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Analysis of Biopharmaceutical Formulations by Time Domain Nuclear Magnetic Resonance (TD-NMR) Spectroscopy: A Potential Method for Detection of Counterfeit Biologic Pharmaceuticals

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1. ABSTRACT

^{1}H Time-Domain Nuclear Magnetic Resonance (TD-NMR) is used to characterize solutions of antibodies that simulate biologic pharmaceutical formulations. The results from these measurements are compared with those from solutions in which the concentration or identity of the antibody has been altered. TD-NMR is shown to be very sensitive to differences in the amount of antibody in solution, with the ability to detect variations in as low as 2 mg/mL. It is therefore capable, by comparison with data from known formulations, of determining whether a particular sample is likely to be of an authentic biologic formulation. This method expands on the previous use of HPLC, UV/VIS, Near-IR and High-Resolution NMR to detect adulterated pharmaceutical materials. While the sensitivity of the method is high, it is a fingerprinting methodology, illustrating differences but not elucidating their origin. The extracted relaxation times reflect the combined effect of all solutes (antibody, buffer components, etc.) on the solvent (water).

Keywords

Biologic pharmaceuticals, monoclonal antibodies, counterfeit, NMR spectroscopy, relaxation times

INTRODUCTION

Ensuring the authenticity of pharmaceuticals is a necessity for the protection of patients. However, counterfeits of prescription medicines continue to be a concern for the drug industry. Although the fraction of biological pharmaceuticals (biologics) approved by the United States Food and Drug Administration since 2010 represents 24% of the total approvals[1], biologics have an outsize presence in the marketplace. In 2019, 7 of the top 10 bestselling pharmaceuticals were biologics[2]. These materials are tempting and potentially lucrative targets for unscrupulous individuals and organizations.

Biologic drugs targeted for counterfeiting have included erythropoietin[3], bevacizumab (Avastin)[4], pembrolizumab (Keytruda®)[5], nivolumab (Opdivo®)[6], and rabies vaccines (Verorab, Speeda and Rabipur)[7]. While the pharmaceutical supply chain is overall extremely safe, robust and rapid methods are needed to detect counterfeits and characterize drugs whose provenance may not be what is claimed.

In this paper we describe the application of ^{1}H Time-Domain Nuclear Magnetic Resonance (TD-NMR) for the detection of altered and counterfeited biologic pharmaceuticals. TD-NMR has been used in several studies on protein samples. It has successfully been employed to determine the water content of lyophilized proteins[8], and the aggregation of proteins in solution[9-11]. It has also been used to characterize mixtures of drugs in the solid state[12].

Time-Domain NMR determines the spin-spin (T_2) relaxation times of excited ^{1}H nuclei in the sample. In a traditional frequency-domain NMR experiment, the hydrogen nuclei of only solutes are detected. The signal from the solvent, which would otherwise swamp that of the solute, is eliminated via dissolution in a deuterated liquid. In contrast, the TD-NMR experiment uses the sample as is in order to measure relaxation times. The time-domain measurement usually represents the effect of the solute(s) of interest on the relaxation of the solvent. While all components in the sample contribute to the detected TD-NMR signal, the solvent dominates, for the same reason that motivates the use of deuterated solvents in traditional NMR. The observed relaxation time of the solvent depends on its interaction with the other constituents of the sample. Changes in both the structure

and concentration of the other components affect the measured T_2. Differences in size, hydrophilicity, and tendency to aggregate also have an easily detectable influence.

TD-NMR for the solvent lacks the extreme resolution that high-field frequency-domain NMR has for solute molecules. However, in the case of biologics it offers a rapidity that is not possible with the traditional experiment. The atom-by-atom characterization of biologics is relatively slow because of their large sizes. In contrast, speed is required for the robust detection of counterfeit pharmaceuticals, particularly with a methodology that can eventually be deployed outside the laboratory. For the experiments described here, TD-NMR is a fingerprinting measurement, one that is very sensitive to differences between samples. The tradeoff is that the determination of the origin of these differences requires the use of other analytical techniques.

