analytical chemistry

Article

pubs.acs.org/ac

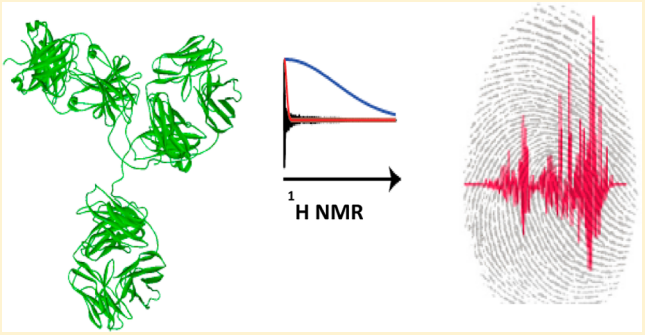
Profiling Formulated Monoclonal Antibodies by ^{1}H NMR Spectroscopy

Leszek Poppe,[†,*] John B. Jordan,[†] Ken Lawson,[‡] Matthew Jerums,[‡] Izydor Apostol,[‡] and Paul D. Schnier[†]

[†]Molecular Structure and Characterization and [‡]Process and Product Development, Amgen Inc., One Amgen Center Drive, Thousand Oaks, California 91320, United States

Supporting Information

ABSTRACT: Nuclear magnetic resonance (NMR) is arguably the most direct methodology for characterizing the higher-order structure of proteins in solution. Structural characterization of proteins by NMR typically utilizes heteronuclear experiments. However, for formulated monoclonal antibody (mAb) therapeutics, the use of these approaches is not currently tenable due to the requirements of isotope labeling, the large size of the proteins, and the restraints imposed by various formulations. Here, we present a new strategy to characterize formulated mAbs using ^{1}H NMR. This method, based on the pulsed field gradient stimulated echo (PGSTE) experiment, facilitates the use of ^{1}H NMR to generate highly resolved spectra of intact mAbs in their formulation buffers. This method of data acquisition, along with postacquisition signal processing, allows the generation of structural and hydrodynamic profiles of antibodies. We demonstrate how variation of the PGSTE pulse sequence parameters allows proton relaxation rates and relative diffusion coefficients to be obtained in a simple fashion. This new methodology can be used as a robust way to compare and characterize mAb therapeutics.



Monitoring protein integrity is critical during drug development to ensure that any batch to batch variations in the product are within acceptable limits.[1] Structural fingerprinting of large therapeutic proteins, such as monoclonal antibodies in a formulation buffer, is challenging, and there is an urgent need for the development of analytical techniques that allow the higher-order structural integrity between protein samples to be quantitatively compared.[2] Recently, hydrogen−deuterium exchange with mass spectrometric detection (HDX-MS) has been demonstrated for the structural characterization of molecules as large as monoclonal antibodies.[3] However, the intrinsic complexity of this analysis highlights the need for more direct approaches, such as NMR spectroscopy, which has been challenging because of the large size of antibodies. For proteins, NMR measurements are exquisitely sensitive to subtle changes in protein structure because the proton chemical shifts and line shapes are sensitive to the spatial arrangement of amino acids.[4] NMR fingerprinting of proteins, however, is typically based on 2D ^{1}H−^{1}H, ^{15}N−^{1}H, or ^{13}C−^{1}H correlation spectra[5,6] and generally requires isotopically labeled protein samples. To date, the acquisition of 2D NMR spectra of formulated monoclonal antibodies has not been practical because of their relatively large size, which causes severe peak overlap and broadening, rendering the correlation spectra uninformative. With smaller molecules, such as a typical organic compound, the 1D ^{1}H NMR spectrum is directly related to its three-dimensional structure and is widely regarded as the molecule's "fingerprint".[7] The same approach is often used for smaller proteins. However, applying conventional 1D proton NMR to formulated monoclonal antibodies has major drawbacks when used for fingerprinting; significant portions of the spectrum are not observable because of solvent suppression or spectral overlap with much stronger signals from the formulation buffer. Additionally, as with 2D methods, severe line broadening compromises the uniqueness of the ^{1}H proton NMR spectrum for large proteins. Standard proton spectra of different antibodies are nearly indistinguishable by spectral similarity measures, such as a typical correlation coefficient. These two major drawbacks of protein fingerprinting by 1D ^{1}H NMR are resolved by the PROtein FIngerprint by Line shape Enhancement method (PROFILE), described in this work. The PROFILE methodology exploits differences in the diffusion of large antibodies and formulation components to generate a highly resolved one-dimensional ^{1}H spectrum of the entire monoclonal antibody (mAb). The subsequent subtraction of the featureless component of this spectrum yields a detailed fingerprint spectrum, suitable for spectral similarity calculations. We demonstrate that the similarity of two IgG1 antibodies, as determined from PROFILE spectra, is commensurable with that determined from the corresponding ^{15}N−^{1}H correlation spectra, where the latter is widely recognized as the *de facto* fingerprint of a protein's three-dimensional structure. Since the PROFILE

