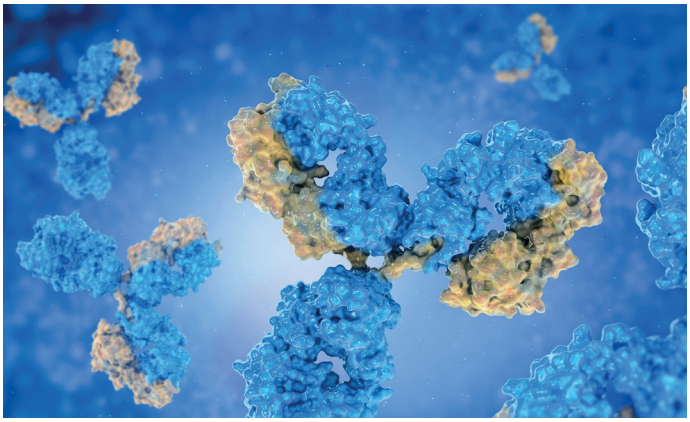


Evaluation of the Higher Order Structure of Biologics at Atomic Resolution with NMR – Overcoming Challenges

F. Baroni,[1*] F. Benevelli,[2*] M. Vogtherr,[3*] C. Fischer,[4*] R. Verani,[1] M. Rossi[5]

*These authors contributed equally to this work
[1]Analytical Development Biotech Department, Merck KGaA, Guidonia Site, 00012 Rome, Italy
[2]Bruker Biospin Business Unit, Bruker Italia, 20158 Milano, Italy
[3]BIO NMR & NMR Spectroscopy Analytical Service, Merck KGaA, Darmstadt Site, 64293 Darmstadt, Germany
[4]Bruker BioSpin GmbH, Silberstreifen, 76287 Rheinstetten, Germany
[5]Global Analytical Pharmaceutical Science & Innovation Department, Merck KGaA, Guidonia Site, 00012 Rome, Italy

Abstract

NMR is a high precision analytical technique and therefore presenting suitable characteristics for robust statistical analysis of results, critical when assessing comparability between batches or similarity between a biosimilar and its originator. Its high sensitivity to relevant structural changes further candidate the method among the best that are able to characterize the Higher-Order Structure of biotherapeutics. Additionally, it is also a robust and non-destructive technique that requires almost no sample preparation.

This feasibility study, performed on three different biotech products of increasing complexity (both in terms of molecular mass and buffer composition), was aimed at verifying the applicability of NMR as a routine technique for HOS characterization during the development of biotherapeutics. 1D and 2D NMR spectra at natural isotope abundance were therefore acquired and comparability among different batches was evaluated in a statistically robust manner.

In all three case studies reported here, the technique presented sensitivity and resolution superior to that of other techniques but, perhaps more importantly, it provided access to unique information in terms of being able to assess batch-to-batch variability.

Overall, while demonstrating the applicability of NMR to R&D routine studies such as characterization for comparability and stress testing, two main challenges are highlighted : i) analysis of intact monoclonal antibody (mAb) molecules and ii) analysis of the molecules in excipients-rich buffers. The solution of the first challenge will lead to a sensible reduction in the costs of analysis, while solving the second challenge will grant the possibility to analyze a molecule under native conditions.

Introduction

Overall, the need for high-resolution techniques is regarded as being urgent for the characterization of Higher Order Structure (HOS: secondary, tertiary and quaternary structure). This is because the HOS of a protein is a fingerprint covering structural quality attributes (QA) potentially linked to the function of the molecule. It is therefore a key determinant in assessing comparability between different manufactured batches, the similarity between a biosimilar and its originator as well as development of an in-depth understanding of the structure-function relationships.

Currently, there is no single approach for a comprehensive evaluation of HOS. Techniques typically used for HOS characterization (circular dichroism (CD), hydrogen deuterium

exchange mass spectrometry (HDX-MS), fluorescence, fourier transform infrared spectroscopy (FT-IR), Raman spectroscopy, and thermal analysis by nano differential scanning calorimetry (Nano DSC)) measure different aspects of the structure, either directly or indirectly. With the exception of HDX-MS, these low-resolution techniques are often not sensitive enough to small, local changes in the protein fold.

For these reasons, the application of 1D and 2D nuclear magnetic resonance (NMR) spectroscopy during the development of biotherapeutics has become the subject of great interest to biotech companies. Improvement during the last decade have made the use of this technique feasible in the pharmaceutical environment,[1,2] assuring an atomic-level fingerprint of a molecule's structure through the characterization of multiple structural aspects with a single method. In fact, NMR grants resolution and sensitivity much higher than other analytical methods.[3]

Whilst 1D NMR is already frequently employed to support submissions to the health authorities for new drug substances (both chemical and biological),[4] 2D NMR of biologics is not yet in routine use to support product characterization. However, the use of 2D data is highly promising and is expected to soon become an additional milestone in characterization packages, due to its superior resolution and to the comprehensive atomic-level fingerprinting of primary, secondary, tertiary and quaternary structure.

Several papers[2, 5-11] have been published in the past few years, describing different approaches to deliver NMR to protein structural characterization of biotherapeutics. These have addressed the challenges arising from long acquisition times, large protein dimensions, complex buffers and the necessity to work with ^{13}C and ^{15}N at natural abundance.

