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# Investigation of the Effects of Albumin Aggregation in $D_2O$ Solutions on NMR Relaxation Times

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**Abstract:** ***This study was conducted in order to reveal the effects of protein aggregation on $T_1$ and $T_2$ relaxation times and to clarify the mechanisms leading to relaxation. The presence of protein aggregation was examined in terms of Inversion Recovery (IR)-$T_1$ signals, Spin Echo (SE) - $T_2$ signals and decay curves. In the experiment, Human serum albumin and Deuterium Oxide ($D_2O$) solvent, which includes 0,1 % residual water, were used. The albumin solutions used for the analysis of concentration were prepared in two groups by adding different amounts of albumin to 1 ml of $D_2O$. The measurements were performed by using Bruker Avance 400 MHz NMR spectrometer. $T_1$ measurements were carried out with the Inversion Recovery method and $T_2$ measurements were carried out with the Carr-Purcell-Meibon-Gill method. With the acquisition of FID and Echo sets and of IR-$T_1$ and SE-$T_2$ curves, it was observed that FID and SE sets were changed as a single exponential. However, as protein was added, the exponential appearance of the FID and SE sets was changed. This presents that the protein aggregation begins to increase in advanced concentrations. IR-FID and SE-FID sets of the samples that are up to 10 % concentration and IR-$T_1$ and SE-$T_2$ curves fit perfectly with the related formulas. This shows that NMR $T_1$, $T_2$ measurements can be performed by using albumin up to 10 % of concentration.***

**Keywords:** NMR, $T_1$, $T_2$, protein aggregation

## 1. Introduction

Spin-lattice ($T_1$) and spin-spin ($T_2$) relaxation times of albumin solutions have been studied in details by Nuclear Magnetic Resonance Dispersion (NMRD) and Nuclear Magnetic Resonance (NMR) techniques. Several methods have been employed to explain the mechanisms of relaxation [1-12]. First of these methods depends on the derivation of rotational correlation time of albumin from the Stokes-Einstein equation [3, 6]. Second method is based on the derivation of correlation times using experimental data via the fit approach [1, 4]. Third method is based on the comparison of $H_2O$ and $D_2O$ relaxation rates [9, 10]. Fourth method is based on obtaining rotational correlation times from $T_1/T_2$ ratios [12]. Correlation times that were obtained in initial NMR studies in the level of microseconds were later measured in the level of nanoseconds in subsequent studies [2, 5, 8-12]. The difference has been explained by the high protein concentration used in previous studies (i.e. 10% or 15%). It has been suggested that albumin aggregation at high concentrations slows down the motion of this molecule, and that the long correlation time measured in microseconds is related to this aggregation [9, 10, 13-18]. Identification of the effects of albumin aggregation on $T_1$ and $T_2$ relaxation times is crucial in terms of clarifying the mechanisms that lead to relaxation.

Deteriorations in the Inversion Recovery (IR) and Spin Echo (SE) signals have been proposed as indicators of protein aggregation [13]. The linear relationship between relaxation times and protein concentration was also observed to be impaired by the occurrence of protein aggregation [9, 10, 13-18]. However, there is no consensus on the concentration where protein aggregation begins to occur [19]. According to some sources, such aggregation occurs at a protein content of 20 g/dL (20%) and even 30 g/dL (30%) [19]. In this case, clarification of the concentration where aggregation occurs is necessary to explain relaxation mechanisms. In order to identify the concentration where albumin aggregation begins, $^1H$, IR, FID (Free Induction Decay) and SE sets of $D_2O$ solutions, and the IR-$T_1$ and SE-$T_2$ curves obtained from these sets were investigated in increasing albumin concentrations.

## 2. Experimental Details

### 2.1 Sample Preparation

The human serum albumin used in this study was purchased from Sigma (Sigma-Aldrich Co., St. Louis, USA). $D_2O$ (Deuterium Oxide) solution used as solvent, which includes 0.1% residual water, was purchased from MERCK (Merck KGaA, Germany). Albumin solutions used for concentration studies were prepared in two groups by adding various amounts of albumin to 1 ml $D_2O$. Albumin quantities were changed up to 0.10 g with increments of 0.02 in one group, and up to 0.5 g with increments of 0.1 in the other group. In the examined sample, there is 0.1 ml of water. For this reason, the spectrum obtained in this study is the water peak. As seen in Figure 1, the water peak appears at 4.703 ppm.