Received: June 18, 2013
Accepted: September 5, 2013
Published: September 5, 2013

   dx.doi.org/10.1021/ac401867f | *Anal. Chem.* 2013, 85, 9623−9629

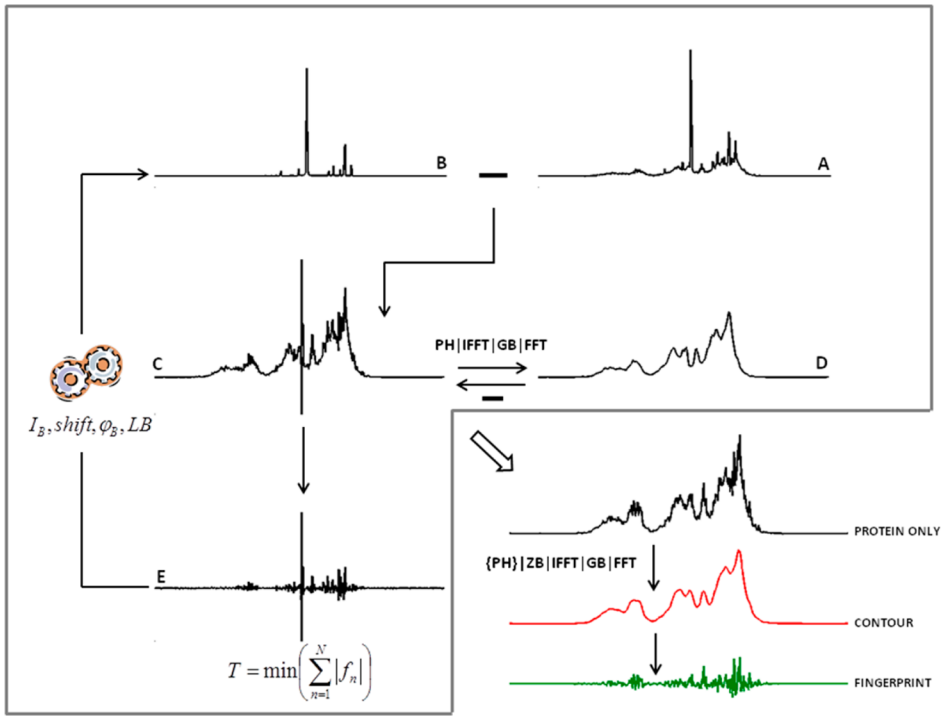


Figure 1. Flow chart of PROFILE data processing. The buffer subtraction algorithm is depicted in the box, where A and B are the absorption ^{1}H PGSTE spectra of an antibody and the corresponding buffer blank, respectively. C–E are the trial spectra during the iterative minimization of the target function T (calculated from the trial fingerprint spectrum E), by varying intensity (I_B), shift, zero order phase correction (ϕ_B) and line broadening (LB) of the buffer spectrum. The optimization was performed using the Matlab direct search algorithm. The resulting protein-only spectrum is subsequently convoluted with Gaussian broadening function to obtain the contour spectrum. Subtraction of the two spectra yields the fingerprint spectrum. In cases where all the buffer signals are suppressed by the PGSTE pulse sequence, the process can start from the protein-only spectrum. (PH, automatic zero order phase correction; ZB, zero order baseline correction; IFFT, inverse fast Fourier transform; GB, Gaussian window multiplication; FFT, fast forward Fourier transform.).

spectra correspond to a "pure" protein, they can be used in a quantitative fashion through ratiometric (based on the ratios of the total spectral intensities) analyses of higher-order protein structure (i.e., reversible aggregation, dynamics, and hydration) of formulated mAbs from different sources.

METHODS

NMR Sample Preparation of Formulated Antibodies. The unlabeled human monoclonal antibodies mAb#1, mAb#2, mAb#3, and mAb#4 were produced recombinantly in Chinese hamster ovary (CHO) cells and purified/formulated at Amgen. Protein samples, in their formulation buffers, at concentrations ~30 mg/mL, were used for the NMR analysis. Typically, 180 μL of the protein solution containing 5% D_2O for the internal lock signal was placed in a 4 mm Shigemi tube matched for the water dielectric susceptibility. The detection volume was adjusted to a sample height of 20 mm. The NMR samples were preheated to 42 or 52 °C in a heating-block to void air bubbles prior to placing samples inside the magnet. This assured high field homogeneity during the entire NMR experiment. For recording H-PROFILE spectra, the IgG1 protein was exchanged to 20 mM sodium phosphate buffer at pH = 7.0, at a concentration of 20 mg/mL.

