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Diffusion-Based Biomolecular Sensing Using Low-Field NMR

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Diffusion-Based Biomolecular Sensing Using Low-Field NMR

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Chemistry from the College of William & Mary

by

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Accepted for Honors

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Abstract

Identification of molecules in complex mixtures is a fundamental problem in analytical chemistry. NMR is widely used for molecular identification in impure samples, but traditionally is an expensive technique that requires a large-scale laboratory setting and extensive training to operate. New relaxometric techniques have been developed for low-cost NMR apparatuses with strong field inhomogeneities, where change in relaxation time $T_2$ of water surrounding the aggregation of paramagnetic nanoparticles around a given analyte is measured. These devices’ strong magnetic field gradients make them suitable for simultaneous measurement of the self-diffusion constant $D$. In this study, the advantages of a two-dimensional $T_2-D$ approach to molecular identification of a protein-specific ligand analyte in complex with its target protein are assessed as a “proof of principle” experiment. Since complexation reduces molecular motion, we expect both $T_2$ and $D$ will decrease, indicating the presence of the ligand by the shape and size of the sensor as a unit rather than its chemical makeup. Per the complex nature of our selected protein-ligand binding interactions (those of bovine serum albumin and naproxen), the detection of a number of unexpected phenomena, including naproxen self-association, non-specific binding interactions, and possibly rapid chemical exchange are reported. A potential method by which low-field, single-sided NMR devices may be used to determine thermodynamic constants is also demonstrated.
Acknowledgments

My work has in one sense been a direct product of the care and patience of a great number of individuals—from faculty with whom I’ve developed very strong relationships to members of the staff whom I may have never met and whose contribution I will never know. I can say for certain that I am beyond grateful for the opportunities provided to me by the Chemistry Department here. It is my hope that the understanding I’ve fostered with its help will serve as a framework for the rest of my working career. Thank you most of all to Prof. Meldrum, or Tyler, as he invariably insists upon me calling him. His guidance has, in many ways, defined the past year-and-a-half of my academic life, and I owe so much of my progress as both a student and an individual to him. Thank you also to my fellow lab students for being such a pleasure to work with throughout the process of completing this research—in particular Justin, for whom I hope this thesis will be a helpful guide if he continues the project in my place. Though we only met a handful of times during my entire undergraduate experience, I feel I must acknowledge the invaluable aid given to me by Dr. Beverly Sher over the course of my preparations to pursue a career in medicine—I cannot predict if I will indeed become a physician in time, but I know that whatever success I find in my future endeavors will be thanks to her inspiration. Lastly, it is my friends and family that give true meaning to my life and work, and my greatest appreciation goes out to them for all their love.
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1 Introduction

1.1 Supplemental Background of NMR Spectroscopy

This study sought to assess as a proof-of-principle experiment the advantages and disadvantages of a so-called “low-field” Nuclear Magnetic Resonance (NMR) approach to molecular identification. Thus, a brief description of the principles of NMR before going into detail about the research performed is appropriate. When relevant, more conventional, “high-field” techniques will be brought in for comparison, but otherwise this explanation will only cover topics necessary to understand this particular study. Further information on the subject is widely available thanks to authors such as Keeler, Blümich, and Cavanagh, whose work is recommended should the reader wish to know more.

Currently, NMR spectroscopy is one of the predominant techniques used to determine the three-dimensional structures of molecules at atomic resolution. In addition, NMR is a powerful technique for investigating time-dependent phenomena, including reaction kinetics and intramolecular dynamics. Nuclear magnetic resonances in bulk condensed phase were first reported in 1946 by Bloch et al. and by Purcell et al. Nuclear magnetism relies upon the notion that all nuclei possess the quantum mechanical intrinsic properties of nuclear spin and spin angular momentum. Most nuclei of interest in NMR techniques possess a spin angular momentum quantum number ($I$) of $\frac{1}{2}$, these include elements such as $^1$H, $^{13}$C, $^{19}$F, and $^{129}$Xe. In this research, only protons ($^1$H) were analyzed, while other elements and isotopes (such as deuterium, known as either $^2$H or D) are said to be NMR inactive. This is a good assumption in grossly inhomogeneous fields such as the one employed by our instrument—the detection coil is tuned specifically to the proton frequency, a range where $^1$H is the only nucleus that produces a
reasonably large signal (the precise meaning of these specifications will be made more clear shortly).

Associated with angular momentum is always a nuclear spin magnetic moment—in effect, each proton generates a small magnetic field. In NMR techniques, this field interacts with an applied field \(B_0\) such that the spin falls into one of two energy states, either aligned with the field (lower in energy) or aligned against the field (greater in energy). To simplify the true quantum mechanical environment (beyond the scope of this research), this interaction is further explained using a vector model: we say that the result of the interaction between individual magnetic moments and \(B_0\) is a summed equilibrium magnetization vector that, at equilibrium, is parallel to \(B_0\), where the energy of the interaction depends upon the angle between the two, and is lowest (most stable) when the magnetic moment is parallel to the applied field \((\theta = 0)\). This vector can be visualized in a three-dimensional coordinate system running along the positive \(z\)-axis (as can \(B_0\)). If, by some means, this magnetization vector is tipped away from its preferred orientation, however, it will rotate about the \(z\)-axis in a kind of motion known as Larmor precession (Fig. 1). In an applied magnetic field \(B_0\) the vector will precess at a constant frequency \((\omega)\) described by:

\[
\omega = -\gamma B_0
\]

where \(\gamma\) is the gyromagnetic ratio, a constant unique to each nucleus. For \(^1\text{H}\), \(\gamma = 42.58\ \text{MHz}\cdot\text{T}^{-1}\) or \(2.675\times10^8\ \text{rad}\cdot\text{s}^{-1}\cdot\text{T}^{-1}\). The frequency of precession of the magnetization vector is what is actually detected in an NMR experiment. Typically, a small coil of wire placed around or underneath the sample detects precession in the form of an induced current (known as free induction decay, or FID) brought about as the spinning magnetization vector “cuts” across the
coil. The precise nature of the magnet supplying the applied field and of the coil varies with the specific NMR technique used; our apparatus will be discussed in more detail in the following section.

The induction of Larmor precession is where the concept of resonance comes into play in NMR techniques—radiofrequency (RF) power is supplied through this same coil, applying to the sample a small magnetic field perpendicular to $B_0$ that oscillates at or near the Larmor precession frequency in the $x$ direction. Because the oscillating field is resonant with the precession frequency, the magnetization vector can be tipped from its equilibrium orientation towards or into the transverse ($x$-$y$) plane even in the presence of a much stronger applied field $B_0$. This pulse of RF power is called an on-resonance pulse, or alternatively a $90^\circ \left( \frac{\pi}{2} \right)$ pulse, and is all that is required for the simplest NMR process: the pulse-acquire experiment. Here, the pulse sequence (Fig. 3) consists only of a $90^\circ$ pulse to induce Larmor precession in the relevant nuclei, followed by a period during which the coil is used to detect the precession’s FID.

1.2 Single-Sided NMR

NMR devices using lower applied field strengths (e.g. 0.5 T, vs. a high-field value of 20 T) have been employed as easy-access, mobile, and/or low-cost analytical tools in areas of study where more advanced apparatuses are inconvenient or unusable. Our device, the NMR-MOUSE, is a single-sided apparatus that uses permanent magnets to produce the applied field $B_0$. Their configuration and resulting magnetic field is described in Fig. 4. More can be learned about the origins and construction of the NMR-MOUSE from the work of Blümich et al in 1996. Our specific device is a newer model with some modifications compared to the original; relevant points about the instrument will be covered later. Being single-sided, a wide range of
materials can be analyzed with the MOUSE; however, this configuration results in severe magnetic field inhomogeneity, as well as a sharp field gradient \((G)\). More advanced phenomena often measured in high-field techniques, such as chemical shift or \(J\)-coupling, cannot be easily measured in inhomogeneous fields. Furthermore, hardware limitations brought about by the single-sided design dictate some modifications to our pulse sequence beyond the simple pulse-acquire experiment: there is a “dead time” period after the initial 90° pulse where residual RF energy has yet to leave the coil, such that the MOUSE does not have time to measure precessional signal and frequency via FID before the signal has decayed (before the magnetization vector has returned to equilibrium). Previous work, known today as Hahn echoes\(^{10}\) and the Carr-Purcell-Meiboom-Gill (CPMG) sequence,\(^{11,12}\) build off the pulse-acquire pulse sequence to circumvent this shortcoming of low-field NMR. Hahn echoes, so-named due to their discovery by E. L. Hahn in 1950, are echoes of the original FID signal created by a refocusing pulse of magnetization (known as a 180° or \(\pi\) pulse). This 180° pulse is produced by the RF coil a certain time after the initial 90° pulse, and flips the bulk magnetization vector along the \(x\)- or \(y\)-axis (depending upon the phase of the initial pulse) (Fig. 5).\(^{10}\) Recalling that the summed magnetization vector describes the overall trend of individual \(^1\)H magnetic moments, it follows that there will be a reconvergence of the magnetization vector back upon the original precessional event: each individual magnetic moment, which after the on-resonance pulse has returned to some unspecified orientation resulting in a summed precessional signal decay, now reverses direction in response to the refocusing pulse. Each individual magnetic moment therefore converges back on its precessional orientation (Fig. 6), with full convergence occurring after a measure of time equal to the delay between the 90° and 180° pulses. Since the delay time

\(^{4}\) Though there is a dead time associated with high-field techniques as well, it is much shorter, and the signal persists for much longer.
between each magnetic pulse can be chosen at will, this precessional “echo” can be made to appear long after the “dead time” of both pulses, and so the echo can be detected by means of the RF coil. This method allows for us to measure the original FID signal amplitude via the Hahn echoes; however, the FID frequency cannot be determined.

