Addressing the Challenge of Higher-Order Structure Assessment of Biologics with NMR

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National Institute of Standards & Technology (NIST)

Non-regulatory agency established in 1901 in the US Department of Commerce.

Mission: to promote US innovation and industrial competitiveness by advancing measurement science, standards & technology

Part of a world-wide network of National Metrology Institutes (NMIs)
NIST Program in Biomanufacturing Metrology

Measurement Science, Standards and Technology for:

✓ Protein Structure
  - Primary, Secondary and Higher-order structure
  - PTMs (glycosylation)

✓ Protein Stability, Aggregation & Particulates

✓ Measurement tools & standards for production cells

NISTmAb RM release - Spring, 2016; Glycan RM in development

Program Coordinator: Dr. Mike Tarlov michael.tarlov@nist.gov
Improved Measurement Science, Standards, Technologies is Key to Biologics Development

• Measurement science and standards can help:
  - Account for bias between different analytical methods or instruments
  - Determine variability of characterization and test methods and setting of product specifications
  - Determine test methods are in control
  - Assess performance of new analytical technologies

• Sound measurement science is also essential for QbD, product understanding, development & manufacturing

Biosimilars Challenge – can’t make a copy of something that is not adequately defined by measurement!
Higher-Order Structure (HOS) is a Distinguishing Feature of Protein Therapeutics

- Proper three-dimensional structure is required for function
- Aberrant three-dimensional structures (misfolding) can result in loss of efficacy or side effects
- Structure/dynamics is related to protein stability, viscosity and aggregation

Challenges

- PTMs: Biologics are glycosylated
- Exist as conformational ensembles
- Highly concentrated formulations

What measurements best define the system?

A number of physiochemical methods have been proposed for structure assessment:

(CD, FT-IR, HDX-MS, NMR, fluorescence, Raman, SEC/IEC/HIC)

- For low resolution techniques (UV, CD, fluorescence) difficulty comparing and combining data

- For high resolution techniques (NMR, HDX-MS) difficulty ranking data importance

- For mid resolution techniques (IR, Raman) both problems

✓ No single solution to the problem: Each method measures different aspects of structure – either directly and indirectly

✓ Need to define standards/SOPs to establish method accuracy and precision
NMR Spectroscopy provides High-Resolution and Atomistic Assignment of Signals

Protein Polypeptide Backbone

$^{1}{\text{HN-}}^{15}{\text{N}}$ Amide Correlation for each amino acid in a protein

Sequence specific assignment of Resonances with heteronuclear correlation methods and stable isotope labeling
Probes of Local Structure & Chemical Environment

Dr. Yves Aubin

Health Canada
Santé Canada
NMR Chemical Shifts are Sensitive to Structure

Cross peaks represent amides and can be assigned to specific amino acids – 2D patterns provide fingerprints.
Methods must be robust and applicable to formulated protein biologic drug products

Isotope Labeling ($^{15}$N-labeling) while cheap – is **NOT** an option

NMR data collected using isotopes at natural abundance

$^{15}$N = 0.37 %

$^{13}$C = 1.11%

Must be Sensitive: **NMR Cryoprobe Technology:**

S/N > 6,000:1 @ 600 MHz; > 10,000:1 @ 900 MHz
Example: Formulated NUFIL Safe™

**1D ¹H Spectrum**

Inset: Amide 'Fingerprint' Region

Residual Water

Acetate

Sorbitol

**2D ¹H, ¹⁵N- HSQC with Coherence Selection**

Excipients & Water Artifacts

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**Formulations**

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<th>Drug Name Company</th>
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2D $^1$H-$^{15}$N NMR ‘Fingerprints’ of Biologics

GM-CSF
Leucotropin

Dr. Yves Aubin
Inter-laboratory Comparability Study (FDA, NIST, Health Canada and MPA-Sweden)

Study on the comparability of NMR spectral 'Finger prints' obtained using 2D NMR

- **4 Sites in North America and Europe**
  - FDA; Health-Canada; MPA-Sweden; NIST

- **4 Fields**
  - 500, 600, 700 and 900 MHz

- **Different Instrument vintages**

- **2 Vendors**
  - Bruker Biospin, Varian/Agilent

*(Filgrastim; Neupogen®)*
Met-G-CSF (19 kDa) – used in cancer patents with neutropenia.