Preparation of ^{15}N Labeled Antibody Samples. Isotopic ^{15}N labeling and purification of monoclonal antibodies mAb#5 and mAb#6 were performed as previously described.[8] The antibody samples were cleaved into the F(ab′)2 and Fc domains using the Fabricator enzyme (Genovis, Lund, Sweden). mAb samples were incubated with the enzyme at a ratio of 1:5 (enzyme/protein) at 37 °C for 1 h, and then further overnight at 4 °C. Complete cleavage was visualized by SDS-PAGE. The enzyme was removed from the samples using a 1 mL Ni-NTA FF column (GE LifeSciences, Piscataway, NJ) pre-equilibrated with 10 column volumes (CV) of 20 mM sodium phosphate, pH 7.0, 50 mM NaCl. The mAb/enzyme solution was manually injected onto the column, and the cleaved F(ab′)2 and Fc were eluted with 5 CV of elution buffer (20 mM sodium phosphate, pH 7.0, 50 mM NaCl, 10 mM imidazole). These fractions were concentrated down to a final volume of 500 μL using an Amicon 10K MWCO concentrator (Millipore, Billerica, MA) and purified into the F(ab′)2 and Fc fractions by gel filtration chromatography using a Superdex-200 16/60 Hi-Load column (GE LifeSciences, Piscataway, NJ) in 20 mM sodium phosphate buffer (pH 7.0, 50 mM NaCl). Baseline resolution was achieved for each peak, and 1 mL fractions were collected throughout the purification. Each collected fraction was assessed by SDS-PAGE, and the appropriate fractions were pooled to comprise the final F(ab′)2 and Fc samples. Final NMR samples were prepared by concentrating the F(ab′)2 and Fc fractions and by final dilution to a sample concentration of 100 μM.

NMR Experiments. All experiments were performed on a Bruker Avance III spectrometer operating at 800.13 MHz for proton using a TCI cryogenic probe. The PROFILE spectra were recorded with the PGSTE experiment,[9] where the duration (δ) and the strength (g) of bipolar gradients and the diffusion delay (Δ) were adjusted for optimal elimination of resonances from the buffer. Signal acquisition was programmed

dx.doi.org/10.1021/ac401867f | Anal. Chem. 2013, 85, 9623–9629

to start exactly at the peak of the echo pulse to ensure a flat baseline and to avoid the need to perform first order phase correction.[10] The echo peak time was observed to be sample dependent and required a small (up to 2 μs) heuristic adjustment. The acquisition time and relaxation delay were 1.02 and 2.5 s, respectively. The gradient pulses were 56.6 G/cm at 100% power, as calibrated with a standard sample of 1% H_2O/99% D_2O doped with gadolinium chloride (Cambridge Isotopes, Andover, MA). The PROFILE spectra (32k data points) were recorded at 42 or 52 °C, typically with 1280 scans, yielding S/N for the protein-only spectra of greater than 2000:1.

The H-PROFILE experiment, where H stands for hydrodynamic, consists of PGSTE experiments recorded with different sets of parameters for various pulse sequence delays and gradient strengths. For comparison, we propose here a ratiometric procedure (Supporting Information eqs S2−S6), with carefully adjusted parameters of the PGSTE pulse sequence (as described in the Supporting Information). This protocol yields accurate values for R_1 and R_2 relaxation rates and the ratios of translational diffusion coefficients, D_t, for two protein samples. In cases where the protein tumbling in solution can be well approximated by the Stokes−Einstein law, one can further deduce the relative hydrodynamic radius R_H and the relative viscosity, η, as described in the Supporting Information. The H-PROFILE spectra were recorded with 128 scans at 42 or 52 °C with the parameters indicated in Supporting Information Figure 8S. The ratiometric analyses are feasible even in cases when the protein is not perfectly stable at elevated temperatures and slowly precipitates (this is checked by comparing signal intensities from the same experiment before and after the total NMR acquisition time). If this is the case, and the precipitation rate is approximately linear, the H-PROFILE spectra are recorded twice for each set of pulse program parameters, in ascending and descending order, and the average spectra are used for the analysis. This protocol assures accurate determination of the relaxation and hydrodynamic parameters. To make sure that the results of ratiometric analyses are not influenced by thermal convection or static field gradients,[11] we measured the H-PROFILE spectra in 3 mm Shigemi tubes with 10 mm sample height and found no difference from the measurements in 4 mm Shigemi samples with 20 mm sample height.

The 2D $^{15}N-^{1}H$ TROSY spectra[12] were recorded on the same spectrometer using a standard pulse program from the Bruker library. Each spectrum was recorded with 80 and 20 ms acquisition times in the t_1 and t_2 dimensions, respectively, and with a 1 s relaxation delay and 512 scans per each t_1 increment for a total acquisition time of 20 h per spectrum. The spectra were apodized with square cosine window functions in both dimensions, linear predicted to 512 points in the t_1 dimension, and Fourier transformed to give 1024 × 512 point final data matrices. The S/N, calculated as the signal intensity divided by the RMSD of the noise from traces with the maximum cross-peak intensity (~10.4 ppm/110 ppm for F(ab′)2 and FL, and ~11.3 ppm/124 ppm for Fc proteins), were 90 and 30 and 95 for both mAb#5 and mAb#6 samples, respectively. The cross-correlation coefficients between two 2D spectra were computed as the maximum values of the normalized cross correlation sequences for the two corresponding spectral vectors using the Matlab function *xcorr*.

