Structural Mapping on the NIST Standard Monoclonal Antibody by 2D NMR Fingerprinting Methods

Robert G. Brinson, Ph.D.
April 13, 2015
• Non-regulatory agency within U.S. Department of Commerce

• Founded in 1901 as National Bureau of Standards

• NIST responsible for US physical standards, test methods, & calibrations

**Unique Mission within the Federal Government**

to promote U.S. innovation and industrial competitiveness by advancing
measurement science, standards, and technology

in ways that enhance economic security and improve our quality of life
Measurement Science, Standards and Technology for:

- **Protein Structure**
  - Primary, Secondary and Higher-order structure
  - PTMs (glycosylation)
- **Protein Stability, Aggregation & particulates**
- **Measurement tools & science for bioprocess development**

Future: **NISTmAb Standard Reference Material (SRM)**

Program Leader: Dr. Mike Tarlov [michael.tarlov@nist.gov](mailto:michael.tarlov@nist.gov)
NIST mAb Attributes:
• Humanized mAb (IgG1κ) expressed in murine suspension culture
• Frozen bulk “Drug-like substance”
  • 100 and 10 mg/mL, ≥ 98% purity
  • 12.5 mM L-His, 12.5 mM L-His HCl (pH 6.0)
Definitions:
• In-House Standard: Manufacturer-specific drug substance
• Reference Material: Issued under NIST trademark and established to be fit for intended use in measurement of nominal property values.
• Standard Reference Material: Issued under NIST trademark and assigned one or more specified property values with associated uncertainties and traceability.

Approach for IgG SRM:
• Complete rigorous interlaboratory characterization
  • Results used for book compilation
• Compile reference data, methods, etc.
  • End user accessibility to http://igg.nist.gov/
• Certify for concentration traceable to the kg
• SRM made available to biopharmaceutical community
Application of NMR to the Assessment of Higher Order Structure of Protein Therapeutics

Advantages of NMR:
- Atomic level assignment - Spectral frequency referencing
- Measurement/instrument reproducibility
- Can be Quantitative
- NMR can directly assess ‘as provided materials’

Challenges of NMR: Sensitivity & Resolution
- With new spectrometers the dynamic range has increased, so in principle states/impurities present even at the level of parts per billion can be detected.
NMR Chemical Shifts are Sensitive to Higher Order Structure

Filgrastim: Amide Fingerprints

Unfolded $^{15}$N metG-CSF $^{1}H^{N-15}N$ HSQC spectrum

Native $^{15}$N metG-CSF $^{1}H^{N-15}N$ HSQC spectrum

- 2D correlation spectra provide a unique fingerprint for discreet ensembles of folded proteins
Perception of NMR Spectroscopy of Large Biomolecules

• General perception:
  – NMR is only applicable to biomolecules ~30 kDa or less
  – For applications above 30 kDa, perdeuteration is required

• What is the purpose of the measurement?
  – If desire a high resolution structure, stable isotope labeling absolutely required (\(^2\text{H}, \, ^{13}\text{C}, \, ^{15}\text{N}\))
  – If desire a structural map for comparability, the NMR spectral fingerprint becomes very accessible at natural isotopic abundance (\(^{13}\text{C} \, 1.1\%; \, ^{15}\text{N} \, 0.37\%)
Intact NISTmAb at 900 MHz
Concentration is Critical, 100 mg/mL

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

$^1$H$^N$-$^{15}$N Amide SOFAST-HMQC spectrum at 50 °C
Intact NISTmAb at 900 MHz
Concentration is Critical, 30 mg/mL

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

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

What about lower fields? Intact NISTmAb at 600 MHz

Methyl Spectrum

- In mass spectrometry language, let’s try a “middle down approach”
- Let’s use a protease such as papain to effect cleavage at the hinge region

Goal: Minimal sample manipulation
Fab/Fc fragmentation can be accomplished by facile papain digest

- digest mAb on immobilized papain resin
- Protein A affinity chromatography
- centrifugation over 30 kDa and 100 kDa filters.
Methyl Fingerprint: Domain Cleavage does not alter HOS

Overlay of Methyl spectra of the full length NISTmAb (blue) and the Fab/Fc (2:1) digest product (red).
Amide Fingerprint