Hahn echoes were later employed and improved upon after their discovery to access more information about a sample by two successive pairs of scientists: Carr and Purcell in 1954,\textsuperscript{11} followed by Meiboom and Gill in 1958.\textsuperscript{12} Together, their work culminated in a pulse sequence that came to be known as the CPMG sequence. Their innovation, in short, was to repeat the refocusing pulse many times, alternating its phasing with respect to the 90° pulse each time, in order to repeatedly create Hahn echoes (Fig. 7). These echoes are themselves subject to decay (recall, only a single on-resonance pulse is actually applied in each iteration of the pulse sequence) due to several different phenomena, which are consistent and quantifiable for a given proton source. It is these phenomena around which this research project is designed, namely two properties known as $T_2$ (transverse) relaxation and self-diffusion ($D$). $T_1$ (longitudinal) relaxation is also used in some cases to help determine our required acquisition parameters.

1.3 The Physical Origins of $T_1$, $T_2$, and $D$

$T_1$ Relaxation

Both $T_1$ and $T_2$ refer to relaxometric properties—that is, processes by which the bulk magnetization returns to its equilibrium position. A detailed description of $T_1$ is not necessary to understand the current research; it is satisfactory to understand that it is a measure of the length of time it takes for the energy put into a system to dissipate\textsuperscript{1}. Signal loss due to $T_1$ relaxation follows an exponential decay. Its use in our experiments was to help determine the proper
acquisition parameters for $T_2$ and $T_2$-D tests which are covered in the Experimental Methods section. The textbook on single-sided NMR by Blümich et al.$^{13}$ is a suitable resource for any further questions the reader may have on the subject.

$T_2$ Relaxation

$T_2$ is a term for the characteristic time after which transverse magnetization decays away to its equilibrium value of zero, also known as transverse relaxation.$^{1,†}$ Understanding transverse relaxation requires considering the given sample as a collection of many individual protons (within molecules) with their own magnetic moments, which then can be summed to produce a bulk magnetization vector. In inhomogeneous fields, each magnetic moment in fact experiences a slightly different magnetic environment. Application of a 90° pulse, as discussed previously, will shift the magnetization vectors into the transverse plane, but, due to field inhomogeneity, each individual magnetic moment will precess within this plane about the $z$-axis at a slightly different precessional frequency. This dephasing of precessional frequencies increases over time as individual magnetization vectors become more and more out of sync with each other; consequently, the summed bulk magnetization vector’s $x$-$y$ components will decrease as those of the individual magnetization vectors cancel each other out. The result is the eventual decay of the FID signal amplitude at a rate specific to the proton source. This rate (occasionally referred to as $R_2$) is often described by its reciprocal, $T_2$. In low-field techniques, $T_2$ can be found using CPMG experiments. The equation used to find $T_2$ in this case reads thus:

$$\frac{S}{S_0} = e^{-t/T_2} ; \ln \left( \frac{S}{S_0} \right) = \left( \frac{-1}{T_2} \right) t$$

† $T_2$ is the inverse of $R_2$, the rate of relaxation; thus, while $T_2$ (having units of s) is not technically a rate, it still characterizes how quickly transverse relaxation occurs.
where $S$ is the measured signal intensity, $S_0$ is the greatest signal intensity measured overall (which is used to normalize the data), and $t$ is the time as each CPMG-sequence scan takes place. By taking the natural log of both sides of the initial equation, it can be easily demonstrated that the natural log of the normalized signal intensity decays over time at a rate equal to $-1/T_2$. A plot of this log value vs. time can therefore be generated in a CPMG NMR experiment in order to determine $T_2$ for the given proton source.

D - The Self-Diffusion Coefficient

Signal decay due to diffusion is a result of the field gradient of our single-sided NMR device’s applied magnetic field creating a non-ideal environment for the application of refocusing $180^\circ$ pulses during CPMGs. Under ideal circumstances where $B_0$ is constant throughout a sample, $180^\circ$ pulses reflect individual magnetization vectors along the $x$-axis, which then form an echo of the original FID (as explained above). With a sharp field gradient, however, $B_0$ is weaker at greater heights above our apparatus’ sensing surface, and different depths of a given sample experience different magnetic field strengths (Fig. 8). As a result of molecular motion, then, protons will experience different field strengths as they move about within the sample, and thus will precess at different frequencies at one time during an experiment compared to another. In such conditions, upon application of a refocusing pulse during CPMG, a given proton magnetic moment will reverse direction and converge back on its initial FID orientation at a different rate from the one it experienced previously. Each magnetic moment will therefore no longer converge directly back on its original orientation, resulting in a reduction in intensity of the echo (Fig. 9). This diffusion decay increases over time as additional refocusing pulses are applied.
In order to measure diffusion, the regular CPMG pulse sequence is modified in the following way: a second 90° pulse is applied after the first, after a delay time δ, which rotates the bulk magnetization vector out of the transverse plane; this ensures that no signal decay will occur due to $T_2$ relaxation during a second delay time $\Delta$; finally, a third 90° pulse is applied, which sums with the second to effectively act together as a 180° pulse, resulting in the production of a Hahn echo after an additional time $\delta$ (Fig. 10). This process combines well with measuring $T_2$, since this echo can then be reformed as in a regular CPMG experiment using additional refocusing pulses. The combined pulse sequence is therefore used in multidimensional $T_2$-$D$ experiments, where both transverse relaxation and self-diffusion are measured. In ideal situations, where $\delta \ll T_2$ and $\Delta \ll T_1$, the equation used to find $D$ appears thus:

$$\ln \left( \frac{S}{S_0} \right) \approx -\gamma^2 G^2 \delta^2 \left( \Delta + \frac{2}{3} \delta \right) D$$

where $\ln \left( \frac{S}{S_0} \right)$ is the normalized signal intensity, $\gamma$ is the gyromagnetic ratio, $G$ is the magnetic field gradient of $B_0$. As we did before for $T_2$, the normalized signal intensity can be plotted out, this time vs. $\gamma^2 G^2 \delta^2 \left( \Delta + \frac{2}{3} \delta \right)$, to give a function with slope $-D$.

1.4 Rationale for Exploring a Low-Field, $T_2$-$D$ Approach

As a reference, raw data of a typical $T_2$-$D$ experiment, showing signal decay due both to $T_2$ and $D$ vs. time (specifically, acquisition time for $T_2$ and change in $\delta$ delay time for $D$) is depicted in Figure 11. Low-field NMR instruments must compensate for their poor signal-to-noise ratio by repeating a given pulse sequence many times, summing each iteration or “scan” together to ensure representative data is collected. In the case of $T_2$-$D$ experiments, the delay time $\delta$ is then changed after a certain number of scans (each set of scans of a certain $\delta$ is called
an increment), providing a means to determine both $T_2$ and $D$ according to the equations listed above.

Multidimensional experiments are useful in molecular identification; increasing the number of dimensions to characterize a given sample reduces the chance of signal crowding, making determination of the contents more exact. $T_2$ and $D$ in particular are quite compatible in multidimensional experiments with low-field NMR. Relaxometric techniques do not require a homogeneous field, and thus can effectively be measured by low-field NMR devices. Meanwhile, our single-sided magnet’s high field gradient in fact improves the accuracy of measuring self-diffusion. Furthermore, as stated previously, a combined experiment is convenient, since a composite pulse sequence can be used that acquires the data required to determine both terms. At a more in-depth, conceptual level, however, they share an additional connection: both $T_2$ relaxation and diffusion are related to molecular motion, and thus are related to molecular size. As sample proton sources get larger, they should diffuse more slowly, and relax more quickly (both $T_2$ and $D$ should therefore decrease as the size increases).

This last point provides a theoretical means to use low-field, single-sided NMR as a tool to monitor changes in molecular size as a reporter for changes in structure during chemical events: Molecular identification can be carried out in impure samples if an $^1$H-containing analyte of interest is known to bind to some other molecule of non-comparable size with high specificity. In a typical instance of this technique involving a comparatively small analyte and a much larger “probe” ligand, a $T_2$-$D$ experiment could be run on two samples: a control sample containing the original impure mixture, and a test sample into which the ligand is added to the original mixture. Complexation between the analyte and ligand will occur in the test sample if the analyte is present, causing the analyte to relax and self-diffuse along with the ligand. A corresponding
drop in $T_2$ and $D$ for one proton source found in the control experiment can be understood to indicate the presence of the analyte in question in the impure sample (Fig. 12). Previous studies by Weissleder et al.\textsuperscript{14,15} have relied on a similar principle, using paramagnetic nanoparticles as magnetic relaxation switches. This experiment seeks in part to improve upon this body of work—which relied on single-dimension $T_2$ analyses—by factoring in $D$ to create a more reliable, multidimensional experiment.

1.5 Bovine Serum Albumin & Naproxen as Test Compounds

This technique, in analyzing molecular shape and size rather than chemical makeup, lends itself to a wide range of binding pairs—most readily, perhaps, protein-ligand pairs and other biomolecules. Many protein-ligand pairs are widely available for purchase and are incredibly common as test compounds in scientific research (e.g. avidin\textsuperscript{16} & biotin\textsuperscript{17}). Bovine serum albumin (BSA) and naproxen-sodium were chosen as a test pair for our proof-of-concept experiment (Fig. 13). Both BSA and naproxen-sodium are relatively cheap and easy to acquire,\textsuperscript{18,19} and their interactions are well-characterized (in terms of binding interactions, naproxen-sodium should be entirely analogous to naproxen).\textsuperscript{20,21} Both compounds lend themselves well to analysis in the aqueous (D\textsubscript{2}O), neutral pH, room temperature environment of the test samples as they were originally envisioned. Important chemical properties of the two molecules are listed in Table 1.