Based on this body of work, the National Institute for Standards and Technologies (NIST) recently coordinated an interlaboratory project aiming at establishing a harmonized, routine 2D NMR approach for HOS assessment of mAbs. The results[12] showed the high precision and reproducibility of both ^{1}H, ^{15}N (amide) and ^{1}H, ^{13}C (methyl) spectra generated using 39 NMR spectrometers from different vendors with operating frequencies ranging from 500 to 900 MHz. Overall, this work, highlights the potential of NMR to become part of the characterization package.

Here we report the results of a feasibility study performed on Merck KGaA biotech products, to verify the applicability of NMR as a routine technique for HOS characterization. Using the workflow proposed by NIST[12] as a starting point, 1D and 2D NMR spectra were acquired for three different biologics of increasing complexity (all molecules were at natural isotope abundance and were all expressed in Chinese hamster ovarian (CHO) cells). The three samples were a nanobody (40 kDa molecule in simple buffer), a mAb and a Fc-fusion protein (142 kDa and 177 KDa, respectively in a complex buffer).

Material and Methods

Samples

All the NMR experiments were performed on native molecules at 40 mg/ml. The target concentration was reached either by dilution with formulation buffer (in the case of the nanobody) or by concentration using a 30 kDa cut-off centrifugal filter (in the cases of the mAb and Fc-fusion protein).

The nanobody (40 kDa) was analyzed in its formulation buffer (10 mM Tris, pH = 7.5).

The mAb (144 kDa) was analyzed both in its formulation buffer (10 mM histidine, 8% trehalose, 0.05% tween 20, pH = 5.5) and after buffer exchange, in a simplified buffer (10 mM histidine, pH = 5.5).

The Fc-fusion protein (177 kDa) was also analysed in its formulation buffer (10 mM histidine, 5 mM methionine, 6% trehalose, 40 mM NaCl, 0.05% tween20, pH = 5.9) and after buffer exchange, in a simplified buffer (10 mM succinate, 40 mM NaCl, pH = 5.9).

Nuclear Magnetic Resonance Spectroscopy

The spectra of the nanobody and of the Fc-fusion protein were measured on a Bruker AVIIIHD 600 system equipped with a TCI helium cryoprobe. The spectra of the mAb were measured on a Bruker AVIIIHD 700 system equipped with TCI helium cryoprobes

The nanobody samples (40 kDa) were prepared by adding 30 µl of D_2O to 200 µl of the protein solution in a 3 mm NMR tube. ^{1}H-^{13}C HSQC spectra were measured using the pulse sequence *hsqcetfpgpsi2* from the Bruker library. The number of scan was set to 48, data points in the indirect dimension (F1) to 226, relaxation delay to 1 s and acquisition time to 100 ms for an overall acquisition time of 3 h and 24 min. The ^{13}C spectral window was set from 5 to 35 ppm in order to map only the methyl region. All spectra were measured at 310 K.

The 1D proton spectra of the Fc-fusion protein (177 kDa) were measured with simple solvent suppression (for samples in simplified buffer) and, to partially remove the signals from the excipients, with a diffusion filter[13,15] (for samples in formulation buffer). All samples were prepared by adding 30 µl of D_2O to 200 µl of the protein solution and transferring to 3 mm NMR tubes. Solvent suppression was achieved with an excitation sculpting scheme, while for the diffusion filter a LED sequence with 200 ms of big delta, 4 ms of little delta and 60% gradient strength was chosen. To compensate for different sensitivities of these approaches, the number of scans was set to 128 for the excitation sculpting and to 256 for the diffusion filter. All spectra were measured at 310 K.

Samples of the mAb were prepared by adding 50 µl of D_2O to 500 µl of the protein solution then transferring to 5 mm NMR tubes. The 1D proton spectra were recorded with excitation sculpting water suppression and 128 scans. The 2D ^{1}H-^{13}C correlations were acquired as methyl-TROSY SOFAST-HMQC spectra with 128 scans, 512 data points in F1, 200 ms relaxation delay and 60 ms acquisition time. All spectra were measured at 318 K.

Statistical comparison of 1D proton spectra was performed by the 1D PROFILE[13,14] analysis tool available in the Bruker Software AssureNMR 2.1.2. Statistical comparison of 2D spectra was performed by the newly released BiologicsHOS software (Bruker/MestreLab).

Results

NMR and forced degradation studies

Forced degradation studies (stress testing) during development of biologics are of key importance to the assessment of protein degradation pathways, which in turn is instrumental to the evaluation of drug stability and the related impact on purity, potency, and safety. Knowing the impurity profiles and evaluating the degradation of products under various stress conditions instructs the development of analytical methods, supports specification setting, and provides usable knowledge from formulation studies under the quality-by-design (QbD) paradigm.[18]

Oxidation and deamidation, are often a consequence of stressing conditions and are generally expected to induce HOS perturbations, although these structural modifications are often too small to induce changes in secondary and tertiary structure that are detectable using low resolution spectroscopic methods (e.g. FT-IR, fluorescence and circular dichroism). Furthermore, even if such changes could be detected by these lower resolution methods, they only provide gross information and it would not be possible to identify the regions of the protein subjected to such a subtle structural modification.

In our feasibility study on a comparison of an oxidized sample (obtained after incubation with 0.1% H_2O_2 for 60 min) with untreated samples of correctly folded and misfolded mAb, it is clear that NMR could discriminate between the three samples, detecting differences both in ^{1}H 1D and ^{1}H-^{13}C 2D spectra in terms of chemical shifts and line-broadening (Figure 1).

