Table 33: Use of NMR Fingerprinting in Late-Stage Biologics Development

Facilitator: William Matousek, *Regeneron Pharmaceuticals, Inc.*
Scribe: John Brogan, *Seagen Inc.*

Key Words:

NMR, HSQC, Heteronuclear

Scope:

Nuclear Magnetic Resonance (NMR) spectroscopy has long been applied in the pharmaceutical industry in small molecule development, and given the atomic resolving nature of the method, is considered a necessary tool for organic synthesis and material quality control. In academic circles, NMR has an equally long history of successful application in interrogation of solution state behavior, epitope mapping, and higher order structure determination in larger systems such as protein complexes and nucleic acid constructs. Recent advances in both NMR technology and pulse sequence architecture have made use of NMR in a biopharma setting more viable, both increasing the sensitivity of the NMR probes, thereby decreasing the requirement for isotopic enrichment, while also leveraging non-uniform sampling techniques to decrease the experimental acquisition times, enabling quasi-high throughput workflows. The resulting NMR fingerprint spectra of GMP materials can effectively be compared during late-stage extended characterization efforts, thereby demonstrating secondary/tertiary structural homology between lots and processes, or be used to inform on the risks associated with shifts in specific post-translational modification content.

Topics for Discussion include Emerging Technologies:

- 2D NMR
- Pulse NMR
- Recent journal articles have been publication comparing the two systems
- Discussion of NMR fingerprinting expanding to all applications of the technology

Discussion Notes:

- There is a general feeling of skepticism toward NMR and its use in protein characterization

Brief case study:

- Drug product agitation study where aggregation occurred in the presence of preservative excipients in a multi-dose vial designed to enable multiple withdrawals from the vial
- The preservative (m-cresol, an antibacterial agent frequently used in insulins) is known to cause aggregation
- Molecule under investigation (~40 kDa protein, not terribly large) was known to aggregate under agitation
    - Vigorous agitation was required to show aggregation
    - Study showed that aggregation was not caused by structural unfolding
    - NMR showed that aggregation occurred under low concentration of m-cresol
    - High concentrations of m-cresol were linked to aggregation
- 2D NMR allowed evaluation of completely opaque samples to show slight structural changes in areas where hydrophobic interactions were occurring
- NMR team worked with statistics group and commercial structural assignment software

- Working with NMR group, titrations of different preservatives enables NMR assignment that worked better than traditional techniques
- Work was not included in the CTD, but presented to the HA via an RFI
- Previous criticisms: NMR is sensitive, but what is its application?
- Molecule size has historically been a limiting factor in applicability of NMR technology

Use of NMR in release assays:

- 400 mHz NMR in QC laboratory for vaccine carbohydrate polysaccharide analysis
- What about conjugating material?
    - Polysaccharide data - characterization but not in a release specification
    - NMR not used in ADC release testing

Use of NMR for characterization in vaccine space

- It was heavily used in covid vaccine nRNA sequencing. Detailed were filed under 3.2.S.3.1

Application of NMR in biosimilar space and FDA perspective around openness/requirement of NMR use in large molecule characterization

- Recent entrance into biologic space and FDA acceptance of its use in the place of cell-based assays
- Question to Agency/Attendees: Can you leverage structure/function relationship assigned through NMR?

NMR use in ADC technology

- Start development of small molecule components
- Characterization of impurities, particularly conjugatable/nonconjugatable impuriities
- Negative example - if you've had an impurity in your original process, do you need it to be present in the new process?
    - Just because an impurity may have been present in the original process and now it has been identified through technology improvements - what approach should be taken or what requirements should be met?
    - NMR is powerful in compound identification, but not as useful in the determination of levels without standards for comparison
    - Threshold levels are important and regulators will be concerned

Biosimilars: If an NMR profile with minor impurities is the baseline from the innovator, what are the requirements for biosimilars?

- Some feel biosimilars have morphed into "bio-betters"
- Original impurity profiles will not always align with biosimilar impurity profiles, therefore the resource requirements to evaluate and identify all the impurities is extensive
- Justification that the biosimiliar impurity profile is not going to be any worse than the innovator
- Comparison: Sactuximab non-human cell line with HCPs

Comparability packages for proteins

- NMR has potential to be a better way of meeting comparability needs
- 2D method with non-uniform sampling
- Has it been picked up?
    - Yes, in biosimilars, particularly in the peptide space
    - NMR was specifically requested by agencies with peptides

- Health Canada presentation from Monday CMC Strategy forum included NMR data of oligosaccharides and importance of 3 on and 6 on and discussion of how FC effector function was linked
    - NMR data showed differences and the presenter discussed interactions
    - Structure-function relationship between glycan profile variants
- NMR use alongside MALS
    - NMR weakness is size - if you are above 150 kDa is really pushing the envelope of what is left soluble in solution
    - For polysaccharides, the repeating unit of 4 residues results in signal amplification. The complexity of the side chains becomes the limiting factor
- For mAbs, accelerated stability for highly stable molecules, it's not clear how NMR would provide more help
    - It's difficult to determine site-specific aggregation and that would enable mechanistic understanding of aggregation

Cryo EM does well with large molecules. Has anyone gotten experience with excipient interactions or small-molecule binding datasets?

- Yes to excipients, particularly interactions with peptides

What kinds of questions can we answer with NMR?

- Would NMR be a tool to investigate why some sources of polysorbate are more stable?
- NMR is a great tool for determining what else is in there?
- Is there another protein present?
- Is there bacterial matter in there?
- Highly effective at determining if other small molecules are present
- Highly effective as a screening tool, particularly with automation
- Great example is determination of silicone oil in the DP. Question is at what level?
    - Proved the sensitivity of NMR
    - Halted the product to determine what the signal was - determined that it was in the placebo as well
- NMR is effective at a range of molecule sizes
- For epitope mapping, the size range is less limited by size and more linked to what level of isotope mapping
- HDx mass spec is often proposed in place of NMR

Use of NMR in large proteins in large molecules

- Do you only see the surface residues or can you get into the core of the molecule
    - You can do H-D exchange in the protein by growing the protein in the presence of D
    - Unfolded proteins enable determination of full protein readout
- The technology relies on proving the negative and its difficult to prove that in a simple way
- Presenting complex concepts in quick "three sentence" groups and distilling the concept down to the simplest manner is difficult
- Do to the limitations of NMR, it presents a strong tool in the toolbox that shouldn't be ignored and should be considered in the context of data from other analytical techniques

New direction - application of NMR for prediction?

- Could NMR be used in that area moving forward?
- Effective use of alpha-fold and shift-X to predict structure and chemical shift