PROFILE Data Processing. The workflow of the PROFILE data processing is shown in Figure 1 and in Supporting Information Figure 3S. Here, the ^{1}H PGSTE NMR spectrum of a protein is decomposed into its low pass and high pass spectral components for the subsequent calculation of spectral (dis)-similarity. Protein ^{1}H NMR spectra, especially of samples the size of an antibody, are extremely crowded; to an extent that only a fraction of the total signal is represented by well resolved spectral lines. For proteins the size of mAbs, it appears that only this fine featured portion of the spectrum, which can be obtained by the subtraction of the smoothed from the original spectrum, exhibits significant variance between different proteins. To this end, the original protein spectrum (processed in Topspin as described below) is automatically phased (using both real and imaginary portions generated by Topspin) and baseline corrected (both zero-order), and subsequently smoothed with the Gaussian function (σ = 130 Hz) to obtain the contour (C) spectrum by applying inverse FFT, Gaussian window multiplication, and forward FFT as illustrated in Figure 1 (and in more detail in Supporting Information Figure 3S). The contour spectrum is then subtracted from the original protein spectrum to yield the fingerprint (F) spectrum. The contour and fingerprint are used separately in the calculation of the similarity between two ^{1}H NMR protein spectra (*vide infra*). In order to facilitate spectral comparison between two protein samples, it is desirable to eliminate all nonprotein resonances, which have not been suppressed by the PGSTE pulse sequence. We employed a straightforward subtraction algorithm that minimizes the total absolute intensity in the fingerprint spectrum. This is accomplished using the workflow shown in the boxed area of Figure 1, which depicts an iterative generation of the fingerprint spectrum from the trial protein spectrum. This trial spectrum is a result of subtraction of the optimized (with regard to the intensity, shift, line broadening, and phase) "buffer-only" spectrum from the original protein spectrum. The performance of this algorithm is demonstrated in Supporting Information Figure 4S, which illustrates that any bias introduced in this stage has negligible effect on the similarity measure.

Initial spectral processing was performed using Topspin 3.0 analysis software (Bruker Biospin, Billerica, MA). Spectra were apodized using the mixed Lorentz−Gauss window (LB = −1, GB = 0.005), and residual water signals were eliminated with a 25 Hz wide Gaussian filter function,[13] Fourier transformed and initially phased with automatic zero order phase correction using a Topspin *apk0* function. In the PROFILE spectra, the residual water signal is very small (compare Figure 1S) and primarily originates from back exchange with labile protons from the protein. Therefore, the digital filter width can be set very small as compared to the entire spectral range (~0.2%). Thus, the potential loss of information from the protein spectrum is insignificant for the calculation of the correlation coefficient. In the case of H-PROFILE spectra, the residual water peak can be much larger, requiring wider filters (up to 160 Hz). We found that the hydrodynamic parameters do not vary significantly with the filter widths. All additional spectral processing was performed in the Matlab R2012a (MathWorks, Inc.) programming environment. The hydrodynamic analysis used integral intensities of the H-PROFILE contour spectra obtained from the trapezoidal numerical integration using Matlab's *trapz* function.

PROFILE Similarity Calculation. One can express the protein-only spectrum (S) in Figure 1 as the Minkowski sum of the contour and fingerprint spectra: $C \oplus F = \{c_i + f_i \mid c_i \in C, f_i \in F\}$. Since C and F are independent of each other, it is

dx.doi.org/10.1021/ac401867f | *Anal. Chem.* 2013, 85, 9623−9629

convenient to define the similarity measure between two protein spectra as a product of two correlation coefficients, one for the pair of contours and one for the pair of fingerprint spectra:

$$r_P(S_1, S_2) = r_C(C_1, C_2) \times r_f(F_1, F_2) \quad (1)$$

where r_c and r_f are the maximum values of the normalized cross correlation sequence for two corresponding vectors. This quantitative assessment of spectral similarity is intuitive, as it is measured within the (0,1) interval. The r_c and r_f correlation coefficients were computed as the maximum values of the normalized cross correlation sequences for the two corresponding spectral vectors using the Matlab function *xcorr*.

