Application of 1D and 2D NMR to HOS characterization studies: how to make NMR a routine technique

Fabio Baroni, PhD

Physico-chemical Characterization Lab. – Protein Chemistry Department
Pharmaceutical & Analytical Development Biotech Products
EMDSerono –Guidonia Site, Italy

April 8th -10th, 2019 - HOS 2019, San Mateo (CA)
Outline

1. About us
2. Why NMR in a physico-chemical characterization package
3. Case studies
4. Statistical tools
5. Key messages
6. Acknowledgements
Prescription medicines for the treatment of cancer, multiple sclerosis and infertility, over-the-counter pharmaceuticals for everyday health protection or to provide fast relief for colds and pain, as well as innovations in the allergy areas.

Innovative tools and laboratory supplies for the life science industry that makes research and biotech production easier, faster and safer for patient health.

A wide range of specific chemicals, such as liquid crystals for displays, effect pigments for coatings and cosmetics, or high-tech materials within the electronics industry.
Merck-Group worldwide – 158 locations in 66 countries

Guidonia Site
Merck Serono
Pharmaceutical & Analytical Development
Biotech Products
1

About us

Merck Serono Pharmaceutical & Analytical Development Biotech Products
Protein Chemistry Department – Guidonia Site

Application of 1D and 2D NMR to HOS characterization studies: how to make NMR a routine technique Fabio Baroni /April 8-10 2019
Why NMR in a physico-chemical characterization package

- It offers the highest resolution among techniques for Higher Order Structure characterization (information at atomic level)

- Offers a fingerprint-like similarity approach (FDA Guidance, Dec 2016)

- In the near future, NMR will be included in the characterization packages required by regulatory agencies.

The NIST coordinated an interlaboratory project (24 labs involved, worldwide) aimed to establish a harmonized, routine 2D NMR analytical workflow for HOS characterization of mAbs. Data highlighted for both high precision and high reproducibility of the technique.

Brinson et al. mAbs 2018, DOI 10.1080/19420862.2018.1544454

Application of 1D and 2D NMR to HOS characterization studies: how to make NMR a routine technique Fabio Baroni /April 8-10 2019
Case studies

- Finding a viable NMR setup to work with our molecules at isotopes’ natural abundance (the challenge is 2D NMR!);
- Evaluation of the resolution of the technique: what can we see and what is its added value

1. **Nanobody**
   - Small protein in NMR-friendly buffer – no excipients
     - (40 KDa)

2. **Recombinant mAb**
   - mAb in excipients-rich buffer
     - (144 KDa)

3. **Modified recombinant mAb**
   - Modified mAb in excipients-rich (very rich) buffer
     - (177 KDa)

**Increasing complexity**
Case studies

**NIST Setup** (all experiments have been performed on intact molecules)

- @ 600MHz or 700MHz with TCI Cryoprobe and NUS.
- 250 µL of sample ~ 40 mg/mL
- Experiments at 37°C

**Experiment**
1D $^1$H
2D $^1$H-$^{13}$C HSQC (methyl region)*

**Signals observed**
- every proton of the protein (very high overlap)
- every methyl group of the protein

---


---

Application of 1D and 2D NMR to HOS characterization studies: how to make NMR a routine technique Fabio Baroni /April 8-10 2019
Case study 1: Nanobody - 1D and 2D Spectra (@ 600 MHz)

1D $^1$H Spectrum

2D $^1$H-$^13$C HSQC - Methyl region

Signals from every proton of the molecule

Signals from every methyl of the molecule

High resolution for a comparability exercise (1D&2d)
Case study 1: Nanobody - 1D and 2D Spectra (@ 600 MHz)

What we can see...

1D and 2D confirmed what observed by our previous comparability (influence of thawing on HOS) but provided information on batch-to-batch variability that cannot be seen by other techniques previously applied (fluorescence, NanoDSC, CD).
Case study 2: Recombinant mAb - 1D and 2D Spectra (@700 MHz)

**1D $^1$H Spectrum**

Signals from *every proton* of the molecule

**2D $^1$H–$^{13}$C HSQC - Methyl region**

Signals from *every methyl* of the molecule

**GOOD resolution FOR A COMPARABILITY EXERCISE (1D&2d)**

Application of 1D and 2D NMR to HOS characterization studies: how to make NMR a routine technique Fabio Baroni /April 8-10 2019
Case study 2: Recombinant mAb - 1D and 2D Spectra (@700 MHz)

What we can see...