BSA monomers each possess a single binding sites for naproxen-sodium with $K_D = 3.27\times10^{-8}$ M.\textsuperscript{21} An explanation of the binding calculations used to make our test samples is provided in Appendix E. Additionally, it turns out that at high concentration individual BSA molecules dimerize ($K_D = 10^{-5}$ M).\textsuperscript{22} This, as we will see, results in a splitting of the BSA $T_2$-$D$
signal into a monomer source and a dimer source. BSA dimerization was not observed to have any effect on the binding affinity of naproxen-sodium.
2 Experimental Methods

2.1 General Protocol for Collecting $T_2$-$D$ Results

A general description of the entire protocol for producing results from a single $T_2$-$D$ experiment as a reference is provided below, before going into detail about each individual portion of our experimental process:

1. The test sample is prepared.

2. The NMR-MOUSE, spectrometer, power source, and computer set to acquire data are all turned on; the operating/processing program Prospa is loaded up on the computer in order to operate the magnet. A cable runs from a port on one side of the magnet housing to the “Probe” input port on the spectrometer.

3. With no sample in place, the frequency of the magnetic field $B_0$ is confirmed to be correct (it should be 19.44 MHz) using a test known as the Wobble check (all tests are selected from the NMR-MOUSE drop-down menu). The “Save Data” box is unchecked and the test is started by clicking “Run”. Generally, there should be no reason for the frequency to be incorrect, unless the magnet has not been plugged into the spectrometer. Once the frequency is confirmed, the test is ended by clicking “Abort”.

4. With no sample in place, the signal-to-noise ratio is checked using the MonitorNoise test, and is confirmed to be acceptably low for running experiments (generally, the acceptable range is below 0.65). Again, this test need not be saved, and is stopped and started in the same way as the Wobble.

5. A deionized (DI) water sample (prepared earlier and left at the instrument workstation for repeated use) is then placed on the magnet for use in the Calibration or “Cal” test, which determines the current optimum pulse length $\tau$ (in $\mu$s) by taking a determined number of measurements with a set of incrementally larger pulse lengths (the start, end, and increment size of these $\tau$ are all defined by the user). The calibration need not be saved, but must run to completion after clicking “Run”; the calibration cannot be aborted ahead of time. The relative amount of signal acquired using each possible pulse length is shown in a graph in the Prospa window, with both real data and noise displayed. The optimum $\tau$ is that which shows the largest difference between signal and noise.

6. The test sample is then placed on the magnet and is run through a $T_1$ Saturation Recovery ($T_1$Sat) test in order to determine $T_1$ (in ms). The save data box is left checked and the correct directory is chosen for the test files’ location, including the test’s name ($T_1$) and
experiment number. This will create a folder in the given directory labeled T1 containing subfolders for each iteration of the test performed.

7. The test sample is then run through a CPMG test to determine $T_2$ (in ms). The data is saved in the same manner as the $T_1$ test, but under the name “T2”.

8. At this point, the values for $T_1$ and $T_2$ are entered into an excel spreadsheet designed to help determine the optimum acquisition parameters for a $T_2$-D experiment, in particular the delay times $\delta$ and $\Delta$ (here, ms), the number of echoes, the number of scans, the repetition time (ms), and the number of increments—these all determine the length of time an experiment will take.

9. A $T_2$-D experiment is then run on the sample (referred to in Prospa as an SSE-T2 test). This is saved into the desired directory under the name “T2_D”.

10. At the conclusion of the experiment, the raw data is accessed (for us, this was on a second, more powerful computer) and copied to the desktop to avoid accidental modification of the original files; the data consist mainly of an acqu.par file, which records the acquisition parameters, and three files each containing either real (data2d_Re.2d), imaginary (data2d_Im.2d), or complex (data2d_Cp.2d) data from the experiment. Prospa is again opened. From here, complex data can be dragged into a 2D plot window in Prospa to be processed.

11. In Prospa, the raw data is prepared in order to be run through Inverse-Laplace transformations, which give the final $T_2$-D distribution used to determine results. To prepare, the data’s axes are defined using the Calibrate2d macro; the y-axis (change in delay time $\delta$ in $\mu$s) is mapped to a log scale using the Mapping function; and finally the data is autophased by means of the macro Phase2d.

12. Once prepared thus, “Analyze2DPlotTest” is selected from the NNLS drop-down menu to perform the inversion. A FISTA format is used. The delay value $\Delta$ (in $\mu$s) and the time units used in the calibration mentioned in the previous step (again $\mu$s) are input. The inversion calculations are performed by selecting boundaries for the desired viewing range of the $T_2$-D distribution, along with inputting the desired image resolution, or steps (generally, 100x100 was used in preliminary measurements; 750x750 was used for displaying final data). Upon clicking “Calculate” in this window, the inversion process will begin, taking several seconds to several minutes, depending upon the number of steps. For 750x750 data, the calculations took about 20 minutes to complete.

13. The data should now be displayed as a $T_2$-D distribution. Options in the “View” and “Calculations” drop-down menus provide the ability to either display the coordinates of specific points, or measure by integration what amount of total signal intensity displayed is contained within a box of the user’s selection. For each experiment, the $T_2$ and $D$ values for the point of highest intensity within each signal source were selected by eye, as
well as the peak’s signal fraction. The inverted data was then saved to our lab group’s Z: Drive.

14. The saved inverted data was reopened in another 2D Plot window in Prospa, so that it could be saved again as an image (.png file).

15. MATLAB is used to measure the precise point of greatest intensity for each peak (by-eye measurements are quick and are used to identify basic trends in the data to help guide further decisions), as well as calculate error boundaries by creating a contour where the signal intensity was 90% of the corresponding peak intensity.

16. Lastly, each sample is analyzed by UV/Vis Spectroscopy in order to confirm the concentration of each analyte.

2.2 Preparation of Test Samples

BSA and naproxen-sodium were both obtained from Sigma-Aldrich with no further purification. Initially, naproxen purchased from MP Biomedicals was considered, but it did not effectively dissolve at our desired concentrations (15.9 μg·mL⁻¹ in H₂O at 25°C), which ranged as high as 194.0 mg·mL⁻¹. Naproxen-sodium—identical to naproxen besides a replacement of the carboxyl H⁺ with Na⁺—was found to be a suitable substitute (solubility in H₂O at 25°C is 250 mg·mL⁻¹). For the main experiment, D₂O (Acrōs) was used as the only solvent (as it did not produce proton signal). BSA was stored at 2°C, while D₂O and naproxen were stored at room temperature. Glass scintillation vials were used as containers for test samples. Possessing a diameter of about 1 inch, and a uniform, thin bottom, they ensured our samples were within the magnet’s cross-sectional and vertical scanning range. All containers were capped and sealed with parafilm.

Samples were prepared directly, without the use of stock solutions. To create samples, 4 mL of D₂O were pipetted into a vial, and appropriate masses of BSA and naproxen-sodium were individually weighed out, recorded, and added to the vial. The mixture was capped and shaken gently for 1-5 minutes upon addition of each solid until full dissolution was achieved. At
this point, it was also confirmed using pH strips (Fisherbrand) that the sample possessed a pH greater than BSA’s pI of 5.4, at which point the absence of charge interactions would have caused undesired aggregation of BSA.\textsuperscript{22} When not being tested, samples vials were stored in a drawer at room temperature, as it appeared that exposure to light quickened a process in which the naproxen-sodium came out of solution. Such a development was reversible, needing only slight warming with the hand and a minute or two of gentle shaking; there was no visible change in the samples over the course of the experiment apart from this. Our experiments were likewise carried out in the dark, since the NMR-MOUSE was housed in a sealed climate-control chamber set to constant temperature (20°C) and humidity (50.0 % relative humidity). These measures were taken to account for the possibility that naproxen could be undergoing some photodegradative process over time.

2.3 The NMR-MOUSE

The single-sided PM-5 NMR-MOUSE (Magritek, New Zealand) was used for all experiments. This MOUSE has an applied field strength $B_0$ of 0.4566 T and a field gradient ($G$) of 23.86 T/m. As indicated by the name PM-5, the instrument is rated to give valuable information for any sample within 5 mm of the RF coil in the vertical direction; the RF coil is contained within a raised section of the magnet housing and has a sensing area approximately 1-inch square. One 2–mm plastic spacer was in place between the coil and the sensing surface, providing an ultimate effective vertical sensing range of 3 mm. An image of the device in use is shown in Fig. 14. Our entire experimental apparatus consisted of the MOUSE, a KEA spectrometer, and an operating computer running the program Prospa (all by Magritek). Sample vials were placed directly onto this surface during experiments, ensuring that as much of the test sample was within the effective range of the magnet as possible. The glass underside of each vial
was of suitable thinness and uniformity so as not to complicate this effort. Additional elements of the apparatus, such as a lift controlling magnet height and a platform for larger samples, did not need to be used or considered given this setup.

### 2.4 Acquisition Parameters

A large number of user-defined acquisition parameters were required for each experiment, with some variation depending upon which test was performed. $T_1$-Saturation Recovery and CPMG tests were used to determine $T_1$ and $T_2$, respectively, while the Simulated Spin Echo test (SSE-$T_2$) was used for $T_2$-$D$ experiments. This section will discuss our settings for these parameters for all the sets of experiments carried out, as well as explain our general approach and needs in performing a full iteration of the above protocol; further explanation of the meaning of selected parameters important to our decision-making process is provided in Appendix D. A table is provided that displays the values input for each set of $T_2$-$D$ (SSE-$T_2$) experiments (Table 2); an explanation of our experimental structure is given in the section following this one—for now, only cover the steps necessary for collecting $T_2$-$D$ data for any given sample will be described.