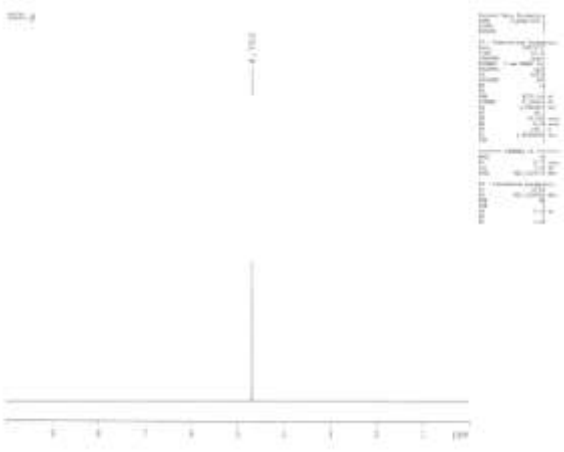

**Figure:1** Spectrum of pure $D_2O$

**2. 2 Relaxation Time Measurements**

The measurements were performed using an Avance Bruker 400 $^1$H-MHz NMR spectrometer. $T_1$ relaxation time was obtained using the Inversion Recovery (IR) pulse step (180-τ-90). The pulse repetition time (TR) of albumin solutions was taken to be higher than $5T_1$ and $T_1$ of each sample was first detected with preliminary experiments. Pulse repetition time of $D_2O$ was selected as 150s. $T_2$ measurement was performed by the Carr-Purcell-Meibon-Gill (CPMG) method.

IR-FID and SE-FID signal sets of the prepared samples were obtained. In IR-$T_1$ and SE-$T_2$ measurements, the waiting time was changed according to the concentration in the sample.

**2.3 Protein aggregation and Relaxation Times-Concentration Relationship**

Towards the end of the 1980s, it was shown that a protein added to a solution could not be fully disaggregated into its individual molecules beyond a certain concentration [4, 12, 14-18, 20, 21]. In such a situation, a high number of protein molecules remain attached to each other. This is called protein aggregation. In this situation, relaxation attention time of the aggregated molecule increases, which in turn suddenly increases the corresponding $1/T_1$ time [8]. This phenomenon abruptly changes the linear relationship between $1/T_1$ and concentration. Although some studies have argued that this effect is manifested in the presence of 2% albumin, other studies have noted that the linear relationship was disrupted after 20% albumin content. Therefore it is highly important to clarify at what concentration this phenomenon occurs.

In cases where protein aggregation occurs, IR and SE curves do not make good fits with $1/T_1$ and $1/T_2$ data, and distortions also occur on these curves [13]. In this case, the obtained $T_1$ and $T_2$ values are wrong. As a result, $1/T_1$ saturates at an early stage and its relationship with concentration becomes non-linear.

The fact that $1/T_1$, relaxation time ratio has a linear relation with concentration was first discovered in the 1960 and later verified in various studies [12, 14-18]. It is useful to derive this relationship again in this study.

The water in protein solutions is either in free phase or protein-bound. However, since NMR signals for these two different phases could not be obtained, it was assumed that water was making rapid chemical exchanges between these two phases. It was concluded that this rapid exchange resulted in $1/T_1$ and $1/T_2$ ratios to average. The measured $1/T_1$ and $1/T_2$ ratios with the assumption that water exchanges rapidly between free water and bound water can then be expressed as follows:

$$1/T_1 = P_f\ 1/T_{1f} + P_b\ 1/T_{1b} \quad (1)$$

$$1/T_2 = P_f\ 1/T_{1f} + P_b\ 1/T_{1b} \quad (2)$$

where, $P_f$ and $P_b$ are the probabilities of water being in free phase and bound phase, respectively. $T_{1b}$ and $T_{1f}$ are the relaxation times of water in free phase and bound phase, respectively.