Alternative analytical methods are available for the characterization of active pharmaceutical ingredients (API). Examples include high performance liquid chromatography (HPLC)[13], UV/Visible light absorbance[14], vibrational spectroscopic methods such as infrared[15] and Raman[16], fluorescence emission spectroscopy[17], 2D-NMR[18], and mass spectrometry[19]. The mixtures usually utilized in biopharmaceuticals, as well as the size of biologics, reduce the power of such methods, particularly as a rapid determinant.

There are several routes to counterfeit biological drugs. The API may be replaced with a bogus one. Other formulations eliminate any material similar to the API, leaving only the excipients, a subset thereof, and/or alternative inactive materials. Dilution of authentic drugs, with a resultant reduction in efficacy, is also of concern. Improper storage of samples, a potential issue with "cold-chain" biologics, may result in biologics that are damaged, even when such occurrences are not intentionally malicious.

The materials used for the TD-NMR experiments described here include four commercially available monoclonal antibodies (mAbs) that target the programmed cell death protein 1, PD-1, and its ligand PD-L1. Analysis of these substances investigates the ability of TD-NMR to detect differences between antibody-based pharmaceuticals, as well as the effects of dilution of an individual antibody-based pharmaceutical. Additional measurements examine pair-wise mixtures of three distinct antibodies at different ratios in solution. The preparations simulate the effects of substitution and dilution of APIs on the measured relaxation times.

MATERIALS AND METHODS

Materials

CM-1, CM-2, CM-3 and CM-4 mAbs were obtained from Selleck Chemicals LLC, distributed by VWR International. As purchased, the antibody solutions had a concentration of 5 mg/mL in a phosphate buffered saline solution, pH 7.2. Samples were tested at the initial concentration and at dilutions prepared with molecular biology grade water (Corning).

Three solutions of antibodies representative of biologic pharmaceutical formulations on the market and under development were prepared. These are designated N1, N2 and N6. N2 is a protected form of N1. The N1 and N6 antibodies were in a buffer solution containing 20 mM histidine, 250 mM sucrose, 50 µM diethylenetriaminepentaacetic acid (DTPA) and 0.05% polysorbate 80 (PS80) (buffer B1). The buffer B2, for N2 was similar, eliminating the DTPA and increasing the sucrose to 260 mM. The initial antibody concentrations were: N1 and N2-40 mg/mL, N6-120 mg/mL. Diluted solutions of N6 were prepared by volume using the same buffer in which the antibodies were originally constituted. Mixtures of antibodies were created by combining appropriate volumes of each of the original solutions.

Instrumentation

All TD-NMR measurements were executed using the Formµla µNMR (Waveguide Corporation). The µNMR has a nominal field of 0.5 Tesla from a fixed magnet, corresponding to a frequency of 21 MHz for ^{1}H nuclei. Samples were placed in a glass tube configured for the instrument with a 2.99 mm outer diameter and 2.45 mm inner diameter. The volume of sample used for each analysis was 15 µL.

Spin-spin relaxation was measured using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence[20, 21]. The 90-degree pulse length was 20 µs, with an echo period of 2 ms and a recycle delay of 10 seconds. The µNMR was

placed inside a temperature-controlled oven maintained at 25 ˚C. A thermocouple located within the instrument showed that the actual temperature of the probe was 27 ˚C. Three to thirteen replicates were measured for each sample.

Data Analysis

The ^{1}H relaxation decay times for each TD-NMR experiment were estimated by fitting the measured decay signal to one- and two-component exponential decay functions. The fitting software used a standard non-linear least squares regression analysis of the detected signal. Laplace Inversions of the signal amplitude used 256 uniformly spaced time-constant points up to a maximum T_2 of 4 seconds, with a regularization parameter of 10^{-4}.

