Table 6: The Application of NMR Spectroscopy towards the Analysis of Biologics

SCOPE:

NMR spectroscopy is one of the most powerful analytical methods for studying molecular structure, with three-dimensional structure determination of both small molecules and proteins in solution possible using NMR spectroscopy. The only other analytical method capable of exploring three-dimensional molecule structure at an atomistic level is x-ray crystallography, however this does not provide information regarding how the molecule behaves in solution. NMR spectroscopy was initially used in the analysis of small molecules, however improvements in NMR hardware, most notably the development of cryoprobes, and access to high field spectrometers >600 MHz, the analysis of proteins by NMR is increasingly popular due to the atomistic information that can be derived from NMR.

One of the major challenges when studying proteins by NMR is the low natural abundance and therefore sensitivity of both ^{13}C and ^{15}N, the NMR active nuclei of N and C. The high prevalence of N and C in proteins requires the acquisition of information regarding these nuclei, if a *de novo* assignment of the protein structure is to be achieved. At natural abundance, the low sensitivity of these nuclei makes acquisition of information very challenging, and thus isotopically labelled proteins are typically required. However, a *de novo* assignment of a protein using NMR is not always the aim and NMR can be used to study unlabelled proteins in many different ways. For example, NMR has recently been shown to be a valuable method for the analysis of protein HOS. This is very important in the development of biosimilars, where HOS is a CQA. Using NMR spectroscopy, the HOS of unlabelled mAbs, with molecular weights >150 kDa can be analysed and compared across biosimilars using methods such as PCA to determine spectral variance. A major benefit of using NMR is that little to no sample preparation is required, and molecules can be analysed in their formulation buffers.

Another limitation in the analysis of proteins by NMR is their slow molecular tumbling in solution. The slower a molecule tumbles in solution, the faster the NMR signal relaxes which leads to broad peaks in the spectrum. However, the development of TROSY based methods can override this issue, by selectively observing only the slowest relaxing component of the net magnetisation. This yields sharp peaks and allows for the study of increasing molecular weights. A second method has also recently emerged to overcome the challenges associated with the slow molecular tumbling of proteins. The chemical shifts of the methyl groups on Thr, Ala, Val, Ile, Leu and Met residues are typically very sensitive to protein structure and their fast molecular motion, compared to that of the protein, yields sharp signals in for example a 2D ^{1}H-^{13}C HSQC spectrum. An advantage of this method over traditional TROSY based methods is that it is not field dependent, whereas TROSY methods are more effective at higher magnetic fields.

Despite some of these challenges associated with the acquisition of NMR spectra on proteins, NMR has been used to extract a highly diverse range of information from proteins. For example, NMR can be used to qualitatively and quantitatively study protein-ligand and protein-protein interactions. DOSY experiments resolve different molecules based on their diffusion coefficients and can thus be used to determine the size of a protein, aggregation or the size of the solvation shell. NOESY experiments can deduce conformational information about protein tertiary structure by correlating protons close in space. NMR can be used to study reaction kinetics, such as enzymatic processes. Finally, although this is not an exhaustive list, NMR can be used to study the dynamics of protein folding and degradation. The possible applications of NMR towards the analysis of protein structure and function is continually growing and will likely continue to grow due to the unparalleled information that can be obtained using NMR spectroscopy.

QUESTIONS FOR DISCUSSION:

1. Is NMR a strongly accepted analytical method by regulatory agencies for analysing biologics? If not, why might this be and how could this be addressed?
2. What is the cost of the NMR hardware required to study biologics (e.g. high field spectrometers >600 MHz and cryoprobes etc.).
3. Could solid state NMR help in the analysis of insoluble or crystalline proteins or insoluble aggregates?
4. Could benchtop NMR assist in the analysis of biologics? This has the obvious advantage of being much more cost and time effective and does not require specialised knowledge to operate. However the low magnetic fields makes the analysis of biologics more challenging. However, are there any questions in the analysis of biologics that do not require the high sensitivity afforded by a high field spectrometer and thus benchtop NMR could be used instead?
5. Is NMR widely used in the analysis of biologics in industry? If not, why might this be?
6. What are the most common questions in the analysis of biologics that NMR could be used to answer? Could a 'high-throughput' method be developed to answer these questions? For example, the confirmation of protein HOS is routinely assessed in the analysis of biologics. Could NMR be used as a routine analysis of protein HOS in a 'high-throughput' fashion without the need for highly specialised knowledge in the data acquisition and analysis (i.e. could an analytical scientist be trained to run these experiments and not require an NMR spectroscopist)
7. Will specialised knowledge always be needed to operate and maintain the NMR spectrometers and analyse the data?
8. What are the advantages and disadvantages of using NMR over other analytical techniques in the analysis of biologics?

DISCUSSION NOTES:

The discussion was made with participants from industry and authorities as well as device manufactures. All participants agreed that NMR methods offer valuable information for the characterization of biologics and it is a powerful tool especially for comparability of biologics. One 2D experiment covers information about the primary, secondary and tertiary structure as well as the linkage of disulfide bonds and glycans. And even a 1D experiment is sufficient to compare the fingerprint of different batches. A 600MHz NMR would be enough and one participant was sure that the operation costs would be comparable to that of NMR and MS devices, especially in relation to the information output. The analysis has to be done with the natural abundant nuclei.

The round table discussion was mainly about the following topics: What are the reasons for the current low usage of NMR for biologics and the low abundance of NMR results in submissions. What is needed to see more NMR in submission for biologics and are the currently used methods for comparability of biologics beside NMR still state of the art. NMR requires experts, expensive devices and huge maintenance costs, but all agreed this is not the main reason for the low abundance of NMR in submissions. Contractor labs might be a possibility to reduce the costs and risk of NMR analysis. One further explanation could be the limited knowledge how NMR works and what the results tell us. This was stated for industry and authorities. It was asked for more pharma related NMR application notes and publications. It was also recommended to simplify the language which is used in the NMR community. This would make easier for non-NMR experts from pharma industry and authorities to understand the methods and the results.

A discussion was started if CD as well as far- and near-UV are still sufficient and if NMR would be additional or substituting. The outcome was not clear, but all agreed that the NMR results would comprise more detailed information. This gain in information includes a certain risk for industry. Changes that might be seen in NMR need to be explained and could endanger the drug approval. This thinking might be an explanation for the low appearance of NMR in submissions. It was hypothesized that the bar set from the authorities might be too low. Additionally, was discussed if NMR is ready for a QC environment and it was reported that the manufactures made large steps to facilitate this step. A

parallel was drawn between the development of MS in QC and that of NMR. All agreed that higher abundance of NMR in the pharma industry is possible but would require more time. It was assumed that benchtop devices for specific applications like aggregation monitoring would be a possible development for the near future and could pave the way for NMR.