$T_1$ and $T_2$ values for a given sample were required due to the exact nature of the mathematics used to determine the $D$. Additional terms not listed in the equation as shown in the introduction include $T_1$ and $T_2$ such that these terms are insignificant so long as $\delta \ll T_2$ and $\Delta \ll T_1$. These relaxation times therefore needed to be known in advance in order to inform our selection of $\delta$ and $\Delta$ during the SSE-$T_2$ test. As implied by the equation for $D$, the reference signal $S_0$ (the greatest signal) is measured at short $\delta$ and $\Delta$. In order to collect sufficient information to distinguish multiple diffusion coefficients in the sample, the SSE-$T_2$ test was repeated across a
range of δ values, where the largest δ-value increment produces the least signal. The specific largest and smallest δ were selected by the user in such a way that the simplified equation for D did not differ significantly from the complex version (less than a 10% difference); in addition, the smallest δ value was chosen in order to ensure the normalized signal intensity was as close to 1 as possible, to maximize the range of signal amplitudes acquired in the experiment. δ Values for other increments were selected by the computer based off of a log scaling, so that the data could be assigned a linear fit in a plot of ln \( \frac{S}{S_0} \) vs. \( \gamma^2 G^2 \delta^2 (\Delta + \frac{2}{3} \delta) \) (again, this should produce a slope of \(-D\)). In addition, the value of the MOUSE’s field gradient (G) had to be entered into Prospa. This was calculated via calibration experiments on plain water, whose D is known for a range of concentrations thanks to the work of Holz et al. (2000). At 20°C, the D for water is 2.023×10^{-9} m² s^{-1}.

### 2.5 Main Experiment—BSA & Naproxen Titration

Initial experiments with BSA-only and naproxen-only samples indicated a disparity between the ideal acquisition parameters for tests of each: in short, BSA required few echoes and many scans, while naproxen required many echoes and few scans. BSA had a relatively short \( T_2 \) (~0.5-7.0 ms, depending upon the degree of dimerization) and therefore needed only 128 echoes with an echotime of 60 μs for its entire signal decay to be visualized, but many scans (at least 2048) were required before its signal became clearly distinguished above the noise. This meant that the length of BSA-only tests was primarily determined by the number of scans. On the other hand, naproxen had a relatively long \( T_2 \) (~80 ms on average) that was best measured with 2048 echoes, but produced adequate signal in only 512 scans; given the correlation between the repetition time between scans and the number of echoes used in an experiment, this implied that
the length of naproxen-only experiments was mostly determined by the number of echoes. This disparity presented a problem of practicality for $T_2$-$D$ experiments on mixes of BSA and naproxen-sodium, as we could not combine many echoes with many scans without our experiment lengths growing to on the order of days.

Three main groups of $T_2$-$D$ experiments were decided upon: a BSA-only set, a naproxen-only set, and a mixed set. Each possessed their own, consistent sets of acquisition parameters, with BSA-only and naproxen-only samples conforming closely to their ideal settings, while a “middle ground” set of acquisition parameters that compromised between the two ideals was used for the mixed samples. The parameters for each group as mentioned previously can be found in Table 2. Performing experiments on these three sets was consistent with the original premise of using $T_2$-$D$ for the purpose of molecular identification: identifiable trends in the effects of mixing BSA and naproxen-sodium on their respective $T_2$-$D$ signals on their own and as compared to the control groups serve to prove that this method can accurately characterize a given sample. To increase the amount of information that could be collected from the experiment, a titration of naproxen by BSA across several samples was performed, instead of a single comparison test. Crucially, the concentration of protons (and therefore the total amount of signal acquired) was held constant at 10 M $^1$H; however, the ratio of BSA:naproxen was modified across separate sample so that the initial amount of signal produced by each conformed to a specific ratio. Nine samples for this group were prepared and tested, which were referred to by this proton signal ratio BSA:naproxen: two blanks (10:0 and 0:10) containing only BSA or only naproxen, and seven mixed samples with signal ratios 1:9, 3:7, 4:6, 5:5, 6:4, 7:3, and 9:1. The actual initial concentrations of both BSA and naproxen-sodium added to each sample are displayed in Table 3, as determined by the masses recorded for each ligand during sample preparation. Five
additional samples for the naproxen-only group and three for the BSA-only group were prepared (along with the BSA and naproxen blanks used in the titration) that matched the concentrations of either molecule for the 1:9, 3:7, 5:5, 7:3, and 9:1 (for Naproxen-only) and 1:9, 5:5, and 9:1 samples (for BSA-only)—this means that each sample had a corresponding total proton signal of 1, 3, 5, 7, or 9 M $^1$H for Naproxen-only and 1, 5, or 9 M $^1$H for BSA-only.

2.6 $T_2$-$D$ Data Processing in Prospa

Raw data from our experiments provided a measure of proton FID echo signal vs. both the change in delay time $\delta$ (μs) across each increment and the change in time during each scan (ms). Further processing in Prospa was required to convert these data into a two-dimensional distribution of $T_2$ and $D$ values, using Inverse Laplace transformations. The detailed mathematics of this type of transform are complex, and beyond the scope of this thesis. It is enough to understand that it operates by applying trial exponential decay functions to the data presented, resulting in a two-dimensional distribution of “goodness of fit” that corresponds to the distribution of $T_2$ and $D$ values.

2.7 UV/Vis Quantification

A Perkin-Elmer Lambda-35 UV/Vis spectrophotometer was used to confirm BSA and naproxen-sodium concentration within each test sample after completion of all relevant tests. Samples were diluted 100x when containing BSA only, and 1000x when containing naproxen with or without BSA. For our main experiments, which used D$_2$O as a solvent, these dilutions were each performed by adding either 10 μL or 1 μL (depending upon the dilution scheme) of sample along with 990 μL or 999 μL H$_2$O to an Eppendorf tube. 700 μL of this dilution were then transferred to a quartz cuvette from Science Outlet (700 μL was the approximate volume of
the main cuvette sample chamber). Pure H₂O was used as a reference sample. As stated previously, [BSA] was determined at 280 nm (ε = 44,331 M⁻¹cm⁻¹), while [naproxen] was determined at 331 nm (ε = 1744 M⁻¹cm⁻¹). The programs UV Winlab and UV Winlab DPV were used to operate the spectrometer and calculate the difference in absorbance between the test and reference samples at the appropriate wavelengths, respectively.

It should be noted that some samples with a high concentration of naproxen (1:9 or naproxen blank samples, generally) came out of solution after several days. This precipitation could be reversed easily for several hours to days by simply warming the sample briefly in one’s hands. There is no indication via UV/Vis or T₂-D that some degradative process occurred that induced an actual change in molecular structure. Nonetheless, all T₂-D and relaxometric experiments were carried out as soon as possible after sample preparation.
3 Results & Discussion

3.1 Overview of Whole Series of $T_2$-$D$ Figures

The full series of our $T_2$-$D$ distribution results for all samples is displayed in Figures 15-31. Table 3 displays the initial and equilibrium concentrations (in both a chemical and proton basis) of BSA, naproxen, and the complex of the two as determined by the recorded masses of BSA and naproxen-sodium for each sample during sample preparation. Also listed in Table 3 is the expected fraction of $^1$H signal produced by both free BSA and complexed BSA, labelled $f([B]_{eq}[BN]_{eq})$. Since free BSA and bound BSA are roughly identical in size (66,463 Da for BSA versus 66715.24 Da for BSA-naproxen complex), there ought to be little difference in their $T_2$-$D$ distribution signals. Furthermore, according to the $K_D$ for BSA-naproxen binding ($3.27 \times 10^{-8}$ M), the concentrations of naproxen-sodium required to achieve the desired proton concentration ratios results in essentially all the BSA being converted to complex (again, see Table 3), whereas only a small portion of the naproxen should be bound (in the 9:1 sample, for instance, 97.5% of naproxen molecules ought to be unbound). Once the data were inverted to a $T_2$-$D$ distribution, a peak-picking routine found the centers of the peaks. Error estimates for both $T_2$ and $D$ were found by creating a contour at 90% of the maximum height of each peak (Fig. 32). This error-bounding process, while not statistically rigorous, provides a consistent means of analysis from signal to signal. The 90% threshold was selected to reduce the influence of “streaks” in the data on our results—samples that did not demonstrate complete signal decay within 1024 echoes (most often high-concentration naproxen samples) did not produce a clear upper bound to the range of transverse relaxation, resulting in a “streak” in the $T_2$ dimension. Streaking could still influence the measurement of a given proton source’s signal fraction,
however, since these values had to be calculated based on a manually-selected region for a given $T_2$-$D$ distribution. The signal fraction of peaks that exhibited streaking was therefore often underestimated (for example, signal 1 from the 0:10 and 1:9 samples).

### 3.2 Naproxen-only & BSA-only Controls

The BSA-only and naproxen-only results distinguish which phenomena observed in the titration samples are the direct result of the interaction between BSA and naproxen, and which result from the changing concentration of each free component. The “0:10” (all-naproxen) and “10:0” (all-BSA) blanks serve as part of both the mixed and non-mixed sample sets.