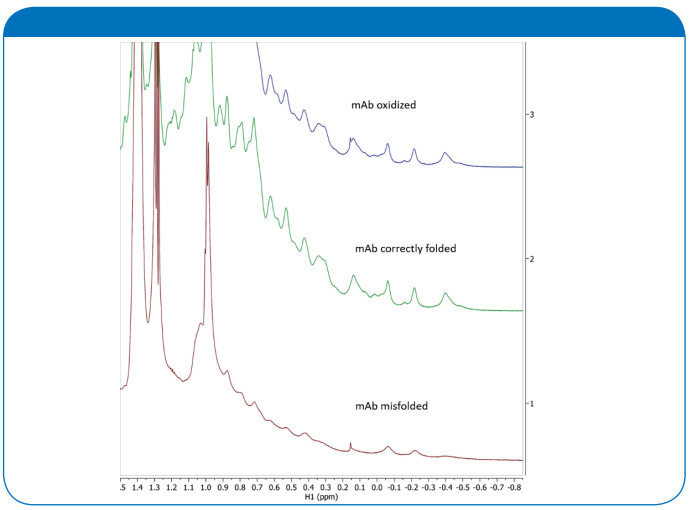


Figure 1. Overlay of 1D proton spectra for oxidized (blue), correctly folded (green) and misfolded (red) mAb.

The experiments presented above were obtained for the samples in simplified buffer. Spectra were also acquired for samples in formulation buffer (Figure 2).

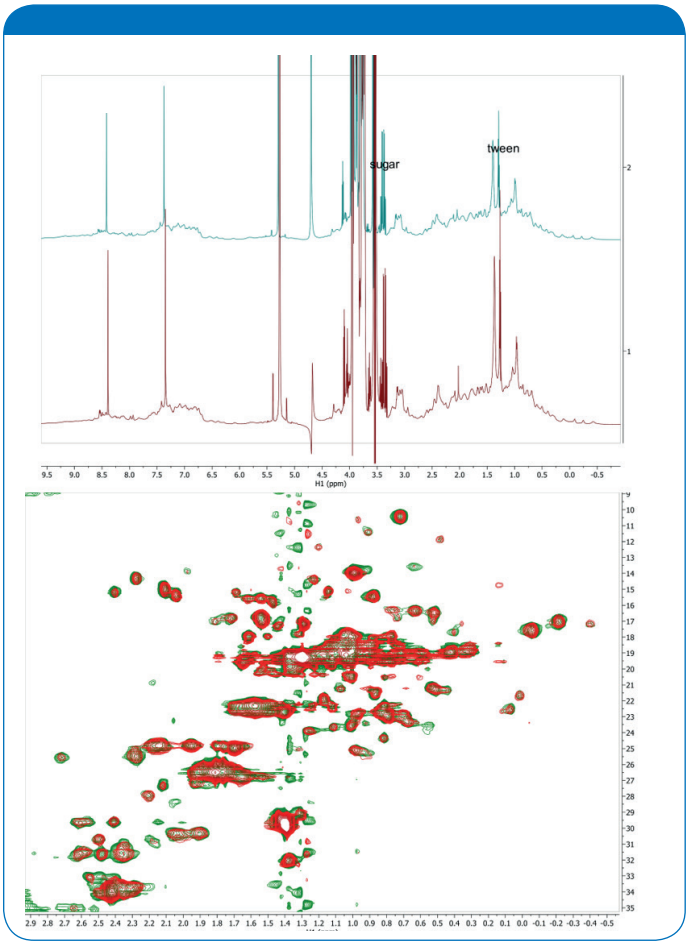


Figure 2. Overlay of 1D proton spectra (upper spectra) and 2D ^{1}H-^{13}C HSQC methyl spectra (lower spectra) of the mAb in formulation buffer (red) and in simplified buffer (green).

Overall, high-resolution 1D and 2D spectra were obtained both in simplified and formulation buffer. As expected, resolution of the protein signals was better in simplified buffer, since the presence of excipients containing aliphatics (mostly polysorbate and trehalose) in the formulation buffer generates intense and broad signals.

The sensitivity of NMR and statistical analysis

The experiments performed on the nanobody highlighted the advantage provided by NMR spectroscopy, in terms of sensitivity to structural changes.

In this case study, possible differences between samples due to different thawing conditions were explored. Previous analysis by other techniques (NanoDSC, SEC-MALS and AUC) have shown that the temperatures of melting (Tms) and degree of aggregation of the nanobody samples were highly influenced by manipulation at 5° C or at 25° C: thawing the samples at 25° C in fact leads to increased aggregation and higher thermal stability. The 2D spectra of a reference batch were therefore compared to other samples from the same batch that where thawed at 5° C and at 25° C (Figure 3).

Overall, the similarity of the ^{1}H-^{13}C HSQC methyl spectra suggests comparability between the three samples (Figure 3A), but also seems to confirm that the different thawing methods influenced local modification of the protein structure, as observed by the chemical shift perturbation (CSP) of certain peaks (examples are reported in figure 3B, 3C, and 3D).

Furthermore, the comparison of the chemical shifts also provided information generally not available from low-resolution techniques - at least not with such high resolution - in terms of batch-to-batch variability (Example shown in Figure 3B).