In the situation of free water molecules are significantly more than bound water molecules, $P_f$ can be taken as $P_f \cong 1$. On the other hand, since $P_f + P_b = 1$ or $P_f = 1 - P_b$ can be written, the following equation is obtained:

$$1/T_1 = (1-P_b)\,1/T_{if} + P_b\ 1/T_{1b} \quad (3)$$

Equation 3 can then be rearranged and expressed as;

$$1/T_1 = 1/T_{if} + P_b\,(1/T_{1b} - 1/T_{1f}) \quad (4)$$

where, $P_b = \frac{N_b}{N}$. $N_b$ denotes the number of bound molecules and N denotes the total number of water molecules. $N_b$ is related to protein concentration. That is, as the number of protein molecules in the solution increases, $N_b$ increases. Therefore, $N_b$=nC can be written. n shows the number of water molecules bound to a single protein molecule. C is protein concentration. Taking all these into consideration, equation 4 can be written as;

$$1/T_1 = 1/T_{if} + \frac{nC}{N}(1/T_{1b} - 1/T_{if}) \quad (5)$$

or as,

$$1/T_1 = 1/T_{if} + K_1 C \quad (6)$$

where, $K_1 = \frac{n}{N}(1/T_{1b} - 1/T_{1f})$. For $1/T_2$ measured with the same approach, the following equation can be derived:

$$1/T_2 = 1/T_{2f} + K_2 C \quad (7)$$

According to equations 6 and 7, during rapid chemical exchange, $1/T_1$-C and $1/T_2$-C relationship is linear.

On the other hand, relaxation time ratios of water molecules are expressed as stated in equations 8 and 9 below.

$$\frac{1}{T_1} = \frac{6}{20}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma^4\hbar^2}{r^6} \left(\frac{\tau}{1+\omega^2\tau^2} + \frac{4\tau}{1+4\omega^2\tau^2}\right) \quad (8)$$

$$\frac{1}{T_2} = \frac{6}{40}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma^4\hbar^2}{r^6} \left(3\tau + \frac{5\tau}{1+\omega^2\tau^2} + \frac{2\tau}{1+4\omega^2\tau^2}\right) \quad (9)$$

$1/T_1$ and $1/T_2$ ratios depend on correlation time τ. Therefore, changes in τ shall affect the relationship of $1/T_1$-C and $1/T_2$-C, and disrupt linearity. In this case, τ remains constant for the concentrations where linear relationship persists [2, 5-8]. In this case, $1/T_1$-C and $1/T_2$-C relation can be written as in equations 8 and 9 under the condition $\omega^2\tau^2 << 1$ including correlation time.

## 3. Results and Discussion

### 3.1. Results

FID, Echo sets, IR-$T_1$ and SE-$T_2$ curves were obtained for albumin contents of 0 g (pure $D_2O$), 0.02 g, 0.04 g, 0.06 g, 0.08 g, 0.10 g, 0.20 g, 0.30 g, 0.40 g, and 0.50 g. FID signal sets, IR-FID sets, and Echo decay ($T_2$) curves obtained in the presence of 0 g, 0.02 g, 0.04 g, 0.06 g, 0.08 g, and 0.10 g albumin were examined. Based on these examinations, it was found that the FID and SE sets of $D_2O$ changed in a single exponential manner. However, as albumin was added, the exponential outlook of the FID and SE sets changed. This is indicative of albumin aggregation in higher concentrations, and is consistent with the literature [9, 10, 13-18].

On the other hand, IR-$T_1$ and SE-$T_2$ curves of samples obtained by addition of albumin to $D_2O$ with increments of 0.1 g/ml were obtained. When protein content exceeds 0.1 g/ml, albumin aggregation becomes more distinct. In this case, IR-$T_1$ and SE-$T_2$ sets do not fit well with single exponential curves. Within the range of these obtained sets, points that were out of the curve were removed. That is, $T_1$ and $T_2$ were taken as approximate values. This suggests that albumin aggregation increases at high concentrations [9, 10, 13-19].

$T_1$ and $T_2$ time data and $1/T_1$ and $1/T_2$ ratio data obtained from the measured IR and SE curves are presented in Table 1 for low concentrations and in Table 2 for high concentrations.