Visual overlay of $^1$H-$^{15}$N HSQC NMR spectra of $^{15}$N-labeled met-G-CSF ‘System Suitability’ Sample

Spectral Finger prints are remarkably consistent across all labs.
Comparability Assessment of the $^{1}H-^{15}N$ HSQC Spectra: CCSD = “combined chemical shift difference”

$\sqrt{0.5(\delta_{H}^2 + (\alpha \cdot \delta_{N})^2)}$

where $\delta_{H}$ and $\delta_{N}$ denote deviations from innovator product in $^{1}H$ and $^{15}N$ dimensions.

$\alpha = 0.1$

some references use $\alpha = 0.14$ (0.20 for Gly)
Small lab-to-lab variations, Health Canada shows temperature variation.
Reference = AVERAGE (FDA500, NIST900, NIST600, MPA600)

HC Data shows sensitivity of temperature offset on $^{15}$N-GCSF shifts

CCSD Analysis: Measurement Variation Observed for the $^{15}$N-GCSF ‘System Suitability’ Sample
Keys to Acquisition and Processing for Comparability

Spectral Resolution:
- Data acquired with comparable resolution calibrated to instrument
- Data processed using the same functions & parameters
- Cross-peaks picked with a common method

How well can peak positions be determined sets the precision of the spectral comparison

Signal to Noise
- Experiments are acquired across labs and platforms using comparable S/N in acquisition

Determines the threshold of detection and lower limit of peak detection.
Can 2D NMR be Applied to Monoclonal Antibody Drugs (150 kDa)?

• **General Perception of NMR Spectroscopy of Large Biomolecules**
  – Practical application to biomolecules < 30 kDa
  – For applications above 30 kDa, isotope labeling, deuteration is required

• **Structure Determination versus Fingerprinting Tool**
  – If desire a spectral map for comparability, the NMR spectral fingerprint may be an accessible option.
NIST mAb Standard as a Platform for Measurement Innovation and Benchmarking

NISTmAb:
- Humanized mAb (IgG1κ) expressed in murine culture
- Frozen bulk “Drug-like substance”
  - 100 mg/mL, ≥ 98% purity
  - 12.5 mM L-His, 12.5 mM L-His HCl (pH 6.0)

NIST plans release as a Reference Material (RM) in Spring, 2016
(contact: john.schiel@nist.gov)
mAbs Are Very Large on the NMR Scale

Theoretical $^{15}$N Linewidths at 900 MHz

- **metG-CSF** (19 kDa)
- **mAb** (150 kDa)
NISTmAb: Initial Attempt at 2D $^1$H-$^{15}$N NMR at Natural Isotopic Abundance

$^1$H$^{15}$N SOFAST-HMQC

~ 700 Signals Expected

Low Sensitivity (Multi-Day Experiment) and Resolution not sufficient.
The high thermal stability of mAbs allows for data acquisition at 50°C where $\tau_c$ is ~ 55% of that at 25°C.
Intact NISTmAb at 900 MHz
Concentration is Critical, 30 mg/mL

$^1$H-$^1$C Methyl HSQC spectrum at 50 °C

$^1$H-$^{15}$N Amide SOFAST-HMQC spectrum at 50 °C

Intact NISTmAb at 900 MHz
Concentration is Critical, 100 mg/mL

$^1$H-$^{13}$C Methyl HSQC spectrum at 50 °C

$^1$H-$^{15}$N Amide SOFAST-HMQC spectrum at 50 °C
mAb Architecture: Flexible Hinges Between Structured Domains

Propose a 2 Step Analysis: (1) 2D NMR Fingerprint of Fragments → (2) Intact mAb Structure Ensemble Determination
• In language of mass spectrometry, a “middle down approach”
• Use the protease Papain to effect cleavage at the hinge region
Biochemical Strategies to Overcome Molecular Weight Limitations

Fab and Fc domains can be prepared by facile enzymatic cleavage of mAbs
NISTmAb Fc and Fab Domain $^1$H-$^{15}$N Fingerprints

Fc Amide Region

198 peaks identified (81%)

Fab Amide Region

357 peaks identified (74%).