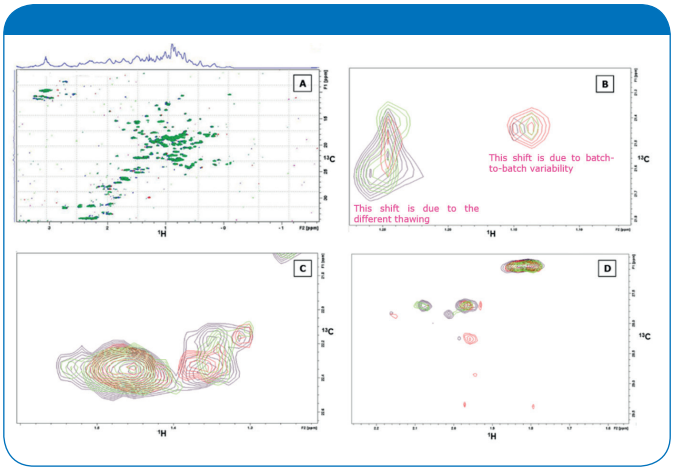


Figure 3. Overlay of 2D ^{1}H-^{13}C HSQC methyl spectra of three nanobody samples: in blue a reference sample; in red, a batch thawed at 25° C and in green the same batch thawed at 5° C. Although overall, there is good overlap **(A),** subtle differences are observed in some regions of the spectra between the sample thawed at 25° C and the others when investigating each signal **(B, C, D)**. Differences are also observed between the reference and the other two samples due to inherent batch-to-batch variability that is not related to the thawing temperature **(B)**.

The results described above highlight a fundamental aspect of the application of the technique to comparability/similarity studies. In fact, since NMR achieves high resolution and good sensitivity to structural changes, it is necessary to use robust statistical tools in order to discriminate between real differences and those that are insignificant. For example, it is known that there is some intrinsic variability that occurs batch-to-batch but this does not affect protein activity, stability and safety.

To determine if the small differences between the 2D NMR spectra of the nanobody were significant, two methods, CCSD[19] and ECHOS,[10] were employed. Both methods provide an objective numerical assessment of structural comparability while pinpointing the spectroscopic reason/s for an observed difference (in terms of chemical shift, number of signals and signals' amplitude). The BiologicsHOS software (Bruker&Mestrelab) was used for this analysis, since it includes both of these methods.

The CCSD[19] (combined chemical shift deviation) method is a targeted analysis based on the comparison of two spectra in terms of chemical shift, thus requiring a peak list as an input. One peak, the reference peak, is used to align the peak positions and amplitudes between spectra.

The positions of all peaks in both dimensions (in the case of the nanobody spectra, the ^{1}H and ^{13}C chemical shifts) are then encoded into a single number, the CCS (Combined Chemical Shift) which is an expression of overall similarity (the lower the value the more similar the spectra). The positions are weighted by Larmor frequencies, and a correction factor α is employed to calculate the CCS as shown in the following equation.

$$CCS = \sqrt{0.5 \cdot (\delta_H^2 + (\alpha \cdot \delta_X)^2)}$$

Where δ_H and δ_X are the shifts (ppm) of the ^{1}H and X nuclei respectively (in the case of the nanobody comparison, X is ^{13}C and α (0.241) is the correlation factor).

In figure 4 shown below, the results obtained from the CCSD comparison of the three spectra of the nanobody are reported.

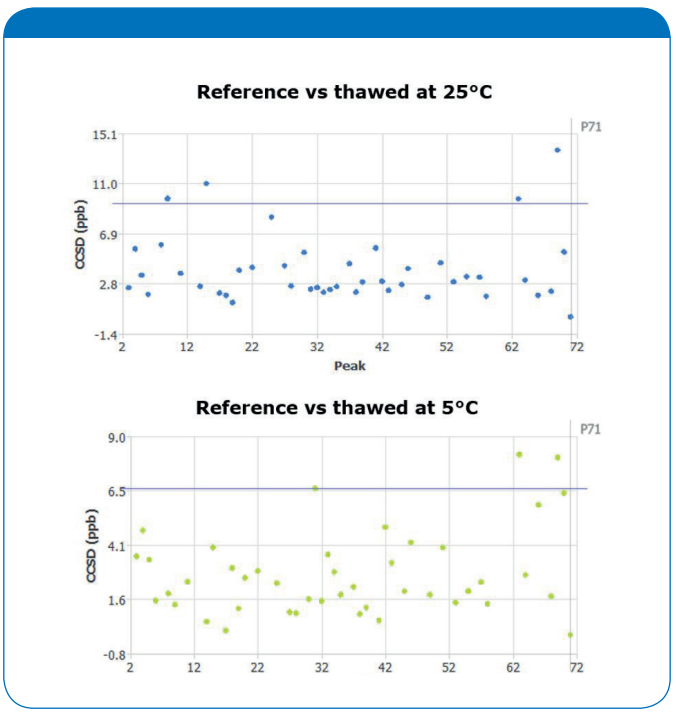


Figure 4. CCSD results on the nanobody samples. Combined chemical shift differences (CCSD) measures the amount that peaks are shifted between two spectra (0 means no shifts i.e. identical samples). The mean values obtained by comparing the reference material to the sample thawed at 5° C or at 25° C are reported in the table. On the left, for each comparison, a plot is shown which highlights which signals between the spectra are the most dissimilar (i.e. those peaks above the threshold shown by the horizontal blue line which is set at twice the standard deviation of the shift differences)

Although the CCSD values are slightly higher in the case of the comparison with the batch thawed at 25° C, overall, the values obtained in both comparisons are small and this is indicative of a high degree of similarity:[19, 20] no statistically significant differences are highlighted for any of the peaks.

