Applications of NMR for assessing the higher order structure of protein therapeutics

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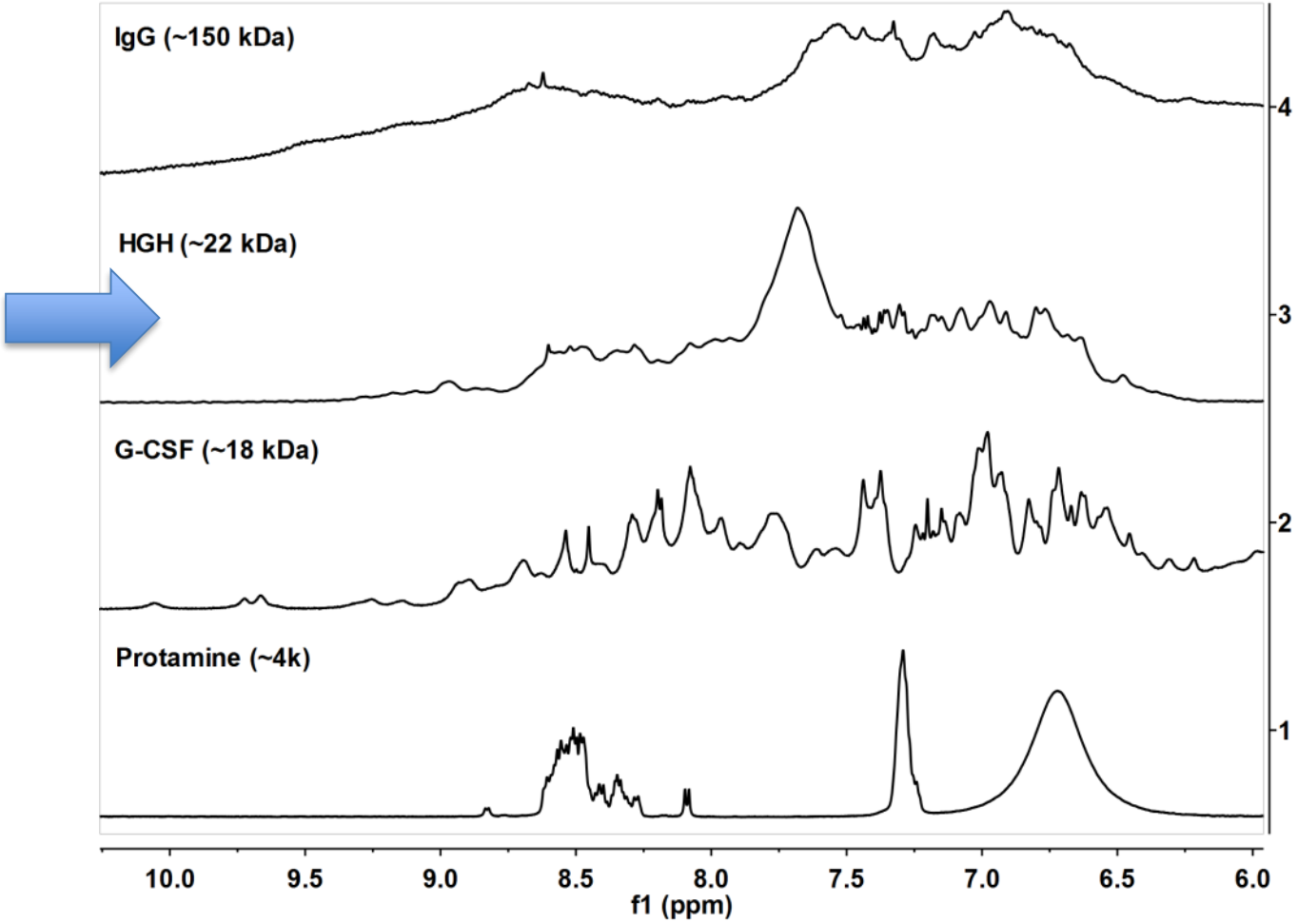
12/05/2016