RESULTS AND DISCUSSION

Data Reduction Methods

Figure 1A presents the decay curve of a typical sample from this study. The figure also shows the result of a fit to a single exponential function with a specific T_2 time. For these samples, a single exponential decay is sufficient to provide a robust quantitative characterization of relaxation. There is the theoretical possibility that more than one solvent environment is present in the sample and that the other constituents make minor contributions to the observed relaxation decay. However, when a second exponential component is included in this analysis, it has only a minor effect on the results. In all cases the primary decay element represents more than 95 percent of the experimental signal amplitude. For the majority of samples, the contribution of the primary component is more than 98 percent. Therefore, the determination of a single overall T_2 relaxation time is sufficient to differentiate between these solutions of antibodies.

The use of a single exponential function for the analysis of the relaxation is motivated by the fact that the solvent, water, dominates the observed decay of excited nuclei. For the solutions of PD-1 antibodies, the hydrogen nuclei in the antibody represent on the order of 0.3 % of the total hydrogens in the solution[22]. Even when larger concentrations of organic compounds are included in the buffer solutions, the solvent contributes more than 95% of the available hydrogen atoms.

We have also examined the Laplace Inversion of the relaxation decay. This inversion is a standard method in TD-NMR for separating the contributions of distinct species or environments[23]. The inversions are dominated by one peak. In the example shown in Figure 1B, the main peak represents 97 percent of the total signal amplitude. The minor peak, corresponding to a faster T_2, contains the remaining 3 percent.

T_2 Relaxation Times of Antibodies in Buffered Solutions

The T_2 relaxation times of four commercial antibodies are shown in Table 1. All samples were at the same concentration by weight in nominally the same buffer. At the highest concentration of antibody, CM-2 and CM-3 are clearly differentiated from each other and from water, even though the latter is the primary constituent of the antibody solutions.

It may appear surprising that the solutions of CM-1 and CM-2, which both target PD-1, have different relaxation times. Crystallographic studies of these antibodies in complex with PD-1 demonstrate that their epitopes and locations of binding on PD-1 are distinct[24]. Although the solution conformation of antibody is not expected to be identical to that in a crystal, the crystal structures are consistent with the results from TD-NMR. X-ray studies have also illuminated differences in binding of CM-3 and CM-4 to PD-L1, supporting the dichotomy between the T_2's of these antibodies[25].

Dilution Studies of Antibodies for PD-1

Figure 2 shows the change in relaxation times of sample N6, a simulated antibody formulation, upon dilution. The dilutions were executed using the same buffer as in the original solution. The measured change in T_2 is thus the

sole result of the concentration of antibody. Even when no antibody is present, the relaxation time is dramatically different from that of pure water. This result reflects the effects of non-antibody constituents within the solution on the dynamics of the solvent.

Two additional sets of diluted samples were prepared from two commercial antibodies. The relaxation times of CM-1 and CM-2 are shown in Figure 3. The diluted solutions differ significantly from those of N6. The antibody concentration is lower: 5 vs. 120 mg/mL at the maximum concentration. In addition, the dilutions, executed with water, affect both the concentration of antibody and of the salts that comprise the buffer. For this reason, the relaxation time is expected to approach that of water as the dilution increases. That is indeed what is observed.

At all concentrations, the two antibodies have clearly distinct T_2 times. Although the concentration of buffer changes across both series, the amount of buffer is the same at each specific concentration. Therefore, the effect of buffer on relaxation is expected to be similar. Except for pure water (concentration zero in this figure), the separation in T_2 between samples remains. This type of dilution, which affects all components in solution, would appear when a counterfeiter dilutes the entire original formulation in order to create more, but less efficacious, doses.

Together, the three dilution experiments demonstrate that the T_2 relaxation times correlate with concentration, of both the antibody and the other components present in the formulation. Divergence of 10 percent or more from the expected composition is easily detected.