The naproxen-only series, for instance, appears to show a major and minor signal. The major peak possesses a signal fraction that ranges from 48-87%; however, in higher concentrations, this value is underestimated by the signal’s streaking in the positive $T_2$ direction. Thus the true signal fraction for each affected major peak in this series of data is likely higher than measured. The major peak appeared across a range of $T_2$ and $D$ values respectively of ~30-130 ms and ~1.0-1.2×10^{-10} m^2s^{-1}. These $T_2$ values do not appear to possess a concentration dependence, however, and are associated with large error boundaries (the magnitude of the range of $T_2$ values within our 90% maximal peak contours was typically between ~70-100 ms). In contrast, a gradual increase in $D$ was observed as the concentration of naproxen decreased (Fig. 33). This trend appeared again during the titration experimental series—thus, some possible sources of this phenomenon will be discussed later. The minor peak did not appear in every sample, but possessed a consistent $T_2$-$D$ distribution when it was encountered: $T_2 = ~0.3$-1.0 ms and $D = ~9.0$-1.1 m^2 s^{-1}. One indication that this signal is not merely an artifact of our inversion algorithm is that its signal fraction increases as the total proton signal decreases in magnitude.
([naproxen] decreases), from 0.67% in the “1:9” 9.0 M ¹H sample to 21% in the “7:3” 3.0 M ¹H sample. This suggests that these samples do indeed contain a second proton source. It is possible that, within our range of naproxen concentrations, self-association of naproxen molecules occurs, resulting in the formation of naproxen aggregates. This is supported by the minor peak’s lower \( T_2 \), and, as is shown later, the increase in naproxen \( D \) as [naproxen] decreases, which is observed both in the major peak of the naproxen-only series and the naproxen signal of the titration series.

No trends in \( T_2 \) or \( D \) appear in the BSA-only control series; however, a splitting of signal from one peak into two is observed as [BSA] increases. At proton concentrations lower than 10 M ¹H only one peak is observed, whereas two are measured in the BSA blank sample (10 M ¹H, or 2.166 mM BSA), a major and minor peak with respective signal fractions of ~63% and ~37% (Fig. 23). The major peak has a shorter \( T_2 \) and smaller \( D \): 2.56 ms and 1.04x10⁻¹⁰ m²s⁻¹ for \( T_2 \) and \( D \) respectively, in contrast with the minor peak’s 31.90 ms and 1.47x10⁻¹⁰ m²s⁻¹. This splitting of signal may indicate that our data depicts the increasing presence of dimerized-BSA, which is known to form at high concentrations as mentioned earlier. Based on the signal fractions of the two peaks, we can estimate the \( K_D \) for our sample to be 9.4x10⁻⁴ M—since dimerized BSA contains twice as many protons as monomeric BSA, the signal fractions indicate that (in a proton basis) the ratio of dimer to monomer is equivalent to 0.315:0.37. This ratio can then be converted to a molecular basis and used to find the equilibrium concentrations of the two forms of BSA given that \([\text{BSA}]_{\text{initial}} = [\text{BSA}]_{\text{eq}} + 2[\text{BSA}_2]_{\text{eq}}\), as shown below:

\[
[\text{BSA}]_{\text{eq}} = \frac{0.37}{0.315} [\text{BSA}_2]_{\text{eq}} \approx 1.17[\text{BSA}_2]_{\text{eq}}
\]

\[
1.17[\text{BSA}_2]_{\text{eq}} + 2[\text{BSA}_2]_{\text{eq}} = 3.17[\text{BSA}_2]_{\text{eq}} = [\text{BSA}]_{\text{initial}}
\]
At this point, $K_D$ can then be calculated as $[\text{BSA}]_{eq}^2/[\text{BSA}_2]_{eq}$. This dissociation constant implies that the dimer:monomer signal fraction should change significantly across our samples, with monomer comprising approximately 70%, 43%, and 35% of the total signal for the 1:9, 5:5, and 9:1 BSA-only samples, respectively. To rule out the possibility of sample contamination (either during production, or by naproxen-sodium after using the same BSA source many times to prepare samples) repletion of this BSA blank experiment with a second 10:0 sample prepared using a different source of BSA gave no change in results.

### 3.3 BSA-Naproxen Titration

*Identifying Peaks via Signal Fraction Trends*

Two distinct signal peaks were observed for all samples containing both BSA and naproxen-sodium, which are referred to here as Signal 1 and Signal 2. A third peak found in the 9:1 distribution, contributing < 10% of the total signal, was considered to be an artifact of the inversion algorithm (Fig. 22). These final results have been re-expressed with appropriate error estimates and on linear axes (Fig. 34). Signal 1 ranged from 20-80 ms and $1.0-1.6\times10^{-10}$ m$^2$.s$^{-1}$ for $T_2$ and $D$, respectively, while Signal 2 ranged from 0.5-4.0 ms and $0.7-1.2\times10^{-10}$ m$^2$.s$^{-1}$ for $T_2$ and $D$, respectively. In order to identify the source of each signal, Figure 35 depicts the signal fraction of both signals as the concentrations of BSA and naproxen change. This change in concentration is represented by the fraction of protons in solution as part of BSA. Signal 1 decreases and Signal 2 increases as the dominant source of protons shifts from naproxen to BSA. This suggests that Signal 1 corresponds to that of free naproxen, while Signal 2 corresponds to the overlapping BSA and BSA-naproxen complex signals. Their rates of change correspond to slopes of -0.6593 for Signal 1 and 0.6067 for Signal 2—as shown in Figure 35, these values are
found using trend-lines that have fixed y-intercepts at 1 and 0, respectively, in order to circumvent the underestimation of naproxen’s signal fraction caused by “streaks” in the $T_2$ dimension at high naproxen concentrations.

*Trends in $T_2$ Relaxation – Possible Evidence of Chemical Exchange*

$T_2$ for both signals changes with changing naproxen and BSA concentrations. Signal 2 (corresponding to BSA) shows an increase in $T_2$ with increasing [BSA] (Fig. 36). Conversely, Signal 1 (the naproxen signal) shows a drop in $T_2$ with increasing [BSA], from a maximum value of ~80 ms in the 0:10 sample to ~30 ms in the 5:5 sample. At this point, $T_2$ remains unchanged despite further increase in [BSA] (Fig. 36). The point at which this leveling off in $T_2$ occurs corresponds to a molecular BSA:naproxen ratio of approximately 1:350. It is possible that both this initial drop and eventual plateau in $T_2$ are due to chemical exchange between bound and free naproxen. Our method of measurement (collecting echoes) is inherently stroboscopic: we measure the signal intensity of an echo once every 60 μs many times (1024) during a single acquisition period to get a summed intensity value, making for an acquisition period ~61 ms long. If the average length of time naproxen molecules remain bound to a given BSA binding site (known as the residence time) is much shorter than this acquisition period, then some sizeable portion of the total available pool of free naproxen should experience binding to BSA at some point during the acquisition period. In this case, the relaxometric characteristics of exchanging naproxen changes over the course of data acquisition; the observed naproxen proton signal should no longer describe simply free naproxen, but instead an average of the $T_2$ of free naproxen and bound naproxen weighted by the average residence time. As the concentration of naproxen decreases relative to BSA, this free naproxen pool decreases and the relative number of naproxen molecules able to bind to BSA increases, sharpening this effect and resulting in a
concentration-dependent drop in $T_2$. At the point where maximal exchange is occurring (all of the naproxen cycles through bound and unbound states during a signal acquisition period), this average should then remain constant regardless of further decreases in [naproxen], giving rise to a plateau $T_2$ value.

The degree of exchange occurring in our titration samples can be approximated in the following way: The residence time for a single naproxen molecule in a given binding site depends upon that site’s $K_D$, such that:

$$K_D = \frac{k_{off}}{k_{on}} , k_{off} = K_D \cdot k_{on} ; \frac{1}{k_{off}} = residence \ time ; k_{on} = 4\pi(D_1 + D_2)(r_1 + r_2)N_A$$

where $K_D$ is in units of M$^{-1}$, $k_{on}$ in units of M$^{-1}$s$^{-1}$, and $k_{off}$ in units of s$^{-1}$; $D_1$ and $D_2$ refer to the self-diffusion coefficients of the two molecules (m$^2$s$^{-1}$), $r_1$ and $r_2$ correspond to their radii (m) assuming a spherical geometry, and $N_A$ is Avogadro’s number. The number of naproxen molecules that cycle through a single, specific binding site is found by dividing the acquisition period time by the residence time; the percentage of naproxen molecules that exchange during a single acquisition period is found by dividing the number that exchange per binding site by the ratio of total naproxen molecules to total BSA binding sites available. The relevant values describing our 5:5 sample can be used to demonstrate this calculation, with molecule 1 corresponding to naproxen, and molecule 2 corresponding to BSA. Though it will become clear that chemical exchange of naproxen through the BSA binding site should be negligible, these results may still be due to exchange if some non-specific binding interactions between naproxen and BSA are occurring. For this sample, $D_1 = \sim 1.2 \times 10^{-10}$ m$^2$s$^{-1}$, $D_2 = \sim 8.6 \times 10^{-11}$ m$^2$s$^{-1}$; $r_1 = 6.41$ Å, $r_2 = 28.8$ Å; $K_D$ for the BSA binding site was $3.27 \times 10^{-8}$ M. From these it can be calculated using the equations listed above that the residence time for the binding site is
5.57×10³ ms. Given the acquisition period time of 61 ms, the number of naproxen molecules exchanging through each binding site is 0.011. Since there are about 700 naproxen molecules to every BSA dimer in solution, maximal exchange ought to be reached when approximately 700 naproxen molecules can exchange per corresponding binding site across all dimers per acquire period. The theoretical percentage of exchanging naproxen molecules, therefore, should be \( \frac{0.011}{700} \times 100\% \approx 1.4 \times 10^{-4}\% \). Given this value, it is clear that exchange through the naproxen-binding site of BSA is minimal. If it is the case, however, that naproxen at our concentration range binds BSA non-specifically (evidence for this is described in Section 3.4), naproxen exchange may still be occurring—the implication of some non-specific interaction being that it could be described by a very large \( K_D \), and the residence time should be much shorter.