The principle of ECHOS[10] (easy comparability of HOS) is a pointwise comparison of two spectra in terms of amplitude of the signals. All points (signals), are included in the analysis provided that in at least one spectrum their amplitude is above the noise level. The statistical comparison is then based on a linear regression where the correlation coefficient R serves as a comparability/similarity indicator, while the value of the residuals calculated for each pair of points gives an indication of the differences between the spectra.

As shown in Figure 5, statistically significant differences are observed between the reference material and the batch thawed at 25° C in terms of signal amplitudes.

Aggregation (caused, in turn by degradation) is expected to influence line-broadening more than the chemical shift. The different results obtained by the CCSD method and by the ECHOS as well as the conclusions drawn are consistent with those obtained by SEC-MALS, AUC and NanoDSC suggesting aggregation of the sample is induced by thawing the sample at 25° C.

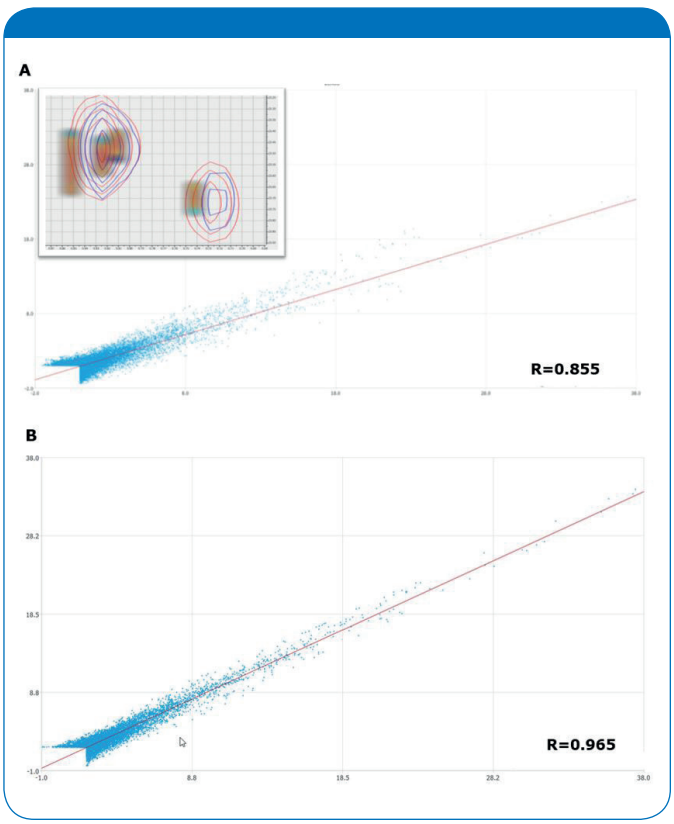


Figure 5. ECHOS analysis of the nanobody samples. The reference batch is compared in **A)** with the sample thawed at 25° C and in **B)** with the sample thawed at 5° C. The first comparison indicates a poorer correlation between the two samples (R=0.855) that can be ascribed to differences in the amplitude of some signals (an example is reported as a heat colour map in the inset).

The improved lowest limit of detection of structural modifications

^{1}H 1D spectra (with excitation sculpting) of samples of the Fc-fusion protein in simplified buffer were measured upon addition of various concentrations of Cu^{2+}. The aim of this test was to evaluate the minimum level of Cu^{2+} that induced a modification of the protein structure that could be detected by NMR.

Briefly, Anhydrous Copper (II) Sulfate was dissolved into H_2O (MilliQ quality) and then added to the protein solution to obtain the element concentrations of 2, 1 and 0.5 ppm. For for each of these titration points, the spectra of two replicates were acquired.

In Figure 6, an expansion (from 7 to 7.5 ppm) of the 1D spectra measured at different copper concentrations is displayed. These spectra show that even upon addition of 0.5 ppm of Cu^{2+}, small variations (i.e. broadening and peak shifts) are observed. It is known that this spectral region contains signals from aromatic and amide protons and the changes observed are indicative of the overall structure.

Paramagnetic ions are known to induce changes in the relaxation behavior of the nuclear spins, thus leading to line broadening. In this case, changes are observed only on some specific peaks (highlighted in the yellow box in Figure 6), indicating that Cu^{2+} interacts locally and selectively.