NMR and the "state" of the protein

HGH vs. G-CSF
Similar size but
different linewidths



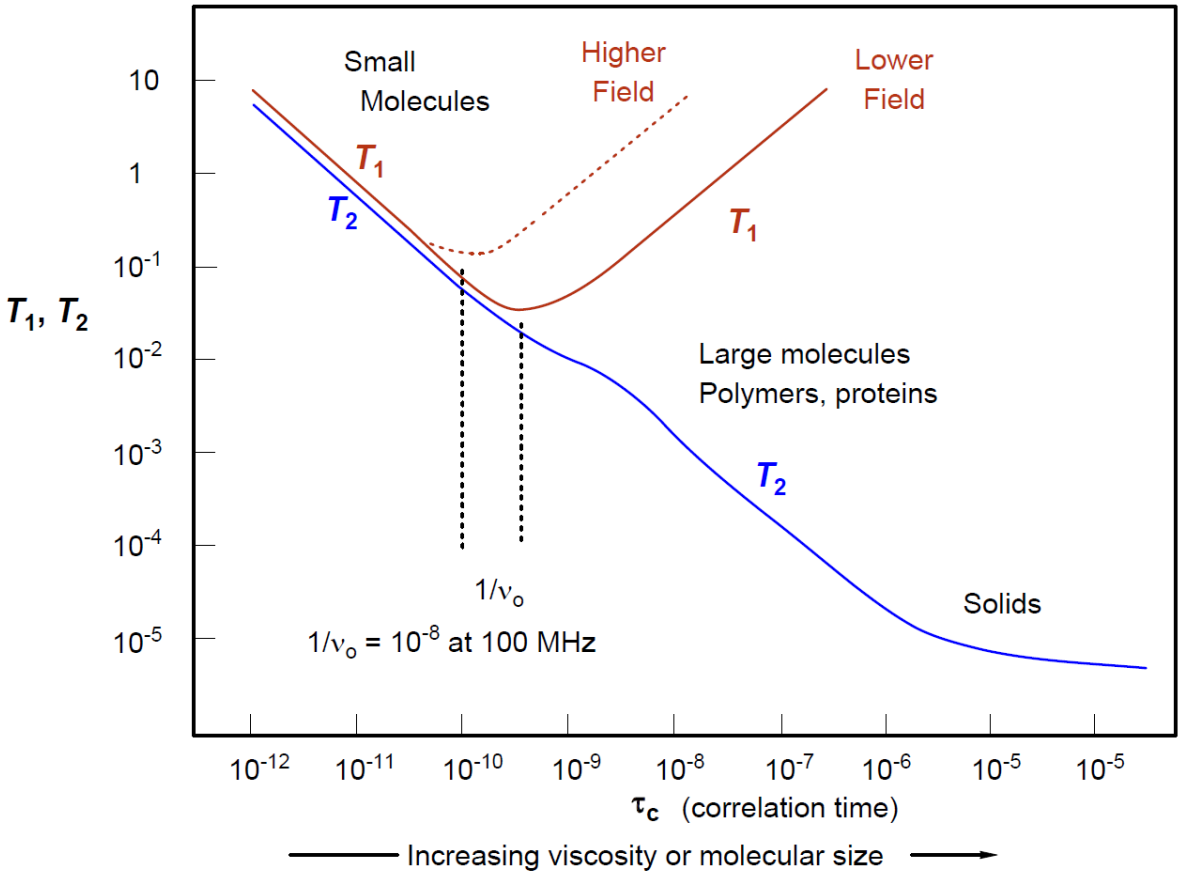
Aubin *et al.,* Chap 13 in Biophysical Characterization of Proteins in Developing Biopharmaceuticals, Berkowitz and Houde eds., Pages 341-383, 2015

HGH

- Self-associated at sub-millimolar concentrations
- Self-association increased with concentration, was reversible and exhibits fast exchange on the NMR timescale.
- Only weak, non-covalent interactions were observed with soluble dimer, trimer and tetramer aggregates present.
- No larger insoluble aggregates were observed.