This hypothesis leads us to question whether other forms of exchange could be taking place beyond those associated with naproxen-BSA interactions and \( T_2 \). The same analysis used to determine the degree of exchange during the \( T_2 \) acquisition period may be applied to the diffusion acquisition period (which was \( 2\delta + \Delta = 2(0.815 \text{ ms}) + 1.0 \text{ ms} = 2.63 \text{ ms} \) at its greatest), which is a much shorter period of time. It is therefore unclear whether non-specific naproxen exchange could influence our results in terms of \( D \) as well as \( T_2 \). Additionally, chemical exchange could occur between the BSA monomer and dimer forms. At BSA concentrations near the \( K_D \) value for dimerization (9.4×10^{-4} \text{ M}), there will be a significant change in the ratio of monomer to dimer as the concentration of BSA changes. At high and low concentrations relative to \( K_D \), the dimer will have a large and short “residence time”, respectively, pushing the effect of chemical exchange on \( D \) and \( T_2 \) to one of two extreme limits. Near \( K_D \), however, both dimer and monomer are present in significant quantities, and the effects
of chemical exchange will be maximized. Investigating the total effects of BSA dimerization on the observed signal will require further study, though it may be complicated by limits on the maximum BSA concentration that is still detectable. High-field NMR, as well as other spectroscopic techniques, may be best able to resolve this issue.

*Trends in Diffusion*

The self-diffusion coefficients for BSA and naproxen respond to changes in concentration similarly to how they did in the naproxen-only and BSA-only control series. Once again, no discernable trend was observed for $D$ of BSA with changing concentration. Likewise, there was an increase in $D$ for naproxen as $[\text{naproxen}]$ decreased (Fig. 37), in near identical fashion to the results of the naproxen-only control sample; the lines of best fit describing the rate of increase in $D$ vs. the fraction composition of BSA both possess strong correlations and similar slopes ($R^2$ values for the titration and control data fits were 0.828 and 0.9604, respectively, and their respective slopes were comparable at $5.39 \times 10^{-11}$ and $3.0 \times 10^{-11}$). This trend is unexpected, as one would expect naproxen to diffuse more slowly with greater $[\text{BSA}]$ on account of the greater portion of naproxen bound and therefore diffusing with BSA. Since the trend is observed in both the naproxen-only and titration series, it is possible that the phenomenon responsible is independent of the presence of BSA. As mentioned before, this could be due to self-association of naproxen into aggregates. With less naproxen in solution, fewer naproxen molecules should self-associate and therefore diffuse faster on average. Two possible factors involving BSA that could explain this trend might also be suitable avenues of further study: changes in the sample’s viscosity with changing proportions of BSA and naproxen, and/or changes in charge concentration. The former possibility rests on the notion that BSA (with many acidic protons) can exchange $^1\text{H}$ with $^2\text{D}$ atoms of the $\text{D}_2\text{O}$ solvent, generating small amounts of HOD. Since
HOD is less viscous than D$_2$O (1.1248 m·Pa·s for HOD as compared to 1.2467 m·Pa·s for D$_2$O at 20°C),$^{26}$ the overall viscosity of the sample will decrease as more BSA is added, resulting in faster diffusion ($D \propto \frac{1}{\eta}$ according to the Einstein-Stokes equations, where $\eta$ is viscosity). Alternatively, the change in diffusion could be a reflection of the changing charge environment within the sample as naproxen is replaced by BSA. At high levels of naproxen (which is weakly negatively-charged in comparison to BSA), the consistent spread of charge could slow down both BSA and naproxen molecules moving throughout the sample, on account of there being less space for these particles to “build momentum” before being repelled back in a different direction. In samples with high [BSA] and low [naproxen], however, most of the negative charge would be concentrated on the large but sparsely distributed BSA molecules, allowing for particles to move more freely.

One final oddity to consider is the anomalously low diffusion rates we observed for naproxen in general throughout these experiments. Despite being a much smaller molecule than BSA, it exhibited a relatively similar $D$ (usually ~1.0-1.5×10$^{-10}$ m$^2$s$^{-1}$). One possible explanation may be the hydrophobicity of naproxen’s largely aromatic structure restricting its movement within our polar solvent D$_2$O. This is unlikely to be a major factor as naproxen anions will repel each other when the charge density gets large, however. Alternatively, it could be the case that the interactions between the polar solvent and the two solutes (naproxen and BSA) are quite different, owing to differences in the molecules’ hydrophobicity and hydrogen-bond-forming capacity. This may be explored by looking at the effects of solvent on the measured diffusion coefficients.
3.4 UV/Vis Concentration Checks

Analysis of our samples by UV/Vis yielded limited success in the context of confirming the concentrations of BSA and naproxen-sodium in test samples containing both solutes. Results consistently put [naproxen] at about 20% below the expected concentration, while [BSA] was measured to be far greater in the mixed samples than in the 10 M $^1$H BSA Blank (the [BSA] for which was 2.126 mM, in comparison to the target [BSA] of 2.166 mM). Additionally, [BSA] for the mixed samples according to UV/Vis measurements decreased as [naproxen] decreased, rather than increased as expected. Calculating these concentrations was complicated by the significant absorbance at 280 nm by naproxen, the wavelength used to measure [BSA]—though BSA possesses a much stronger extinction coefficient (44,331 M$^{-1}$cm$^{-1}$ vs. 2327.6 M$^{-1}$cm$^{-1}$), the BSA absorbance was overpowered by the high naproxen concentration in solution. Based on the lower-than-expected [naproxen], and the fact that [BSA] rises and falls in tandem with [naproxen] in samples where both molecules are present (in total contradiction of our sample preparation scheme), there may be non-specific binding occurring between naproxen and BSA: Being a relatively small, mostly hydrophobic molecule, it is quite possible that naproxen molecules gather around and coat the surface of the BSA present in solution, altering their absorptive capabilities and resulting in an underestimation of [naproxen] that consequently throws off our determination of [BSA]. In the future, we could perform a BSA calibration, where [BSA] is changed in the presence of a fixed amount of naproxen—this may give some indication about non-specific binding or, at the least, the role that naproxen has on changing the extinction coefficient for BSA.
4 Conclusions

4.1 The Experiment as a Proof-of-Principle

This experiment assessed as a proof-of-principle the efficacy of a low-field, relaxometry-and-diffusion-based NMR technique for the task of molecular identification. Our initial perspective on the interactions of a given protein-ligand pair, which predicted a rather simple scenario in which the formation of a protein-ligand complex would be identified as a third proton signal of lower $T_2$ and slower $D$, is not the standard to which our results ought to be held. As was discovered during background research and our own experiments, BSA and naproxen are not a simple, “ideal” binding pair. BSA dimerization and possible naproxen aggregation create a complex equilibrium environment, which our results indicate are likely complicated by rapid chemical exchange and non-specific binding, and may even be affected by the viscosity and local charge density in the solution. Ultimately our results indicate two things: (1) that the two-dimensional aspect of the experiment provided a decided advantage in the identification and tracking of the signals detected; (2) that the interactions of our BSA-naproxen binding pair can be characterized by this method, with observable trends lending themselves to hypotheses that can be tested in the future.

The multidimensional nature of our experimental design is critically important to the interpretation of our results. Since the majority of this analysis considered trends in $T_2$ and $D$ separately, it may not at first be clear why measuring $T_2$ and $D$ together in a combined pulse sequence was preferable to monitoring them one at a time—after all, it is much quicker to measure them separately. The greatest advantage to the two-dimensional process is that it affords the opportunity to instantly match each corresponding $T_2$ and $D$ value for a given proton source
together. This greatly facilitates the identification of peaks: naproxen and BSA peaks could be distinguished from each other in the mixed titration samples by a single factor, their respective contributions to the total amount of “$T_2-D$ signal” measured as concentrations of the two molecules changed. In contrast, the same matching process with two one-dimensional experiments would require a painstaking comparison of separately-determined $T_2$ and $D$ signal fractions for each peak. Our results immediately lend themselves to interpretation thanks to the two-dimensional approach.

Our relaxation-diffusion correlation measurements effectively monitor a multitude of characteristic changes in $T_2$ and $D$ for the BSA-naproxen system. The values of $T_2$ and $D$ can identify the presence of this protein-ligand at a particular concentration. Our BSA-only experimental series suggests that a portion of the BSA in our solutions experience dimerization, while trends in the naproxen-only and titration series hint towards the possible self-association of naproxen at high concentrations. There is evidence (in both the titration experiments and UV/Vis concentration analyses) of non-specific binding of naproxen to BSA. Perhaps the most intriguing result of our research is the possible visualization of chemical exchange by our multidimensional technique, which may be the first time low-field NMR has been used for this purpose. Overall, this experiment provided a wealth of information that opens up several avenues of future study.

4.2 Continuing & Future Studies

This experiment serves as an initial exploration of our protein-ligand system. Having completed that task, there are a number of adjustments to the core experimental design that may be prudent to test. First, a drawback of our focus upon a constant concentration of protons within each sample is the large amount of naproxen required to produce signal comparable to BSA.
While the proton source ratios ranged no more than one order of magnitude, naproxen dominated the samples in terms of molecular concentration—for instance, the molecular ratio of BSA to naproxen in our 1:9 sample was nearly 1:3200. In order to monitor the binding interactions of BSA and naproxen such that the fraction of naproxen binding to BSA changes dramatically across samples, we may wish to repeat our experiment with a different set of proton source ratios, such as 1:100, 1:50, 1:10, 1:2, 1:1, etc. In this way we could observe a greater range of binding affinities.

