      |
| $\tau_{\beta \leftrightarrow \gamma}^{ex}$ | 3.4        | 1.7        | 2.4        | 1.8      |
| $\tau_{\alpha_1 \leftrightarrow \alpha_2}^{ex}$ | 5.4        | 1.7        | 3.3        | 5.5      |
| $\tau_{\alpha_1 \leftrightarrow \beta}^{ex}$ | 770        | 650        | 910        | 420      |

Table 3. For NLE, the fractional contribution of the four components to the total signal at the different temperatures studied. It is observed that the abundance of $\alpha_1$ and $\alpha_2$ increases from 10 to 37°C, whereas the $\gamma$-component and $\beta$-component decrease from 25 to 37°C. This change appears to be well correlated with the negative thermal expansion coefficient of elastin over this temperature range. The numbers shown in the table were determined over a mixing time of 0.5 ms.

|                | NLE (10°C) | NLE (25°C) | NLE (37°C) |
|----------------|------------|------------|------------|
| $\alpha_1$     | 0.352      | 0.353      | 0.368      |
| $\alpha_2$     | 0.321      | 0.324      | 0.330      |
| $\beta$        | 0.198      | 0.204      | 0.197      |
| $\gamma$       | 0.129      | 0.120      | 0.105      |

It is well known that elastin undergoes an inverse temperature transition at approximately 15–25°C [1]. During the inverse temperature transition, elastin goes from a soluble state to an insoluble state with increasing temperature. Microscopically, hydrophobic association of $\beta$ turns occurs during the phase transition, and, as a consequence, elastin’s volume decreases macroscopically with increasing temperature. In Urry’s description, water molecules that were originally in close proximity to the protein are pushed out and become more mobile free water. Experimentally, we find that the exchange rates of all of the components appear to increase from 10 to 25°C. This observation appears to be consistent with the reduction in volume of the elastin fiber over this temperature range. Moreover, referring to table 3, the intensity of the diagonal peak for $\gamma$ at $t_m = 0.5$ ms is observed to decrease with increasing temperature when going from 10 to 37°C. This observation indicates that the number of $\gamma$ molecules decreases on heating, which reveals that the $\gamma$-component is indeed water that is in close proximity to the protein. We observed that the intensity of the $\alpha_1$- and $\alpha_2$-components increases from 10 to 37°C, which we attribute to water that has been squeezed out of the fibers as they are shrunk upon heating. The observation that the intensity of the $\gamma$-component decreases with increasing temperature is also consistent with previous findings of our laboratory using deuterium double quantum filtered (DQF) NMR [26]. The DQF NMR experiment allows one to probe the degree of anisotropic motion of nuclear spins by monitoring the growth and subsequent decay of double quantum
coherence created by a partially averaged quadrupolar interaction and well-defined RF pulse sequence. In that work, the highly bound deuterium water molecules were observed to decrease in number at about 10–25°C, concomitant with a jump in the residual quadrupolar interaction ($\omega_Q$) at 10°C. The observed jump in $\omega_Q$ appeared to be well correlated with the inverse temperature transition of elastin. When we raised the temperature further beyond 10°C to 37°C, we observed a subsequent decrease in $\omega_Q$, indicative of a decrease in motional anisotropy of the $\gamma$-component. Considering elastin and all waters of hydration as a closed thermodynamic system, the net entropy of all waters should increase with increasing temperature in order to obey the second law of thermodynamics. This is true because the local ordering of the protein increases during the phase transition, thereby decreasing its entropy. Referring to table 1, we observed an increase in $T_1$ of all of the components. The components $\alpha_1$, $\alpha_2$ and $\beta$ all have relatively long $T_2$ times and are thus in the fast-motion regime (i.e. $\tau_c \gg \omega_o$, where $\tau_c$ is the correlation time and $\omega_o$ is the Larmor precession frequency). In this limit, the spin-lattice relaxation time is inversely proportional to the correlation time of motion, $T_1 \propto \tau_c^{-1}$ [27]. The observed increase in $T_1$ of all components with increasing temperature reveals a decrease in correlation time, indicative of an increase in motion and entropy. Lastly, referring to table 2, the exchange times for all components decrease as the temperature is raised from 10 to 25°C. This observation is consistent with the decrease in $T_2^{app}$ of all components from 10 to 25°C shown in table 1, as the observed $T_2^{app}$ times are dependent on the exchange rates and the relaxation rates of other compartments, according to equation (9).

4. Conclusion

In this work, we have investigated the exchange of water in hydrated elastin by using 2D $T_1$–$T_2$ and $T_2$–$T_2$ correlation experiments. We studied the exchange of water in bovine nuchal ligament elastin and aortic elastin. We investigated the temperature dependence of the exchange rate of water in nuchal ligament elastin. The experimental data indicate that there are four exchanging sites. A model is proposed that quantifies the exchange between the sites by assuming that they exchange independently. The dynamics of water in nuchal ligament elastin and aortic elastin appear to be similar, indicating that the structural morphology may be identical. The temperature dependence of exchange rates indicates an increase in entropy in water at about 25°C where elastin undergoes the inverse temperature transition.

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