Mixtures of Antibodies

Our final study examined pair-wise mixtures of three antibodies, samples N1, N2, and N6. N1 and N6 target different proteins. The results are shown in Table 2. Since N2 is a protected form of N1, it is expected to be spectroscopically similar to the unprotected version. The initial concentration of N6 was three times higher (120 mg/mL) than those of N1 and N2 (40 mg/mL). The solutions were combined in pairs, varying the relative volumes of each. The combinations model the situation where a non-authentic sample includes a similar, but not identical, material.

Similar to what was observed in the dilution experiments, the addition of the antibody to the solution reduces the relaxation time by 43 to 80 percent relative to pure buffer B1 (zero percent concentration for all three antibodies). For the highest concentration solutions of N1 and N2, which have a similar but not identical constitution, the T_2 times show a small but detectable difference. Interpretation of the results from mixtures of these two samples, however, is not as straightforward. The T_2's for the mixtures all fall between the values of the separate antibodies, but they do not reflect a consistent relationship with the relative concentrations. An additional complication is the small difference in composition of the buffer for N2, with its slightly elevated amount of sucrose and lack of DTPA. Because the concentration of DTPA in buffer B1 is very low (15 µM), we do not expect its presence to have a significant effect on the relaxation times. The change in the amount of sucrose could have an effect on the same order of the differences between samples seen here. This supposition is a further manifestation of the complex interactions between antibody, solvent and other components of the solution that can affect T_2. Regardless of these considerations, this mixture series provides an assessment of the limits of TD-NMR for these types of materials. Differences in T2 of less than 0.1 seconds should be examined carefully to establish their relevance.

When N6 is mixed with N1 or N2, the reduction in T_2 relative to N6 alone is more easily understood. Addition of either N1 or N2 increases the relaxation time. Because the initial concentration of N6 is three times that of the other two antibodies, the admixture of N1 or N2 to N6 reduces the total antibody concentration, which was not the case for combinations of N1 and N2. The majority of the effect on T_2 is thus the result of dilution of N6. Indeed, given the similarity of the relaxation times of N1 and N2, it is not surprising that when the amounts of N1 and N2 are the same in combination with N6, the T_2's are very close (within 0.02 seconds).

Implications for Detection of Counterfeit Biologic Pharmaceuticals

As discussed above, typical non-authentic versions of biologic pharmaceuticals either lack the active pharmaceutical ingredient (here, pure buffers) or have been diluted to reduce the API and/or other elements of the formulation. As the experimental results demonstrate, time-domain proton nuclear magnetic resonance is

sensitive to each of these types of alteration. The dilution measurements show that TD-NMR can detect differences in concentration of antibody that are less than 2 mg/mL, a change that is well below the concentration in most formulations of antibody-based pharmaceuticals. Although the method is an indirect one, determining the effect of the API and excipients on the solvent, it is very sensitive to the changes likely to appear when non-authentic materials are encountered. The limitation remains that the relaxation time is diagnostic for differences in materials, but does not determine the origin of the effects, including possible aggregation, that lead to these differences.

A subset of antibody-based biologic pharmaceuticals are provided as lyophilized powders[26]. Since TD-NMR is sensitive to concentration, care is required in reconstituting the antibody in solution. The protocols must ensure that the same concentration is reached if the sample were authentic. Only then can the agreement or dichotomy in T_2 be relied upon for categorization.

Although not the focus of these experiments, the instrument used here is small and portable. Thus, investigations of whether biologics are authentic can be taken outside of the analytical laboratory. This factor increases the utility of the TD-NMR method for forensic analysis.

Comparison of TD-NMR Spectrometers

The dilution series for sample N1 and the mixtures of N1, N2 and N6 were also analyzed using a second ^{1}H TD-NMR system. The trends discerned here were also found with the alternate unit. However, there were variations in T_2 between the two instruments of more than 0.1 seconds (up to 0.17 seconds) in T_2 for four of the twelve samples in the mixtures.