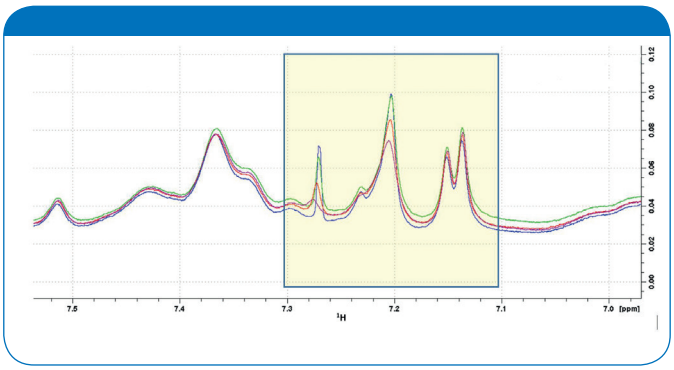
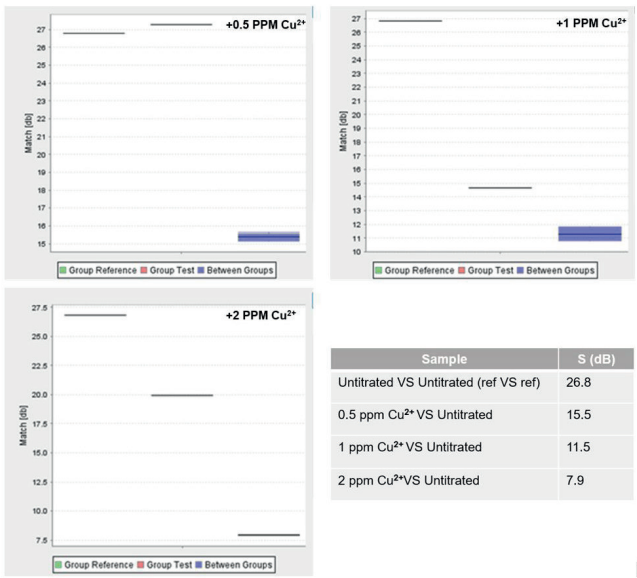


Figure 6. Overlay of 1D ^{1}H spectra (excitation sculpting) of the Fc-fusion protein in simplified buffer supplemented with copper. 0 ppm (blue), 0.5 ppm (green), 1 ppm (red) and 2 ppm (purple). Some peaks in the aromatic-amide region start to shift and broaden even upon addition of 0.5 ppm of copper (highlighted in the yellow box).

Eventually, to verify that the observed differences were significant, the 1D PROFILE was used for statistical comparison (results are displayed in Figure 7), using the two spectra acquired for each sample. The analysis confirmed that the observed perturbations to the structure were statistically significant (shown by the fact that between groups variability did not overlap with in-group variabilities) even upon addition of the lowest concentration of 0.5 ppm of Copper. Overall, this indicates a decrease of similarity as a function of the conentration of Copper (the S value decreases from 15.5 to 11.5 to 7.9 as the Copper content is increased from 0.5, to 1 and then 2 ppm).



Sample	S (dB)
Untitrated VS Untitrated (ref VS ref)	26.8
0.5 ppm Cu^{2+} VS Untitrated	15.5
1 ppm Cu^{2+} VS Untitrated	11.5
2 ppm Cu^{2+}VS Untitrated	7.9

Figure 7. Statistical analysis (1D PROFILE) on the Fc-fusion protein supplemented with copper. Variability is quantified by the value S(dB), the logarithmic scale of similarity:[13,14] the higher the value, the higher the similarity. In all three statistical analyses (graphical plots), the variability between groups does not overlap with in-group variabilities, indicating statistically significant differences between spectra. In the table are reported the S(dB) value calculated for each sample when compared to the reference.

Similar results were obtained when analyzing the Fc-fusion protein in its formulation buffer. In this case, 1D proton spectra were acquired with diffusion filtering (this was applied due to its suitability for removing signals originating from the low molecular weights buffer excipients (Figure 8). Although the spectra obtained presented a worse signal to noise ratio compared to those acquired with excitation sculpting in a simplified buffer, their resolution was enough to highlight the same differences as describe above (Figure 9).

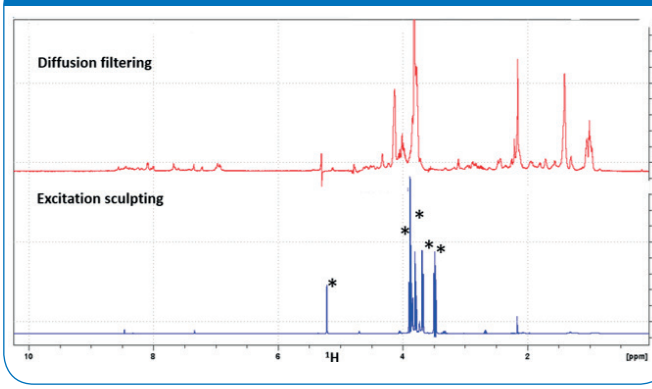


Figure 8. 1D ^{1}H spectra of the Fc-fusion protein in formulation buffer. Comparison of 1D spectra of the sample that has not been supplemented with Copper which was obtained with excitation sculpting and diffusion filtering. The latter approach enables the removal of most of the signals from the excipients (indicated by *) leading to spectra with worse S/N overall but which highlight the spectral feature due to the protein.

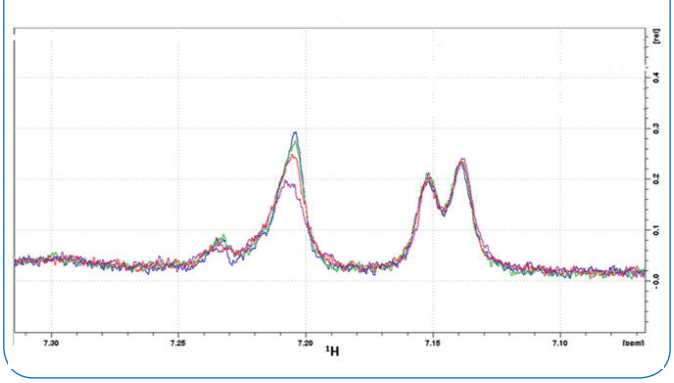


Figure 9. Overlay of 1D ^{1}H spectra (diffusion filter) of the Fc-fusion protein in formulation buffer supplemented with copper. 0 ppm (blue), 0.5 ppm (green), 1 ppm (red) and 2 ppm (purple). Differences are mostly observed in the amide-aromatic region.