RESULTS AND DISCUSSION

Antibody therapeutics are typically formulated in buffered solutions containing pharmaceutical excipients such as sugars (4−10%) and polysorbate (0.01−0.1%). Strong signals from these additives can partially mask protein signals and substantially limit the information content in an NMR spectrum. Because any sample processing prior to analysis (e.g., buffer exchange, filtering, etc.) may induce changes in the material and compromise comparative analysis, it would be highly beneficial to have a means to quantitatively assess protein structure in formulation buffers. The PGSTE experiment can separate spectral signals based on differences in the translational diffusion coefficients of molecules in the NMR sample.[14] This can be used to virtually eliminate almost all signals originating from water and all excipients while leaving the entire protein spectrum only a few-fold attenuated, as demonstrated in Supporting Information Figure 1S. This is similar to the principle of DOSY-NMR spectroscopy,[15] which aims to separate the spectra for a mixture of compounds based on differing translation diffusion coefficients. The loss of the protein signal intensity can be compensated by longer accumulation times. Because polysorbate forms large micelles, it was not possible to completely suppress these signals. However, the significantly reduced intensity allowed for a "clean" subtraction of the buffer control spectrum, as described in the Methods section. Thus, the data used for the comparative analysis represents the entire ^{1}H NMR spectra of "pure" protein. For the antibody samples, we noted that recording the spectra at elevated temperatures (52 °C is 15 °C below the lowest T_m for the IgG1/IgG2 antibodies studied here) substantially improved spectral resolution and differentiation of translational diffusion coefficients between the buffer and the protein signals (Supporting Information Figure 2S). The temperature induced changes in the spectrum are completely reversible, for when the sample is cooled back to room temperature the spectrum reverts to its original (Supporting Information Figure 2S). At 42 and 52 °C NMR spectra for all samples remained unchanged after 24 h. Thus, the PGSTE experiment generates the ^{1}H NMR spectra, which after additional processing steps are suitable for the comparative analysis of the different antibody samples.

A common way to determine spectral similarity is to compute the correlation coefficient between two signals. Ideally this measure should be sensitive to small (but meaningful) differences between the spectra. We accomplish this by constructing a new similarity measure (r_p, as described in the Methods section) which is particularly suitable for ^{1}H NMR spectra of antibody samples. We compare here the similarity of two different IgG1 molecules based on the PROFILE spectra and the r_p measure with the similarity based on the corresponding ^{15}N−^{1}H spectra and a standard correlation measure (see Methods section). Since the ^{15}N−^{1}H TROSY spectra for the intact antibodies exhibit severe overlap and require very long acquisition times to attain adequate signal-to-noise (see Figure 6S), we enzymatically cleaved both antibodies into their respective F(ab′)2 and Fc fragments using the Fabricator enzyme. Both fragments gave highly resolved ^{15}N−^{1}H correlation spectra with signal-to-noise ratios greater than 50, and suitable for the calculation of the spectral correlation coefficients (Figure 2). It appears that the similarity

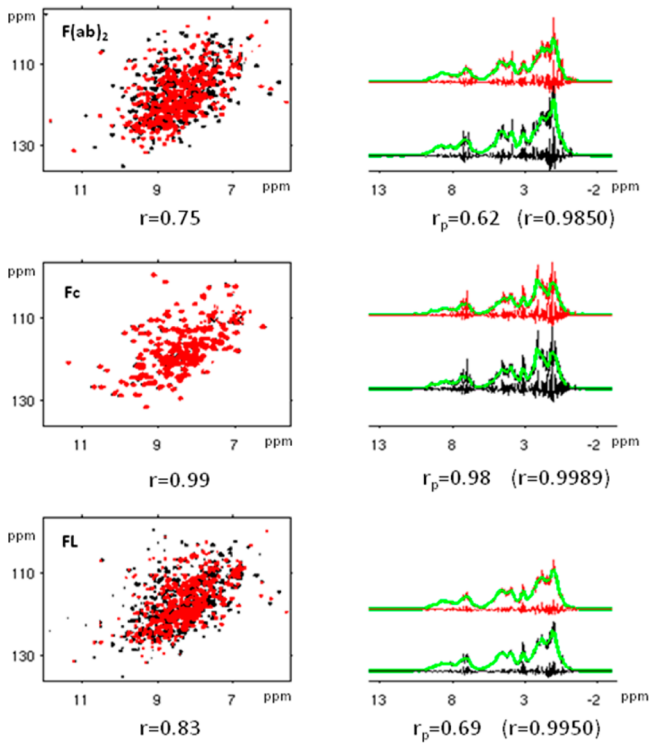


Figure 2. Similarity analysis of the ^{15}N labeled mAb#5 (black) and mAb#6 (red) samples by 2D ^{15}N−^{1}H TROSY (left) and PROFILE spectra (right), both recorded at 42 °C and at 800 MHz. The correlation coefficients (r and r_p) are shown, with values in parentheses corresponding to the protein-only ^{1}H spectra (not analyzed using the PROFILE method).

(as defined here) between the two 1D PROFILE spectra is in very good agreement with the similarity between the corresponding 2D ^{15}N−^{1}H spectra. In addition, the relative r_p and r values shown in Figure 2 indicate that the PROFILE metric may be a more robust measure for differentiating two antibody spectra. For example, the TROSY spectra of the two Fc fragments are indistinguishable by the standard r measure (r = 0.99) but exhibit small differences in their r_p measure (r_p = 0.98), an observation consistent with visual inspection of the 1D ^{1}H spectra. This is an important observation, since the ^{15}N−^{1}H correlation spectrum establishes the roots for modern protein NMR structure determination[16] and, therefore, is widely recognized as the fingerprint of a protein's three-dimensional structure. As shown here, not only is the PROFILE approach equivalent for smaller proteins (≤100 kD), but it has clear advantages when applied to larger molecules, such as

dx.doi.org/10.1021/ac401867f | Anal. Chem. 2013, 85, 9623−9629

intact mAbs, where the acquisition of heteronuclear experiments is currently not practical.