**Table 1:** $T_1$, $T_2$ time data, and $1/T_1$ and $1/T_2$ ratio data obtained from the measured IR and SE curves for low concentrations.

| Concentration (g/ml) | $T_1$(s) | $1/T_1(s^{-1})$ | $T_2$(ms) | $1/T_2(ms^{-1})$ |
|---|---|---|---|---|
| 0 g/ml | 15.58 | 0.0642 | 277.8 | 0.00036 |
| 0.02g/ml | 9.724 | 0.1028 | 750.9 | 0.00133 |
| 0.04g/ml | 9.419 | 0.1062 | 688.5 | 0.00145 |
| 0.06g/ml | 7.723 | 0.1295 | 430.4 | 0.00232 |
| 0.08g/ml | 6.377 | 0.1568 | 350.9 | 0.00285 |
| 0.10g/ml | 5.229 | 0.1912 | 293.6 | 0.00341 |

**Table 2:** $T_1$, $T_2$ time data, and $1/T_1$ and $1/T_2$ ratio data obtained from the measured IR and SE curves for high concentrations.

| Concentration (g/ml) | $T_1$(s) | $1/T_1(s^{-1})$ | $T_2$(ms) | $1/T_2(ms^{-1})$ |
|---|---|---|---|---|
| 0.10g/ml | 5.229 | 0.1912 | 293.6 | 0.00341 |
| 0.20 | 2.256 | 0.4433 | 126.7 | 0.0079 |
| 0.30 | 2.03 | 0.4926 | 78.62 | 0.0127 |
| 0.40 | 1.743 | 0.5737 | 59.33 | 0.0169 |
| 0.50 | 1.251 | 0.7994 | 47.36 | 0.0211 |

Using the data in Table 1 and Table 2, the graphs of $1/T_1$ and $1/T_2$ ratios plotted against concentration are shown in Figures 1, 2, 3, and 4, respectively.