Discussion

A critical aspect that influences signal resolution in an NMR experiment is the increasing the dimension of the protein, since line broadening increases due to progressively slower molecular tumbling. The NIST approach described in the interlaboratory study[12] foresaw that effect as a potential issue, therefore recommending digestion of the mAb with papain (a cysteine protease). This is a justifyable approach-since it has already been demonstrated in other publications that the isolated Fab and Fc regions obtained from such a digestion maintain the structure found in the intact mAb[7,9].

Although this approach would indeed speed up the analysis and increase spectral resolution, it requires significant manipulation of the sample and adds additional costs to an analysis (due to the cost of the enzymatic digestion of several milligrams of protein) and is therefore undesirable. Since the aim of this feasibility study was to establish an NMR method suitable for routine use in which multiple samples may be analysed easily and quickly (e.g. for comparability and similarity studies), it was decided to always perform the analyses on intact molecules.

1D fingerprints of the three proteins were obtained by acquiring their proton spectra. High-resolution data were obtained for all the proteins tested and the acquisition time, which was of course dependent on the dimension of the protein, ranged from 10 to 40 minutes.

Although considered to be the gold standard for HOS evaluation, ^{1}H-^{15}N HSQC spectra were not acquired due to their long acquisition times since this is generally considered to be incompatible with a routine analytical method. Therefore, 2D ^{1}H-^{13}C methyl spectra were chosen instead as they are much quicker to obtain and are an equally informative alternative[9,12] to obtaining a full-spectrum 2D fingerprint of the molecules. High-resolution 2D ^{1}H-^{13}C methyl spectra were obtained both for the nanobody at 600 MHz (taking 3.5 h) and for the recombinant mAb at 700 MHz (taking 1.5 h).

In both the mAb and the Fc-fusion protein, the use of the formulation (i.e. complex) buffers led to spectral interference from the aliphatic components of the matrix. In 1D spectroscopy, these undesired signals were easily removed by gradient diffusion filtering [13,15] without any significant loss of information, although in ^{13}C 2D spectroscopy, some baseline artifacts and obscuration of the protein signals could not be avoided.

The easiest solution to the problem of interference due to excipients in 2D ^{13}C carbon spectroscopy is dilution of the protein in a simplified buffer, thereby eliminating, the undesired signals from buffer components containing aliphatic functional groups. In the case of recombinant mAb, spectra in simplified buffer showed improved resolution, but artifacts due to glycerol (introduced from the filter used in the concentration step) and to polysorbate (which was incompletely removed by buffer exchange) were still present.

The second solution would be the use, also in 2D spectroscopy, of pulse sequences which are similar in principle to diffusion filtering and are capable to suppressing signals from excipients. Development of such approaches should be preferred, since, for a drug, product testing would be more desirable to analyze the product as manufactured and formulated. A promising method recently proposed, for example, demonstrated that selective pulse techniques combined with SMILE-based signal subtraction[16] could mitigate interference from commonly employed aliphatic excipients, with minimal loss in sensitivity.[17]

All the analyses presented in this work highlight the fundamental advantage of using NMR spectroscopy in HOS characterization i.e.:superior resolution and sensitivity compared to routine techniques. NMR can "see" every small difference although this inherent advantage must be dealt with carefully as it may overestimate non-relevant differences. The solution is therefore to combine NMR with an appropriate and robust statistical analysis to demonstrate that an observed difference is indeed significant.

Furthermore, even if statistically significant differences are demonstrated in terms of HOS, orthogonal techniques should be always applied to determine if they meaningfully affect the activity, stability and safety of a drug.

The statistical comparison of the 1D NMR spectra was therefore performed using the 1D PROFILE approach,[13,14] while the 2D spectra comparison was performed using either the CCSD[19] or the ECHOS[10] method. These two approaches are complimentary since they are based on the comparison of different properties of a spectrum (chemical shift and amplitude of signals) and are available in the BiologicsHOS software (Bruker/MestreLab) and suitable for use by non-NMR experts.

Conclusions

NMR is a high precision analytical technique and therefore ideal for robust statistical analysis, critical when assessing comparability between batches or similarity between a biosimilar and its originator. Its high sensitivity to structural changes and resolution further candidate the method among the best available to characterize the higher-order structure of biotherapeutics.

As defined by the international guidelines, pharmaceutical development should adhere to the quality by design paradigm, defined as "*systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management.*" (ICH Q8 (R2)).[18] NMR fits perfectly into this scenario since it can provide a unique and detailed understanding of the product and the process by which it is manufactured, starting from the early phase of development.

The study reported here has proven the applicability of 1D and 2D NMR spectroscopy to intact molecules with acquisition times reasonable for routine analysis. While 1D NMR can only indicate if samples differ significantly from a reference, 2D NMR methods (which are also more sensitive to small changes due to the higher dispersion achieved) can be used to identify the specific regions of a molecule that has been changed. Additionally, if an assigned peak list is available then changes to specific residues can be determined.