• All data was collected on a 900 MHz spectrometer at 50° C at a concentration of ~0.5 mM in 25 mM L-histidine (d3), pH 6.0.
• Total experimental time ~24 hrs using standard SOFAST or BEST pulsing techniques
• Performing these rapid pulse experiments do not require an expert operator

• A traditional spectral fingerprint (HSQC) experiment would take approximately 90 hrs!!!
Collection of Rapid Methyl $^{13}$C Fingerprints

- $^{13}$C at natural abundance is intrinsically more sensitive than $^{15}$N
  
  natural abundance: $^{15}$N = 0.37 % versus $^{13}$C = 1.11%

- Rapid pulsing is not commonly applied to $^{13}$C data collection but can greatly reduce the experimental time

- Non–uniform sampling (NUS) of data can cut experimental time by a further 50 % for 2D data collection
Non Uniform Sampling (NUS)

• In NUS experiments, only a subset of $^{15}$N or $^{13}$C ($t_1$) increments are recorded leading to reduction in experimental duration.

• Decreased sampling at long $t_1$ time points can lead to reductions in experimental noise.

But...

• Reduced sample schedule can lead to introduction in artifacts and imprecision in peak position!

• You must be able to collect a clean uniformly-sampled spectrum for NUS to be successful.

That is, you must have a good NMR sample

• Data is reconstructed using Iterative soft thresholding

Overlay: NUS versus Traditional Data

Blue = Traditional
Red = 50% NUS

gHSQC experimental time: 270 minutes
sfHMQC experimental time: 68 minutes
50% NUS-sfHMQC experimental time: 34 minutes!

How Do We Correlate Spectral Fingerprints?

• Chemical shift analysis
  – NMR Round Robin Study on Filgrastim

• Point-by-point comparison
  – Correlation plots

• If many spectra, a full multivariate analysis can be done
  – NMR Round Robin study on Filgrastim
Statistical Comparability of Drug Product Spectra

Easy Comparability of Higher Order Structure (ECHOS) by NMR was developed for facile objective comparison of protein therapeutic spectra.

Amezcua and Szabo J. Pharm Sci. 102 2013
**Fc Region: Total Correlation between NISTmAb and another IgG1κ poly Ab**

- Second IgG is from human myeloma plasma
- Visually, the spectra look very similar
- Performing an in-house assessment of the Fc region of this second IgG1κ

\[ R = 0.93 \]
• While the Fc regions from the NIST and Sigma mAb’s are highly similar, the Fabs are dissimilar
Concluding Thoughts

• NMR Fingerprinting of Fc and Fab regions is very practical
  – Allows the tracking of higher order structure, including glycosylation
  – This NMR Fingerprinting method is applicable at 600 MHz, the “workhorse” NMR spectrometer
• Data collection for $^{13}$C Methyl can take less than one hour using non-uniform sampling
• Non-uniform sampling can also be applied to amide spectral fingerprints.
Call for Participants: mAb NMR Interlaboratory Study

Goal: Establish standard measurement protocols for the comparability of higher order structure of mAb therapeutics to meet industrial and regulatory requirements

• The aims of the study are to:
  1) Demonstrate that NMR is suitable to assess HOS of the mAb molecule class
  2) Demonstrate comparability on a molecule of high interest to industry and regulators
     • A $^{15}$N-labeled system suitability Fab sample will be measured followed by the unlabeled drug-like NISTmAb Fab domain.
     • Results will be published in a notable peer-reviewed journal
     • The data from the larger study will be blinded by a third party vendor (yet to be determined) to avoid bias for or against any industrial partner.

Projected timeline: **Spring 2015, recruitment**
   Summer 2015, Completion of MTAs
   Fall 2015, Sample Distribution
   Winter 2015-2016, Data Collection

• If interested in participating, please contact Robert Brinson (brinson@ibbr.umd.edu)
Acknowledgements

**NIST-IBBR**
John Marino, Ph.D.
Luke Arbogast, Ph.D.

Zvi Kelman, Ph.D.
Todd Hoopes, Ph.D.

**NIST-Gaithersburg**
John Schiel, Ph.D.
Trina Formolo, Ph.D.

Support
NIST Biomanufacturing Initiative. NMR instrumentation supported by NIST, ARRA, the Keck Foundation and the NCRR/NIH.