These data demonstrate that analysis by PROFILE can detect relatively small structural changes in formulated antibody samples. We further explored the utility of this approach by estimating how much unfolded protein could be detected in the PROFILE experiment. Since it is hardly possible to generate protein samples with well-defined mixed populations of folded and unfolded species, we used combined experimental and simulated data to gain insight. Figure 3 shows the response of

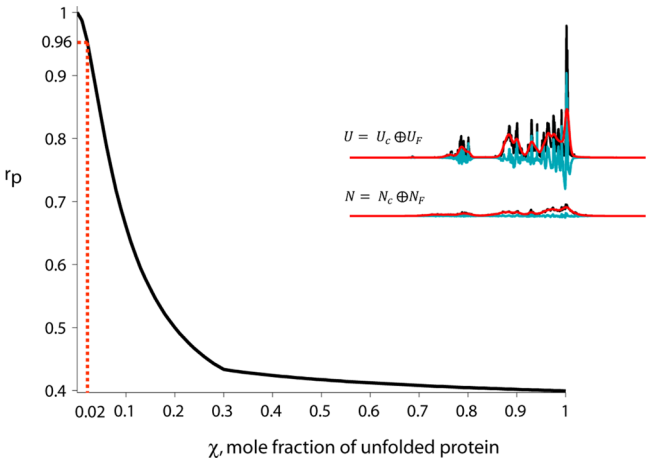


Figure 3. Correlation coefficient, r_p, between the spectrum of the native protein (N) and the spectrum of the mixture of the native (N) and the unfolded (U) proteins, where χ is the fraction of U. The samples of U and N were obtained by splitting 2 mL of mAb#3 (IgG2) (30 mg/mL) into two 1 mL portions. To the one portion was added solid dimethyl urea up to 5 M concentration. NMR sample preparation, NMR experiment, and data processing were the same for both preparations (see the Methods section). The inset shows ^{1}H spectra (black) of both samples plotted on same scale, where subscripts C and F refer to the contour (red) and fingerprint (blue) components, respectively. The r_p coefficient was calculated as $r(N_C, (1-\chi)N_C + \chi U_C) \times r(N_F, (1-\chi)N_F + \chi U_F)$, where r is the correlation coefficient.

the PROFILE correlation coefficient to the fraction of unfolded protein and demonstrates that the sensitivity of the PROFILE analysis should be better than 2%. This estimation must be supported by a sufficiently high signal-to-noise ratio in the PROFILE spectra. The simulation in Supporting Information Figure 5S demonstrates that random noise has a negligible impact on the correlation coefficient when S/N is greater than 50:1 in the fingerprint spectrum. The fingerprint spectra for samples analyzed in Table 1 have S/N values at least 200:1.

Table 1. Mean Values for Correlation Coefficients for Pairs of Spectra Shown in Figure 4[a]

	mAb#1	mAb#2	mAb#3	mAb#4$_1^A$	mAb#4$_2^A$	mAb#4^B
mAb#1	0.99	0.71	0.45	0.49	0.50	0.48
mAb#2		0.99	0.52	0.43	0.44	0.39
mAb#3			0.99	0.69	0.69	0.68
mAb#4$_1^A$				0.99	0.99	0.96
mAb#4$_2^A$					0.99	0.95
mAb#4^B						0.99

[a]The standard errors were less than 1% based on at least ten different experiments for the same protein sample.

Notably, the spectral correlation measure r (and r_p) is sensitive to the overall shape of the spectrum but is insensitive to global relative shifts and intensities of two spectra. While the second characteristic eliminates possible bias due to the instrumental drift in spectra, the overall spectral intensity in the PGSTE experiment depends on various relaxation and translational diffusion rates. The intensity information can be regained by recording the PGSTE spectra with different sets of judiciously calculated parameters (see Supporting Information), such that the integral intensities of the resulting spectra can be used in the ratiometric analysis to quantify differences in the diffusion coefficients and effective proton relaxation rates across different protein samples.

We demonstrate here the performance of the PROFILE methodology for a representative set of six different pharmaceutical samples of four antibody molecules: mAb#1, mAb#2, mAb#3, mAb#4$_1^A$, mAb#4$_2^A$, and mAb#4^B, where the 1,2 and 3,4 molecules correspond to IgG1 and IgG2 subtypes, respectively. The subscripts correspond to different manufacturing lots, and the superscripts correspond to two different formulations. The PROFILE spectra for each of these samples are shown in Figure 4. The spectra were processed and