Despite these differences, it is clear that TD-NMR is sensitive to both gross and subtle changes in formulations of antibodies. Because of the minor deviations observed between spectrometers, sample comparisons should utilize the same instrument.

Conclusion

This study demonstrates that TD-NMR spectroscopy is a useful alternative quantitative analytical forensic technique for the examination of biological pharmaceutical formulations. The analysis proves to be very sensitive, although the cause of what is observed is not directly available from the measurements. The comparison of authentic and suspect samples is rapid and the reduction of the data straightforward, and often automatic. The minimal amounts of material required for the analysis, in the low µLs, is a further advantage of the method.

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22. The relative number of hydrogens between antibody, buffer and water was calculated from the molecular formula of each species and its molar concentration in solution.

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Figures Captions

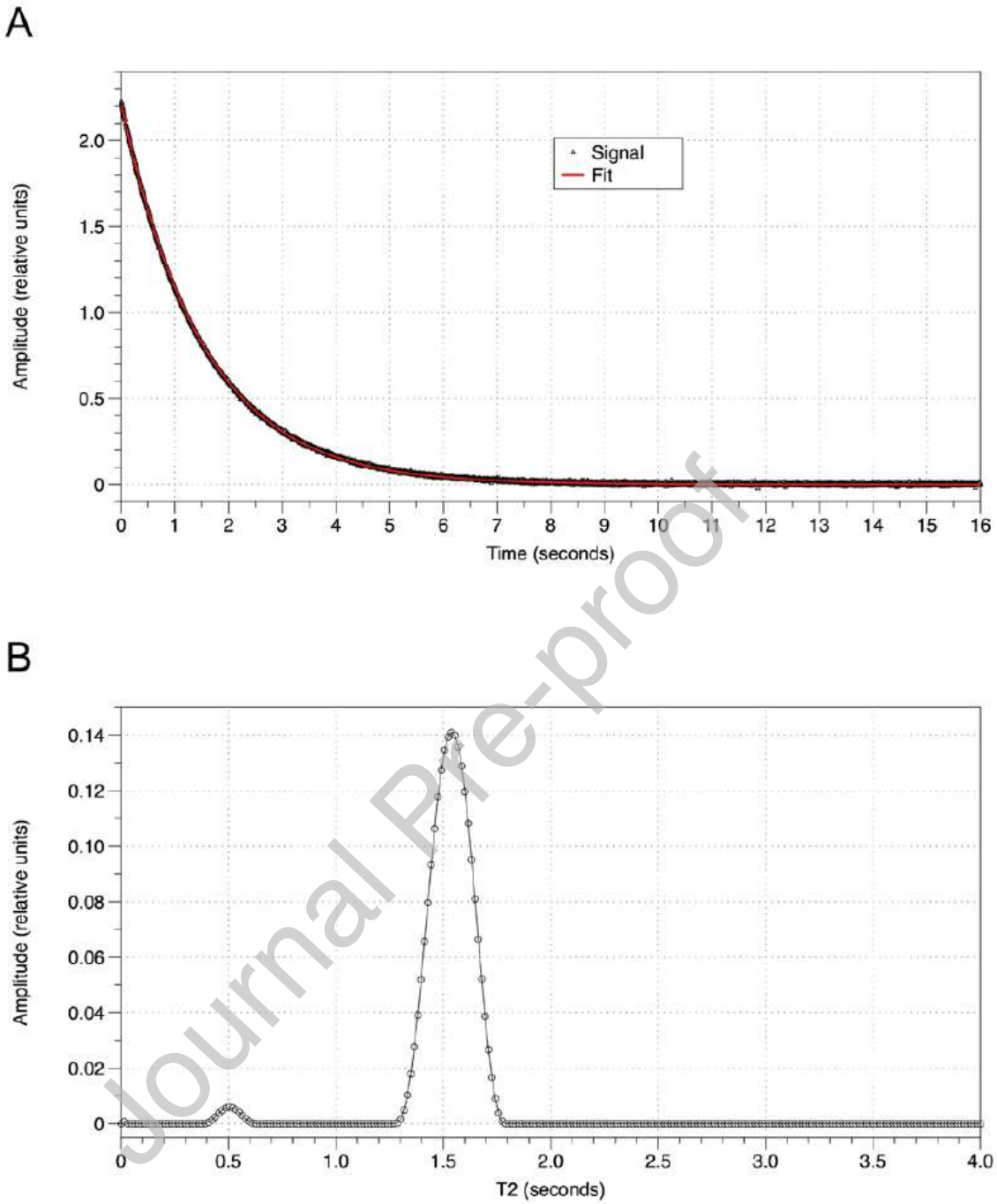

Figure 1. A) Spin-spin (T2) relaxation of CM-1 (5 mg/mL) in a phosphate buffered saline solution. Signal (diamonds) and fit to a single exponential function (red line). B) Laplace Inversion of the relaxation. The primary peak, centered at approximately 1.6 seconds, represents 97 percent of the total signal.

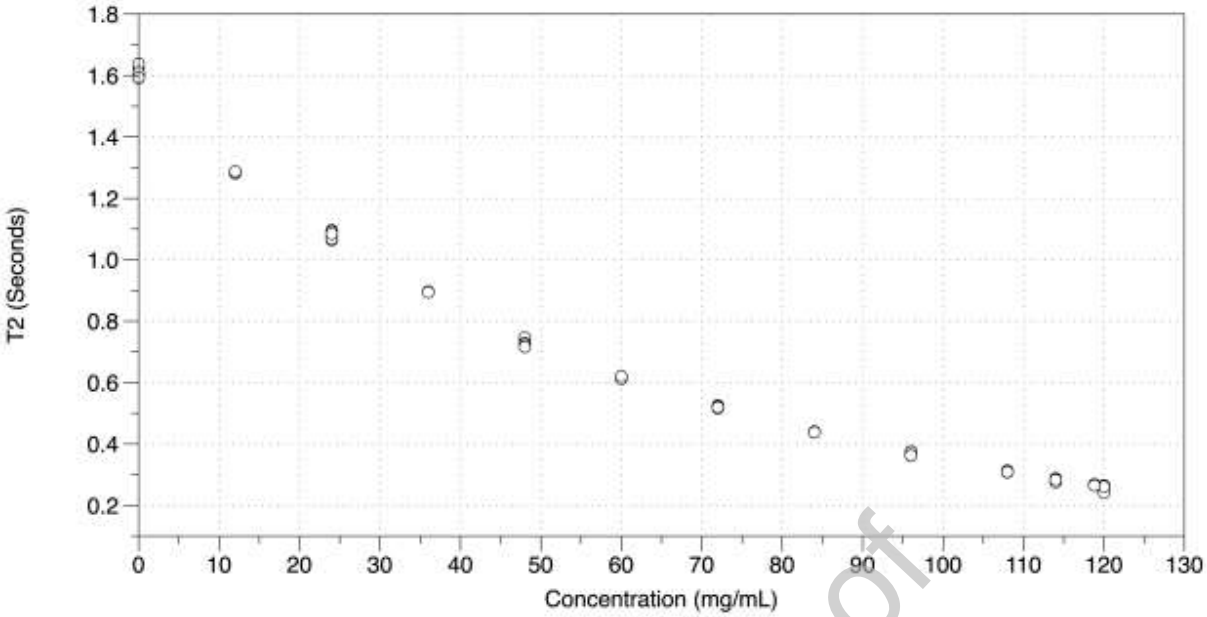

Figure 2. Effect of dilution with buffer on the T2 relaxation time of sample N6. The T2 time of each replicate for a given dilution is shown.