While our current research provides several opportunities to understand our BSA-naproxen system better, we may seek to perform similar experiments with a different ligand pair. A simpler system may lend itself better to characterization by our $T_2$-$D$ technique. The association of avidin and biotin, for instance, is many times stronger than that of naproxen and BSA, their binding together being almost permanent ($K_D \sim 10^{-15}$ M).\textsuperscript{16,17} Such a pair would likely therefore not experience chemical exchange, allowing for us to probe other influences on the $T_2$-$D$ distribution in greater detail. Biotinylated reagents can also be engineered at a range of sizes—we considered using a polyethylene glycol chain with “sticky” biotinylated ends as a possible ligand for avidin, for example—providing great control over the degree of change in $T_2$ and $D$ associated with protein-ligand interactions. Other ligands and NMR hardware may be engineered to contain other NMR-detectable nuclei (notably, $^{19}$F), that could provide high sensitivity and remove the need for an aprotic solvent. This may make detection in impure, aqueous systems viable.

Our results allowed for what may be a novel pursuit for techniques involving low-field, single-sided NMR devices: the measurement of thermodynamic binding constants such as $K_D$. Of particular note is the potential for measuring $K_D$’s associated with the “multimerization” of
proteins, as this is no more difficult to accomplish than the calculation of a $K_D$ for any other type of binding interaction given the appropriate $T_2-D$ data. In this way, our technique could complement conventional methods for measuring thermodynamic constants such as isothermal titration calorimetry (ITC), for which the determination of $K_D$’s of “multimerization” is a complex task. Some research in this area has been performed such as that of Lucas et al.\textsuperscript{27} in 2004.

We were surprised by and rather taken with the possibility of monitoring chemical exchange of a number of forms with this technique. Further research is required to confirm the actual presence of exchange. First, this could be carried out with our $T_2-D$ experimental method by comparing results across a range of temperatures, which should influence the exchange rate. Another option would be to change the echotime used in our experiments, modifying the rate at which we acquire signal echoes and thereby controlling the amount of exchange able to take place during each acquisition period. Experiments of this nature can be planned out in advance based on calculations such as those shown above in order to effectively probe for a specific form of exchange, such as that of naproxen binding to BSA or that of BSA monomer-dimer interactions.
5 References


Appendix A – Abbreviations

NMR – Nuclear Magnetic Resonance
FID – Free Induction Decay
RF – Radiofrequency
CPMG – Carr-Purcell-Meiboom-Gill
BSA – Bovine Serum Albumin
UV/Vis – Ultraviolet/Visual (range of electromagnetism)
DPV – Data Processing Viewer
MOPS-K – 3-(N-morpholino)propanesulfonic acid
NP – Naproxen
ITC – Isothermal Titration Calorimetry
Appendix B – Figures

Fig. 1: Vector Model for Larmor Precession

Initially, the magnetization vector runs parallel to the magnetic field along the $z$-axis. If the magnetization vector is titled away from the $z$-axis, it executes a precessional motion in which the vector sweeps out a cone of constant angle to the magnetic field direction. The direction of precession shown is for a nucleus with a positive gyromagnetic ratio and hence a negative Larmor frequency.

If the magnetic field along the $z$-axis is replaced quickly by one along $x$ (for example, during a $\frac{\pi}{2}$ pulse), the magnetization will then precess about the $x$-axis and so move towards the transverse plane.

Here, the horizontal line serves to denote the sequence of events over time, left-to-right. RF power from the coil (represented by the gray bar) is applied to the sample in the form of an on-resonance ($\pi/2$) pulse. The resulting FID signal is shown to have a specific amplitude, frequency, and decay rate. The dotted line refers to the “dead time” associated with single-sided NMR devices (see Fig. 5).
Cross-sectional representation of the NMR-MOUSE magnetic elements; the applied field $B_0$ is summarized by the black vector and results from the opposite orientations of magnets on either side of the apparatus. As depicted by the gradient triangle top-left, this field direction results in a gradient in which $B_0$ weakens in the vertical direction above the magnet. The placement of the RF coil is shown by the solid-blue concentric rings—just beneath the surface of the apparatus’ scanning area. A corresponding blue vector depicts the direction of the RF field $B_1$ used to cause rotation of magnetization away from $B_0$. 

Fig. 4: NMR-MOUSE Magnet & RF Coil
This figure represents the sequence of magnetic pulses used to create a Hahn echo,\textsuperscript{10} using the same format shown previously in Fig. 3. As discussed in Section 1.2, the initial FID cannot be measured with single-sided devices, on account of the “dead time” before measurements can be taken due to residual energy in the RF coil. Here, an initial $\frac{\pi}{2}$ pulse is followed after a set time by a “refocusing” $\pi$ pulse, creating an “echo” of the original signal an equal length of time later. Only signal amplitude can be measured from the echo; no frequency data is accessible.
Fig. 6: Refocusing of Individual Magnetic Moments via a 180° Pulse

Shown here are the magnetization vectors of three magnetic moments (red, green, and blue), just as Larmor precession about $B_0$ begins immediately following a 90° pulse (left). Each moment precesses in the $x$-$y$ plane; however, field inhomogeneity results in each moment experiencing a different field strength, causing each vector to precess at slightly-different frequencies (the magnitude of the vectors here denotes field strength experienced—for example, red rotates the furthest because its field strength is greatest). Dephasing of the individual spins therefore occurs (second from the left). A 180° pulse is applied to rotate these vectors about the $x$-axis (third from the left) some time after the application of the initial excitation pulse. An equal time length later, full reconvergence of the magnetization vectors is observed (right) at the original orientation, providing an opportunity to measure an “echo” of the FID.
The CPMG pulse sequence.\textsuperscript{11,12} As described above, an initial $\frac{\pi}{2}$ pulse is followed after a set time by a $\pi$ pulse to create a Hahn echo. This $\pi$ pulse is then repeated a given number of times, as determined by the user. The echoes (represented by the highlighted signal spikes) experience signal decay over time, which can be quantified in order to determine transverse relaxation, $T_2$. 
Fig. 8: Movement of Sample Particles within a Magnetic Field Gradient

Implication of diffusion within a magnetic field gradient. Because the precession frequency is directly proportional to magnetic field strength, the precession frequency for any given proton changes as it moves about within the sample.
The three arrows depict three individual magnetization vectors as they precess about the $z$-axis in the $x$-$y$ plane, distinguished from each other by color (red, green, and blue). The magnitude of each magnetization vector is dependent upon the corresponding proton’s placement within the magnetic field gradient $G$. The top row represents perfect refocusing in the absence of diffusion (see Fig. 6). The bottom includes diffusion within $G$—dephasing and consequent signal decay results (right) from each particle experiencing a different applied magnetic field $B_0$ at the time of refocusing (third from the left) with respect to immediately after excitation (left).
In similar fashion to earlier figures, this represents the activity of the RF coil during a single $T_2$-$D$ scan. To allow for the measurement of signal decay due to diffusion, the first $\pi$ pulse of an iteration of the pulse sequence is split into two $\frac{\pi}{2}$ pulses. The first of these $\frac{\pi}{2}$ pulses (and second pulse in this sequence) rotates the bulk magnetization vector out of the transverse plane, “storing” it along the longitudinal axis; thus, for the entire storage delay period $\Delta$, no signal decay due to $T_2$ relaxation occurs. $T_1$ relaxation may still occur, but since its effects are negligible as long as $\Delta \ll T_1$. Application of the second $\frac{\pi}{2}$ pulse of the two (and third pulse overall in this sequence) rotates the magnetization back into the $x$-$y$ plane, facilitating an echo of the original FID to be created that can be measured to detect signal decay resulting from diffusion. After this, a regular CPMG sequence can be performed to measure $T_2$. The delay time $\delta$ describes two periods of time: (1) between the initial excitation $\frac{\pi}{2}$ pulse and the start of the diffusion-related sequence, and (2) between the end of the diffusion-related sequence and the first signal echo. The effect of $T_2$ is negligible during each delay period $\delta$ if $\delta \ll T_2$. 

Fig. 10: The $T_2$-$D$ Pulse Sequence
This plot displays the decay in signal intensity as a result of both $T_2$ and $D$, with $T_2$ relaxation causing signal decay over the length of each acquisition period of echoes, and diffusion causing signal decay as the delay time $\delta$ increases. The resulting values for $T_2$ and $D$ can be calculated using the equations provided in Section 1.3. Note the exponential decay in signal along both axes.
Fig. 12: Changes in $T_2$-$D$ Resulting from Analyte-Ligand Complexation

This figure depicts the theoretical changes in $T_2$-$D$ distribution signals of naproxen and BSA (labelled above) as a result of complexation. Assuming the resulting size increase is the only difference, the BSA-naproxen complex should produce a third signal that is lower in both $T_2$ and $D$ (diffusing more slowly, and relaxing more quickly).
Figure 14: BSA & Naproxen-sodium

This depicts the chemical structure of naproxen-sodium (top) alongside the crystal structure for dimerized BSA (bottom).\textsuperscript{19,20} When forming a crystal, BSA monomers each bind three naproxen (shown here); this is likely not the case when in solution, but no other structure for BSA with naproxen is available.\textsuperscript{21}
Figure 14: The NMR-MOUSE Apparatus Setup