1D and 2D spectra highlighted differences in the tertiary structure between Batch Y and Batch Y oxidized. These differences were not detected by the characterization panel applied to investigate the molecule’s variants as the HOS perturbations induced by oxidation are too small to be detected by our current routine techniques.
Case study 3: Modified mAb - 1D and 2D Spectra (@ 600 MHz)

1D $^1$H Spectrum

2D $^1$H-$^{13}$C HSQC - Methyl region

Signals from every proton of the molecule

 Signals from every methyl of the molecule

resolution FOR A

COMPARABILITY EXERCISE

ONLY IN 1D
These peaks become larger and shift with the addition of Cu$^{2+}$.

Upon titration of the modified mAb with copper (0.5, 1, 2 ppm), differences were observed in the NH/aromatic region of the protein, in the 1D spectra, even with the addition of 0.5 ppm of Cu$^{2+}$.

What we can see...

Which is the minimum level of Cu$^{2+}$ that induces a detectable modification of the protein structure?

NMR is the technique presenting the lowest limit of detection of structural modifications upon copper addition, compared to previously investigated techniques (Far-UV CD LOD: 5.5 ppm; Near-UV CD LOD: 4 ppm).
The importance of statistical tool

Since NMR possess such great resolution and sensitivity to structural changes it is mandatory the use of robust statistical tools. Are these tools available?

1D SPECTRA COMPARISON: Bruker’s AssureNMR™-Profile module

ProfileNMR confirms that the observed differences are statistically significant, starting from the addition of 0.5 ppm of copper.

L. Poppe et al., Anal. Chem. 2013, 85, 9623-9629
L. Poppe et al., Anal. Chem. 2015, 87, 5539-5545
Since NMR possess such great resolution and sensitivity to structural changes it is mandatory the use of robust statistical tools. Are these tools available?

**2D SPECTRA COMPARISON: Bruker’s in-development software**

*ECHO Method*

This value is an expression of correlation between spectra.

- The lower the worst (in this example MS2 vs MS3)

*CCSD Method*

This value is an indication of variability between spectra (calculated on all peaks).

- The higher the worst

**MS1**: Reference batch

**MS2**: batch thawed +25°C

**MS3**: batch thawed + 5°C


Further improvements to employ effectively NMR in R&D routines

- Improving excipient’s signal suppression in 2D spectroscopy
- Optimization of the NIST method on intact mAb, to reduce costs of analysis
- Definition of a standard to be used as system suitability sample

Key messages

- It was possible to obtain 1D 1H and 2D 13C NMR spectra of all the proteins tested (40, 144, 177 Kda respectively) with resolution adequate for comparability exercises. A magnetic field of at least 700 MHz is suggested, especially for mAb.
- Complex buffers lead to diminished resolution. The problem at present is well address in 1D spectroscopy where simplified and complete buffer can be used. In \(^{13}\text{C}\) 2D spectroscopy simplified buffers work better.
- Due to the extremely high resolution and sensitivity, a statistical approach is mandatory to correctly interpret the NMR data: Bruker Biospin’s AssureNMR software package provides robust and well-performing tools for such interpretation: PROFILE (1D spectra) and an in-development software for 2D spectra.
- 1D and 2D NMR can be applied in routine R&D studies. Not only does the technique possess sensitivity and resolution not comparable to that of other techniques currently employed in HOS characterization, but it also offers unique information, especially in terms of batch-to-batch variability.
Acknowledgements

**MS-Guidonia Site**
PCH Lab

Valter Altarocca  
Daniela Bonetti  
Cristian Ferrao  
Ludovica Lanzoni  
Francesca Militano  
Aniello Regine  
Paola Ringhieri

**MS-Darmstadt Site**

Martin Vogtherr  
Cristofer Saal

**Special thanks to:**

Alessandra Pistacchio  
(PhD student)

Mara Rossi  
(Protein Chemistry Department Senior Manager)

Roberta Verani  
(Physico-chemical Characterisation Lab. Manager Ad Interim)

Luisa Iozzino  
(Physico-chemical Characterisation Lab. Manager)

Abhejeet Satwekar  
(Lead Innovation scientist)

Tobias Haas  
(Director of Pharmaceutical & Analytical Development – Biotech Products)

Tommaso Iodice  
(MS-G Site Director)

Dr. Francesca Benevelli  
Dr. Christian Fischer

Application of 1D and 2D NMR to HOS characterization studies: how to make NMR a routine technique Fabio Baroni /April 8-10 2019