900 MHz spectrometer at 50° C, ~0.5 mM protein in 25 mM L-histidine ($d_3$), pH 6.0.

Total experimental time ~24 hrs using SOFAST or BEST pulsing techniques
Standard spectral fingerprint (HSQC) experiment would take ~ 127 hrs!

How Can We Correlate NMR Spectral Fingerprints?

Data Analysis:
• Visual Inspection

• Combined Chemical Shift Deviation

• Point-by-point comparison
  – Correlation plots

• If many spectra, a full multivariate analysis (PCA) can be done
Visually, the spectra look highly similar. Pearson linear correlation coefficient calculated between normalized datasets. Statistically highly similar.
While the Fc regions from the NIST and Sigma mAb sample spectra are highly similar, as expected the Fabs are highly dissimilar due to sequence variation.
Duplicate G-CSF $^1$H-$^{15}$N datasets collected with varying number of scans.

NISTmAb and Sigma Fab $^1$H-$^{13}$C datasets processed with varied indirect acquisitions.

- Maximum possible $R$ between similar samples dictated by experimental $S/N$.
- Minimum possible $R$ between different samples dictated by acquisition time (Resolution).

$$R = \frac{A[S/N - 1]}{1 + A[S/N - 1]}$$
Alternative HOS Fingerprints using Methyl $^1$H-$^{13}$C Correlation

Side Chain Methyl Groups

- $^{13}$C at natural abundance is more sensitive than $^{15}$N natural abundance: $^{15}$N = 0.37% versus $^{13}$C = 1.11%
- Methyl groups have intrinsically favorable relaxation
- Non–uniform sampling (NUS) of data can cut experimental time by a further 50 % for 2D data collection

Isoleucine

Valine

Leucine
NISTmAb: 2D $^1$H-$^{13}$C NMR Fingerprinting at Natural Isotopic Abundance

$^1$H-$^{13}$C SOFAST-HMQC

Using a cutoff of peak S/N ≥ 10:1, ~210 peaks of the 221 expected signals (95%) can be observed.

Methyl Fingerprints at Natural Abundance at 900 MHz
Non-Uniform versus Uniform Sampling

Blue = Standard
Red = 50% NUS

SOFAST/NUS Spectra in ~ 30 minutes (9x faster than standard experiment)

Wagner Lab (Harvard) NUS Protocols - Implemented using a Bruker AVANCE console
$^{13}$C-Methyl and $^{15}$N-Amide Datasets Yield Highly Similar Statistical Correlations

$R^2 = 0.926$
Do the Fab/Fc Domain Fingerprints Represent the Structure in the Intact mAb?

Are the 2D NMR Fingerprints of the Fragments Sufficient to define to a first order the HOS?
$^{1}H-^{13}C$ correlation spectra of intact NISTmAb and the Fab/Fc (2:1) NIST mAb fragments

While line-width/resolution is different, much of the map remains constant post digestion
2D NMR can be used for structure assessment: primary, secondary and HOS of protein biologics

2D NMR fingerprints for comparability assessment:
- Simple & Robust
- Lab to Lab Reproducibility
- Natural Abundance (Label-free technique)
- Tailored Correlations (Signal Filtering/Selection)
- Signal assignment to specific amino acids

2D NMR Fingerprinting of mAbs and mAb fragments is possible
- Allows the tracking of structure, including glycosylation
- Applicable at 600 MHz, the “workhorse” NMR spectrometer
- $^{13}$C Methyl maps can take less than one hour using NUS

Combining NMR with other methods like Small Angle Scattering could provide a full description of the HOS
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