Shown here is our experimental apparatus, including: (a) the PM-5 NMR MOUSE (Magritek), (b) test sample, (c) Kea Spectrometer (Magritek), (d) power source. The sample rests directly on top of the magnet housing.
The following series of $T_2$-$D$ distributions display our data from: (1) the BSA-naproxen titration series, (2) the BSA-only control series, and (3) the naproxen-only control series. The BSA and naproxen blanks (samples 10:0 and 0:10) can be considered to be part of both the titration and control experiments.
Figure 16: 1:9 BSA:Naproxen $T_2$-$D$ Distribution
Figure 17: 3:7 BSA:Naproxen $T_2$-D Distribution
Figure 18: 4:6 BSA:Naproxen $T_2$-$D$ Distribution
Figure 19: 5:5 BSA:Naproxen $T_2$-$D$ Distribution
Figure 20: 6:4 BSA: Naproxen $T_2-D$ Distribution
Figure 21: 7:3 BSA:Naproxen $T_2$-$D$ Distribution
Figure 22: 9:1 BSA:Naproxen $T_2$-$D$ Distribution
Figure 23: BSA Blank $T_2$-$D$ Distribution
Figure 24: 9:1 BSA-only $T_2$-$D$ Distribution
Figure 25: 5:5 BSA-only $T_2$-$D$ Distribution
Figure 26: 1:9 BSA-only $T_2-D$ Distribution
Figure 27: 9:1 Naproxen-only $T_2$-$D$ Distribution
Figure 28: 7:3 Naproxen-only $T_2$-$D$ Distribution
Figure 29: 5:5 Naproxen-only $T_2$-$D$ Distribution
Figure 30: 3:7 Naproxen-only $T_2$-$D$ Distribution
Figure 31: 1:9 Naproxen-only $T_2$-$D$ Distribution
This plot demonstrates the selection of our error-bounds (red) overlaid on top of the corresponding $T_2$-$D$ distribution results. The contour is selected by determining first the point of greatest intensity for each peak, then finding each point in the peak that corresponds to 90% that intensity.
This plot shows the change in $D$ for naproxen with change in concentration for the naproxen-only series major peak. For consistency with other figures, the $x$-axis is labelled as the fraction of BSA-containing proton sources contributing to the total signal—this is not a literal description, as there is no BSA present in any samples of the naproxen-only series. It corresponds directly to $1 - \left( \frac{M \text{ } ^1H \text{ of naproxen}}{10} \right)$. An increase in $D$ is observed as the concentration of naproxen decreases.
This plot features the point of greatest intensity for each signal detected in BSA-naproxen titration samples on linear axes. Signal 1 is shown as a white circle (the 0:10 naproxen blank sample, which only produced one signal, is labelled with a larger white circle in order to tell it apart), while Signal 2 is shown as a black circle. The appropriate error bars for each sample have been included—large errors in the positive $T_2$ direction for high [naproxen] samples (which are shown in Fig. 35 to correspond to Signal 1) are the result of signal “streaking”. Signal 1 trends upwards in $D$ and downwards in $T_2$ as BSA is added and naproxen removed. Signal 2 remains roughly constant in terms of $D$, while gradually increasing in terms of $T_2$ with increasing levels of BSA (see inset).
This plot demonstrates the change in signal fraction for the two signals measured during our titration experiment as BSA is added and naproxen removed. As shown in Fig. 33, the x-axis refers to the percentage of protons in solution whose source is either BSA or the BSA-naproxen complex (the signals for which should overlap). Signal 1 (white circles) displays a regular decrease in relative intensity as [naproxen] decreases and [BSA] increases, while Signal 2 (black circles) displays the opposite. From this we can determine that Signal 1 corresponds to naproxen, and Signal 2 corresponds to BSA (either with or without naproxen in a complex). The data for the two signals have both been fit with lines that have fixed y-intercepts at either 0.0 or 1.0. The fixing counteracts the inaccuracy in the fit brought about by the underestimation of high-concentration naproxen sample signal fractions thanks to “streaking”. Additionally, Signal 1 and Signal 2 for the 10:0 BSA Blank sample (far right) are displayed, though their only possible source is BSA; the splitting of BSA signal may indicate the appearance of BSA dimer at higher concentration.
Figure 36: $T_2$ vs. Fractional Signal Composition of BSA

Naproxen (Signal 1) and BSA+BSA-naproxen complex (Signal 2) $T_2$ values are displayed here with changing concentrations of naproxen and BSA. Naproxen $T_2$ drops as [naproxen] decreases and [BSA] increases until the 5:5 sample, at which point it holds steady at ~30 ms. The BSA signal (see inset) is shown to increase in $T_2$ as [BSA] increases.
This figure displays the diffusion values for naproxen (white circles) and BSA+BSA-naproxen complex (black circles) with changing concentration. For comparison with these titration samples, the values for the naproxen-only major peak (red squares) are included (see Fig. 33). Both naproxen signals show similar trends, increasing in $D$ as [naproxen] decreases. No trend in $D$ is observed for BSA or BSA-naproxen complex.
Appendix C – Tables

Table 1: Chemical Properties of BSA and Naproxen-sodium

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<th>Bovine Serum Albumin (BSA) Monomer</th>
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<td>#¹H per molecule</td>
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<td>Solubility in Water (mg·mL⁻¹)</td>
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<td>Approximate Geometry</td>
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Table 2: Acquisition Parameters for Main Experiment

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### Table 3: Concentrations of BSA & Naproxen-sodium Used

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<tr>
<td>9:1</td>
<td>9010</td>
<td>1001</td>
<td>0.1111</td>
<td>3.923E-06</td>
<td>975.8</td>
<td>9035</td>
<td>0.9025</td>
</tr>
<tr>
<td>10:0</td>
<td>10010</td>
<td>0</td>
<td>0</td>
<td>10010</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
Appendix D – Details of Selected Acquisition Parameters

1. **Repetition Time (ms)**: referred to as rep. time; it determines the amount of delay between the administration of each scan, in order to allow for RF power to dissipate out of the coil and prevent overheating. The rep. time must scale linearly with the number of echoes used. Because it is relatively much larger than most other reasons for delay, rep. time is thought of as analogous to the length of each scan, and is therefore critical in deciding or determining how long an experiment will take.

2. **Scans (#)**: denotes the number of full pulse sequences of the chosen form to be administered in the experiment (CPMG) or increment (T1Sat or SSE-T2). More scans results in a greater sum of signal amplitude data, and thus clearer, more reliable results; this, however, also lengthens experiments.

3. **Increments (#)**: abbreviated as inc. This is required for T2-D (SSE- T2) experiments, which record diffusion data across a range of diffusion delay (δ) times. Each experiment consisted of a certain number of increments, which each took a certain number of scans, each of which took as long as the repetition time to complete.

4. **NrEchoes (#)**: denotes the number of echoes to be collected in the given experiment. Proton sources with slower decay times require more echoes in order to visualize the entire decay curve (it is ideal to see full signal decay in the last half of the data collection window). Since echoes are acquired with even time spacing (see echotime), this number can be used to determine the approximate length of each scan.

5. **Echotime (μs)**: denotes the length in time of each echo acquired. When multiplied by the number of echoes, this provides the approximate length of each scan. For all our experiments, the echotime was constant at 60 μs.

6. **T1 Estimation (ms)**: also known as T1 est. In order to determine T1 accurately, an estimate value close to the true one had to be provided. This was accomplished by running several T1Sat experiments, starting with an initial arbitrary guess T1 est value which was then replaced with the results of each successive experiment, until these results consistently agreed with each other. By convention, the minimum rep. time can be found by multiplying T1 est by five.

7. **-90/-180amp (dB)**: these two values characterized the power of each magnetic pulse administered. For all our experiments they remained constant at -10/-4.

8. **Pulse length (μs)**: abbreviated as τ. It determines for how long each magnetic pulse is administered. The optimum value had to be found before each experiment using a calibration (Cal) test.
Appendix E – Binding Interactions of BSA & Naproxen-sodium

Naproxen-sodium binds to a single binding site within a given BSA monomer with $K_D = 3.27 \times 10^{-8} \text{ M.}^{21}$ Equilibrium concentrations for naproxen, BSA, and the BSA-naproxen complex may be calculated using the classic “ICE Diagram” method, as will be shown below. This same process can be employed to calculate equilibrium concentrations for monomeric and dimeric BSA. The binding of naproxen to BSA may be displayed thus:

$$N + B \rightleftharpoons BN$$

where $N$ is naproxen, $B$ is BSA, and $BN$ is the BSA-naproxen complex. The corresponding ICE diagram displays the initial, change in, and equilibrium concentration for each molecule:

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>B</th>
<th>BN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>$N_0$</td>
<td>$B_0$</td>
<td>0</td>
</tr>
<tr>
<td>Change</td>
<td>$-x$</td>
<td>$-x$</td>
<td>$+x$</td>
</tr>
<tr>
<td>Eq.</td>
<td>$N_0-x$</td>
<td>$B_0-x$</td>
<td>$x$</td>
</tr>
</tbody>
</table>

where $N_0$ and $B_0$ are the initial naproxen and BSA concentrations, respectively. By definition, $K_D = \frac{[N][B]}{[BN]}$. The equations developed in the ICE diagram for the equilibrium concentrations of each molecule can then be plugged into the definition of $K_D$ to produce a quadratic equation:

$$3.27 \times 10^{-8} \text{M} = \frac{(N_0 - x)(B_0 - x)}{x}; 0 = x^2 - (N_0 + B_0 + 3.27 \times 10^{-8})x + N_0B_0$$

The quadratic formula can then be used for any desired initial concentrations of naproxen and BSA to find the value of $x$, from which the equilibrium concentrations of all three molecules can be calculated as shown in the ICE diagram. As per the nature of the quadratic formula, two
solutions for $x$ ought to be found; however, one of these solutions ought to be larger than the amount of $B_0$ available ($B_0$ being the limiting reagent in every case during our experiment). Because $K_D$ for this interaction is very small, it turns out that essentially all the BSA is converted to BSA-naproxen complex. In the 9:1 sample from our titration series, for instance, which features an initial concentration of 77.01 mM naproxen and 1.951 mM BSA, the equilibrium concentrations for BSA, naproxen, and the BSA-naproxen complex are $8.496 \times 10^{-10}$ mM, 75.1 mM, and 1.951 mM (rounded), respectively.