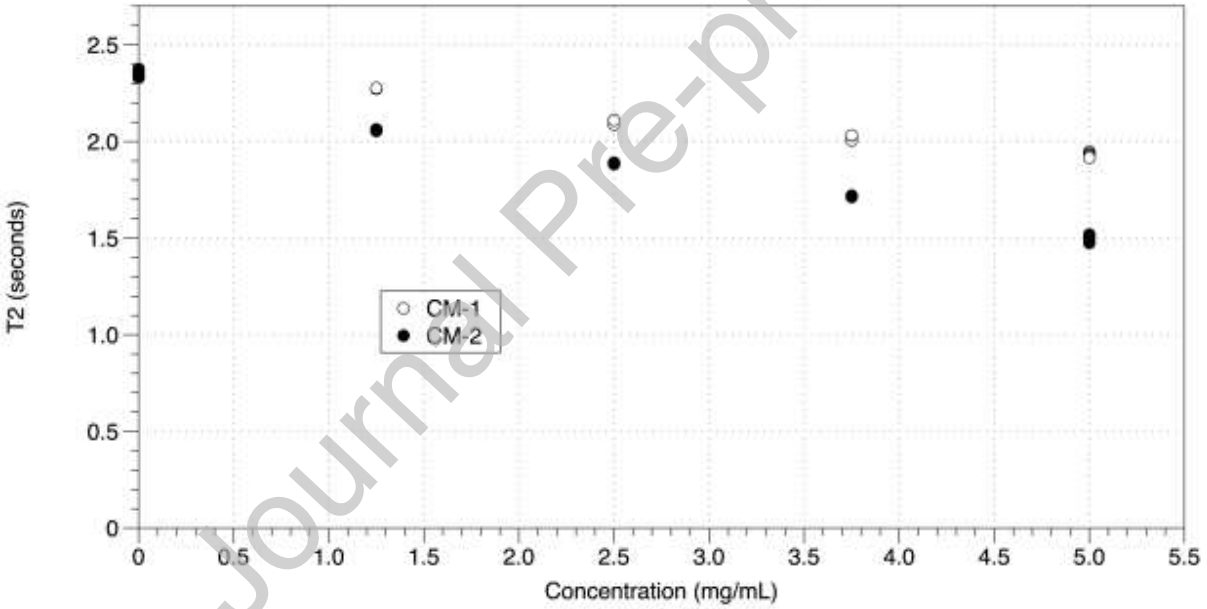

Figure 3. Sensitivity of spin-spin relaxation time (T2) to dilution at low concentrations. Water was used for the dilutions, thereby affecting the concentration of all non-solvent species in the sample. Each replicate is plotted separately.

Table 1. Spin-spin (T2) relaxation times of antibodies for PD-1 and PD-L1

Sample	Target	T2 (sec)	Standard Deviation (sec)
CM-1	PD-1	1.93	0.010
CM-2	PD-1	1.50	0.025
CM-3	PD-L1	1.67	0.002
CM-4	PD-L1	2.00	0.039
Water		2.36	0.020

Table 2. T2 relaxation times for mixtures of antibodies in buffered solution.[1]

N1 (volume percent)	N2 (volume percent)	N6 (volume percent)	N1 concentration (mg/mL)	N2 concentration (mg/mL)	N6 concentration (mg/mL)	T2 (sec)	Standard Deviation (sec)
0	0	0	0	0	0	1.61	0.019
0	100	0	0	40	0	0.81	0.004
100	0	0	40	0	0	0.92	0.009
20	80	0	8	32	0	0.91	0.005
50	50	0	20	20	0	0.87	0.011
80	20	0	32	8	0	0.90	0.007
0	0	100	0	0	120	0.26	0.002
0	80	20	0	32	24	0.69	0.004
0	50	50	0	20	60	0.47	0.002
0	20	80	0	8	96	0.33	0.001
80	0	20	32	0	24	0.69	0.007
50	0	50	20	0	60	0.48	0.001
20	0	80	8	0	96	0.35	0.002

1. The first sample (0% concentrations of all antibodies) is of buffer B1.