Solutions to two main challenges have been highlighted in the work reported here: the necessity to further improve methods for the analysis of intact mAbs and methods for the analysis of biologics in excipients rich buffers. The solution of the first challenge will lead to a sensible reduction of the costs of analysis, while solving the second one will grant the possibility to analyze a molecule in native conditions, making NMR an essential tool in biologics characterization.

Acknowledgments

The authors would like to acknowledge Ian Clegg, Carlo Pergola, Angelo Palmese, Abhijeet Satwekaar and Luisa Lozzino for insightful discussion and for supporting this work, and Christian Richter from BMRZ (Frankfurt/Main) for technical support.

References

[1] Kiss, Róbert, Ádám Fizil, and Csaba Szántay Jr. "What NMR can do in the biopharmaceutical industry." *Journal of pharmaceutical and biomedical analysis* 147 (2018): 367-377.

[2] Japelj, Boštjan, et al. "Biosimilar structural comparability assessment by NMR: from small proteins to monoclonal Antibodies." *Scientific reports* 6 (2016): 32201.

[3] Wen, Jie, et al. "A comparison between emerging and current biophysical methods for the assessment of higher order structure of biopharmaceuticals." *Journal of pharmaceutical sciences* (2019).

[4] Ruggiero, Andrea, and Sarah K. Branch. "Pharmaceutical Industry: Regulatory Control and Impact on NMR Spectroscopy." *NMR in Pharmaceutical Science* (2015): 413.

[5] Brinson, Robert G., et al. "Application of 2D-NMR with room temperature NMR probes for the assessment of the higher order structure of filgrastim." *Journal of pharmaceutical and biomedical analysis* 141 (2017): 229-233.

[6] Aubin, Yves, Geneviève Gingras, and Simon Sauvé. "Assessment of the three-dimensional structure of recombinant protein therapeutics by NMR fingerprinting: demonstration on recombinant human granulocyte macrophage-colony stimulation factor." *Analytical chemistry* 80 (2008): 2623.

[7] Arbogast, Luke W., Robert G. Brinson, and John P. Marino. "Mapping monoclonal antibody structure by 2D ^{13}C NMR at natural abundance." *Analytical chemistry* 87 (2015): 3556.

[8] Hodgson, Derek J., Houman Ghasriani, and Yves Aubin. "Assessment of the higher order structure of Humira®, Remicade®, Avastin®, Rituxan®, Herceptin®, and Enbrel® by 2D-NMR fingerprinting." *Journal of pharmaceutical and biomedical analysis* 163 (2019): 144.

[9] Arbogast, Luke W., et al. "2D ^{1}H, ^{15}N correlated NMR methods at natural abundance for obtaining structural maps and statistical comparability of monoclonal antibodies." *Pharmaceutical research* 33 (2016): 462.

[10] Amezcua, Carlos A., and Christina M. Szabo. "Assessment of higher order structure comparability in therapeutic proteins using nuclear magnetic resonance spectroscopy." *Journal of pharmaceutical sciences* 102 (2013): 1724-1733.

[11] Singh, Surinder M., et al. "Effect of Polysorbate 20 and Polysorbate 80 on the higher-order structure of a monoclonal antibody and its fab and fc fragments probed using 2D nuclear magnetic resonance spectroscopy." *Journal of pharmaceutical sciences* 106 (2017): 3486.

[12] Brinson, Robert G., et al. "Enabling adoption of 2D-NMR for the higher order structure assessment of monoclonal antibody therapeutics." *mAbs*. Vol. 11. No. 1. Taylor & Francis, 2019.

[13] Poppe, Leszek, et al. "Profiling formulated monoclonal antibodies by ^{1}H NMR spectroscopy." *Analytical chemistry* 85.20 (2013): 9623.

[14] Poppe, Leszek, et al. "On the analytical superiority of 1D NMR for fingerprinting the higher order structure of protein therapeutics compared to multidimensional NMR methods." *Analytical chemistry* 87 (2015): 5539-5545.

[15] Franks, Joshua, et al. "Spin diffusion editing for structural fingerprints of therapeutic antibodies." *Analytical chemistry* 88 (2015): 1320.

[16] Ying, Jinfa, et al. "Sparse multidimensional iterative lineshape-enhanced (SMILE) reconstruction of both non-uniformly sampled and conventional NMR data." *Journal of biomolecular* NMR 68.2 (2017): 101-118.

[17] Arbogast, Luke W., et al. "Selective suppression of excipient signals in 2D ^{1}H–^{13}C methyl spectra of biopharmaceutical products." *Journal of biomolecular* NMR 72 (2018): 149

[18] ICH Q8 (R2) – *Pharmaceutical Development*, 2009.

[19] Williamson, Mike P. "Using chemical shift perturbation to characterise ligand binding." *Progress in nuclear magnetic resonance spectroscopy* 73 (2013): 1-16.

[20] Ghasriani, Houman, et al. "Precision and robustness of 2D-NMR for structure assessment of filgrastim biosimilars." *Nature biotechnology* 34.2 (2016): 139.

Bruker BioSpin

info@bruker.com
www.bruker.com

© Bruker BioSpin 01/20 T178189