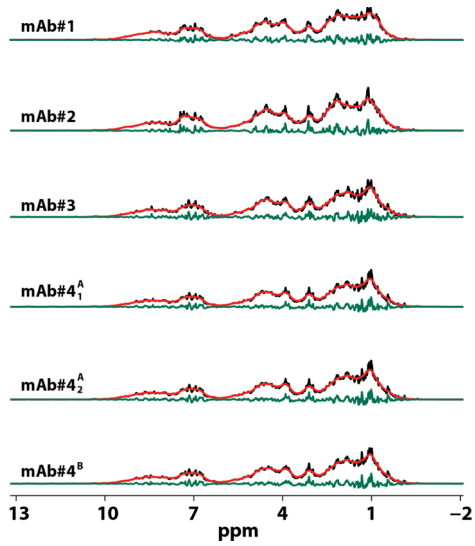


Figure 4. ^{1}H NMR spectra (black) of six antibody samples recorded at 52 °C with the PGSTE experiment. The red and green components correspond to the contour and fingerprint spectra, respectively. mAb's **#1** and **#2** correspond to two different IgG1 molecules, and mAb's **#3** and **#4** correspond to two different IgG2 molecules. The subscripts for mAb#4 correspond to two different lots of the same formulation and superscripts to two different formulations of the same protein. Only spectra for mAb#4 were processed with the subtraction of the buffer-only spectra. The PGSTE parameters were: g = 56.6 G/cm, δ = 1 ms, Δ = 170 ms.

compared according to the workflow described in Figure 1 and Supporting Information Figure 3S. The r_p values listed in Table 1 clearly differentiate PROFILE spectra from the different antibody samples and even from the same antibody in different formulation buffers. The statistical analysis of these correlations showed that 0.99 ± 0.01 is the identity threshold, i.e. where two PROFILE spectra are indistinguishable in terms of the r_p measure. Accordingly, sample mAb#4$_1^A$ appears indistinguishable from mAb#4$_2^A$, but clearly different from mAb#4^B. The differences of the PROFILE spectra for the mAb#4$_2^A$/mAb#4^B pair of samples were further characterized using H-PROFILE,

dx.doi.org/10.1021/ac401867f | Anal. Chem. 2013, 85, 9623−9629

where the hydrodynamic analysis (see Supporting Information) showed that mAb#4$_2^A$ appeared to have an increased (by ∼10%) hydrodynamic radius (*vide infra*).

Proton chemical shift profiling of monoclonal antibodies can be complemented by ratiometric experiments, which yield various proton relaxation rates and molecular diffusion coefficients, representative to the hydrodynamic properties of a protein sample. The relative assessment of these parameters may add additional dimensionality to the comparative analysis of different protein samples. The details of this methodology are described in the Supporting Information. Below, we present the results of such analysis (designated as the H-PROFILE) for an IgG1 sample measured at two different temperatures (Supporting Information Figure 8S). The R_1(42 °C), R_2(42 °C), R_1(52 °C), R_2(52 °C), and D_t(42 °C)/D_t(52 °C) parameters were measured: 2.03 ± 0.01 s^{-1}, 152 ± 2 s^{-1}, 1.98 ± 0.01 s^{-1}, 128 ± 3 s^{-1}, and 0.84 ± 0.01 s^{-1}, where the standard errors were obtained from repeated experiments with different pulse sequence parameters. For larger proteins, the spin−lattice relaxation R_1 is largely affected by the presence of water ^{1}H nuclear spins.[17] Thus, changes in R_1 may also reflect changes in protein hydration. Moreover, the transverse relaxation rate R_2 is (in the slow motional limit) proportional to the overall rotational correlation time and is largely affected by internal motions.[18] From the ratios of transverse relaxation rates and diffusion coefficients, one can obtain the relative hydrodynamic radii R_H(42 °C)/R_H(52 °C) = 0.99 ± 0.01 and viscosities η(42 °C)/η(52 °C) = 1.20 ± 0.02. At this temperature range, the 20% decrease in the viscosity for the protein solution is consistent with the decrease of the viscosity for the pure water. The same analysis applied to the mAb#4$_2^A$/mAb#4^B pair of samples in Table 1 resulted in R_2(mAb#4$_2^A$)/R_2(mAb#4^B) = 1.00 ± 0.02 and D_t(mAb#4$_2^A$)/D_t(mAb#4^B) = 1.26 ± 0.02, and corresponding hydrodynamic radii and viscosity ratios of 1.12 ± 0.02 and 0.71 ± 0.02, respectively. The observed differences in the "apparent" hydrodynamic radii likely represent differences in the inter- (e.g., reversible aggregation) and/or intramolecular (e.g., conformational changes) protein dynamics. These different H-PROFILE results may be due to the change of the formulation, the change of the process, or both these factors. Notably, measurements of the diffusion coefficient D_t alone may not differentiate two samples due to the possible R_H-η compensation. This unique sample characterization by H-PROFILE has the potential to serve as an early control for colloidal stability, a measure important in biopharmaceutical development and manufacturing.[19]