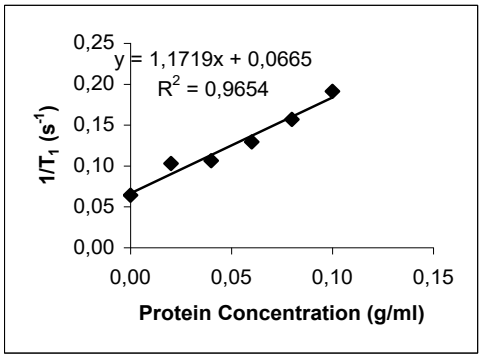


**Figure 1:** $1/T_1$ versus low protein concentration (g/ml)

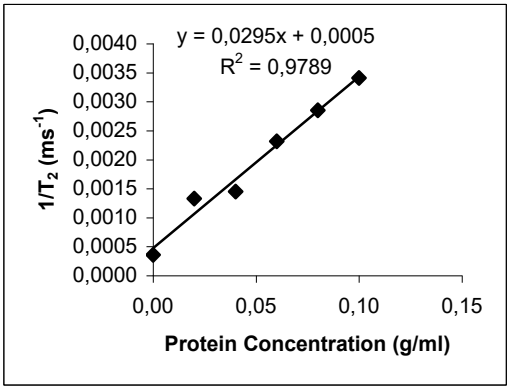


**Figure 2:** $1/T_2$ versus low protein concentration (g/ml)

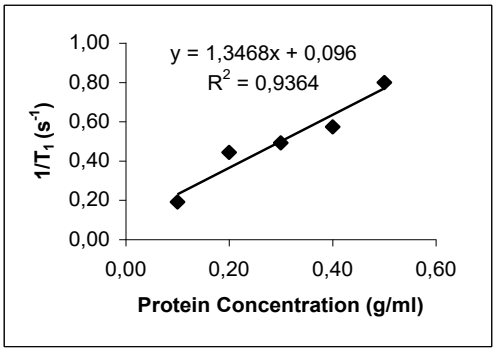


**Figure 3:** $1/T_1$ versus high protein concentration (g/ml)

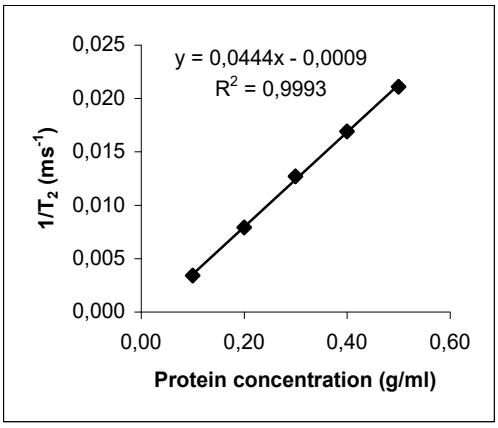


**Figure 4:** $1/T_2$ versus high protein concentration (g/ml)

### 3.2 Discussion

There are two types of proton pools in protein solutions; the first of these is the pool consisting of protein protons, and the other one is the pool consisting of water protons. For this reason, protein solutions give multiple NMR peaks. But only water proton peak is obtained in NMR spectrum because protein protons tend to relax very rapidly [4]. This signal also changes in the manner described in equations 1 and 2 for increased waiting times. Here, the signal shows a single exponential decay. In the case where the signal decays in a single exponential manner, IR-$T_1$ curve increases with increasing t according to the following equation;

$$M_z = M_0(1-2e^{-t/T_1} \quad (10)$$

whereas, SE–$1/T_2$ curve decreases with increasing t according to the following equation;

$$M_{xy} = Ae^{-t/T_2} \quad (11)$$

where, t is waiting time. There are an IR-FID, IR-$M_z$, SE signal and $M_{xy}$ magnetization for each waiting time. In single exponential exchange, the change in FID and SE sets is exponential. IR-$T_1$ and SE-$T_2$ curves fit the equations 1 and 2 well. The IR-FID sets obtained for low protein concentrations exhibit single exponential behavior. Similarly, SE sets obtained for protein concentrations also exhibit single exponential behavior. This observed behavior indicates that protein aggregation is not effective at concentrations up to 0.1 g/ml. This phenomenon is also confirmed by IR-$T_1$ and SE-$T_2$ curve fits. In these figures, IR-$T_1$ perfectly fits equation 1, while SE-$T_2$ perfectly fits equation 2. This proves that at low concentrations, there is no albumin aggregation. In equations 8 and 9, $\tau$ is constant when there is a linear relationship between $1/T_1$ and $1/T_2$ with $\tau$.

In protein solutions, it has been proven that $\tau$ is derived from the tumbling of the molecules [4, 9-13]. On the other hand, under the presence of aggregation, molecules adhere to each other and cause larger molecules to be formed. Since larger molecules move more slowly, the correlation time $\tau$ increases. As a result, $1/T_1$ and $1/T_2$ suddenly increases. In the presence of high protein concentration, decay in the exponential behavior of the FID and SE sets is indication of albumin aggregation. The fact that IR-$T_1$ and SE-$T_2$ curves cannot fit equations 1 and 2 confirms this phenomenon.

Separately, at low concentrations, the linear relationship of $1/T_1$-C and $1/T_2$-C indicates that $\tau$ remains constant, and no aggregation occurs. In the presence of high concentration, the observed increase beyond 0.1 g/ml indicates that $\tau$ is elongated and aggregation occurs.

## 4. Conclusion

IR-FID and SE-FID sets of samples with up to 10 % concentration, and the IR-$T_1$ and SE-$T_2$ curves seem to be suitable. They fit the equations related to the data perfectly. This indicates that NMR $T_1$ and $T_2$ measurements can be performed using albumin up to a concentration of 10 %.

The exponential appearance gradually disappears in both signal sets and IR-$T_1$, IR-$T_2$ curves for concentrations exceeding 10 %. Nevertheless, by discarding some data at 50 % concentration measurements, average IR-$T_1$ and SE-$T_1$ curves can be obtained. Signal distortions become more evident on concentration curves of $1/T_1$ and $1/T_2$ after 20 % concentration. This indicates that the relationship is significantly weak between concentrations of 10 % and 20 %, and multi-effective beyond 20 %. While the data show a linear relationship at low concentrations, this linear relationship is disrupted at 20 % and at exceeding concentrations. This shows that albumin aggregation is multi-effective.

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