CONCLUSIONS

Here we demonstrate a new NMR methodology, PROFILE, which can provide a spectral fingerprint of recombinant monoclonal antibodies by detecting and quantifying subtle structural differences between two samples. The PGSTE experiment allows the acquisition of highly resolved ^{1}H proton spectra of the entire protein, affording a detailed comparison of the 1D ^{1}H spectra of mAbs in order to assess structural similarity. Additionally, the H-PROFILE method allows the effective relaxation rates (R_1, R_2) and translation diffusion coefficients (D_t) to be easily obtained and compared for two protein samples or one protein sample under different conditions (e.g., concentration, formulation, or temperature). Ultimately, these values can be used to establish differences (or equivalence) in terms of protein structure, hydration, dynamics, and intermolecular interactions, which is not currently possible by other analytical methods. The PROFILE spectra report on the higher-order structure of glycoproteins, which are highly characteristic of individual samples and are therefore suitable for the proof of conformance or "high similarity"[2] to a reference standard. We demonstrate this in a comparability study of monoclonal antibodies, arguably the most challenging class of proteins. Applications to other kinds of protein therapeutics are straightforward and require only adjustments of sample temperature and pulse sequence parameters. Our preliminary studies indicate that, in certain cases, where the intramolecular mobility of the attached glycans is on the subnanosecond up to single digit nanosecond time scale, PROFILE can readily discern different glycosylation patterns on the same intact protein, the most frequent and challenging source of variability across different manufacturing processes.

ASSOCIATED CONTENT

Supporting Information

1D ^{1}H spectra of an IgG1 molecule recorded with different pulse sequences and at different temperatures, flow chart of the PROFILE and H-PROFILE data processing protocol, demonstration of the buffer subtraction algorithm, figures demonstrating the effects of S/N on correlation coefficients, PGSTE pulse sequence, H-PROFILE spectra for an IgG1 sample at two different temperatures, and derivation of the relaxation rates and relative hydrodynamic parameters from the PGSTE experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: lpoppe@amgen.com.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We would like to acknowledge Mike Toupikov, Victor Fung, Jennifer Liu, and Thomas Szyperski for insightful discussions, and Peter Grandsard and Philip Tagari for supporting this work.

REFERENCES

(1) Chirino, A. J.; Mire-Sluis, A. *Nat. Biotechnol.* **2004**, *22*, 1383−1391.
(2) Berkowitz, S. A.; Engen, J. R.; Mazzeo, J. R.; Jones, G. B. *Nat. Rev.|Drug Discovery* **2012**, *11*, 527−540.
(3) Houde, D.; Arndt, J.; Domeier, W.; Berkowitz, S.; Engen, J. R. *Anal. Chem.* **2009**, *81*, 2644−2651.
(4) Wüthrich, K. *NMR of Proteins and Nucleaic Acids*; John Wiley & Sons: 1986.
(5) Otting, G.; Wüthrich, K. *J. Magn. Reson.* **1986**, *66*, 359−363.
(6) Aubin, Y.; Gingras, G.; Sauvé, S. *Anal. Chem.* **2008**, *80*, 2623−2627.
(7) Freedberg, D. I. *Dev. Biol. (Basel)* **2005**, *122*, 77−83.
(8) Zhang, X.; Luo, Q.; Apostol, I.; Luo, S.; Jerums, M.; Huang, G.; Jiang, X. G.; Gastwirt, J.; Savjani, N.; Lewis, J.; Keener, R., 3rd; Wypych, J. *Anal. Biochem.* **2013**, Feb 15, *433* (2), 137−49.
(9) Cotts, R. M.; Hoch, M. J. R.; Sun, T.; Markert, J. T. *J. Magn. Reson.* **1989**, *83*, 252−266.
(10) Zhu, G.; Torchia, D. A.; Bax, A. *J. Magn. Reson., Ser. A* **1993**, *105*, 219−222.
(11) Sinnaeve, D. *Concepts Magn. Reson.* **2012**, *40A* (2), 39−65.
(12) Pervushin, K. V.; Wider, G.; Wüthrich, K. *J. Biomol. NMR* **1998**, *12*, 345−348.

(13) Marion, D.; Ikura, M.; Bax, A. *J. Magn. Reson.* **1989**, *84*, 420−425.
(14) Van Zijl, P. C. M.; Moonen, C. T. *J. Magn. Reson.* **1990**, *87*, 18−21.
(15) Johnson, C. S. *Prog. NMR Spectrosc.* **1999**, *34*, 203−256.
(16) Cavanagh, J.; Fairbrother, W. J.; Palmer, A. G., III; Rance, M.; Skelton, N. J. *Protein NMR Spectroscopy, Principles and Practice*, 2nd ed.; Elsevier Inc.: 2007.
(17) Hallenga, K.; Koenig, S. H. *Biochemistry* **1976**, *15* (19), 4255−4264.
(18) Palmer, A. G., III. *Chem. Rev.* **2004**, *104*, 3623−3640.
(19) He, F.; Woods, C. E.; Becker, G. W.; Nahri, L.; Raznikov, V. I. *J. Pharm. Sci.* **2011**, *100* (12), 5126−5141.