Jensen *et al.,* Proteins, Vol 73(1), 161-172, 2008

Correlation time and relaxation



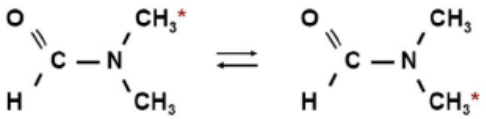
Reich, U.Wisc. Chem. 605

Things that give you fat lines

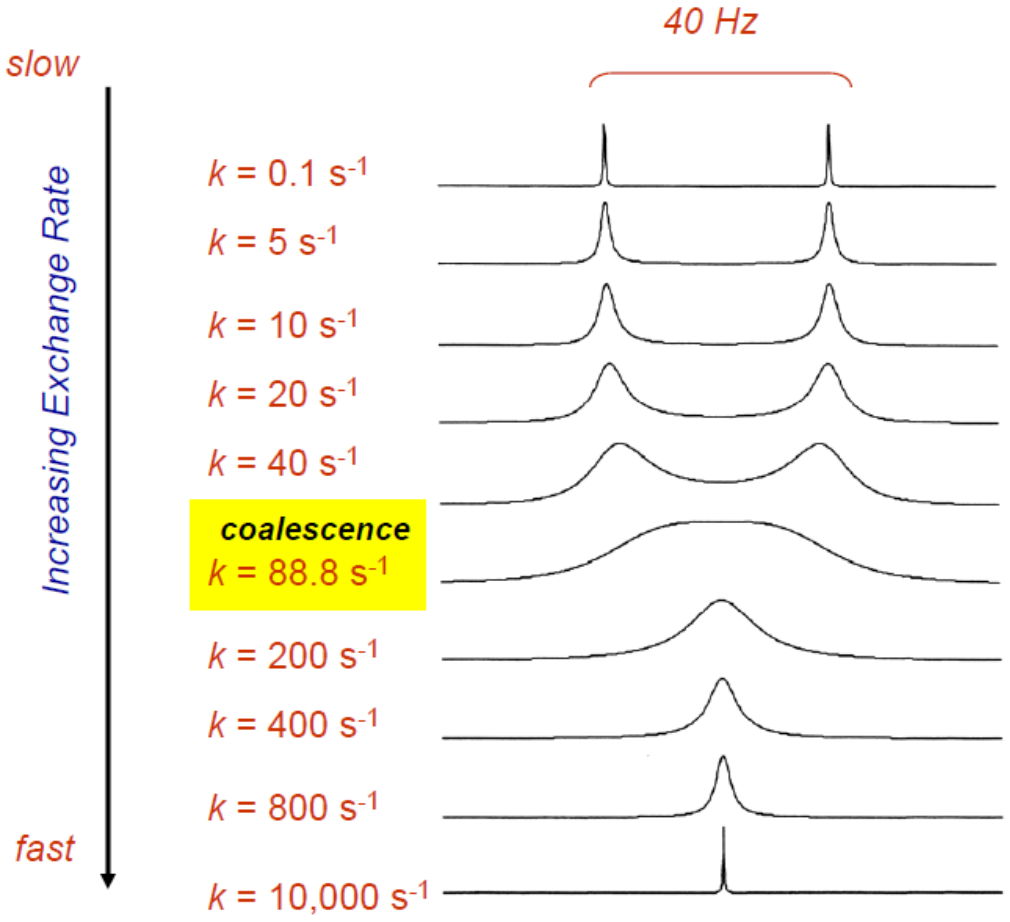
- Slow rotational tumbling
- Solution viscosity
- Chemical Exchange between states
  - Intra-molecular (e.g., cis-trans isomerism)
  - Inter-molecular (e.g., with bulk solvent and/or with aggregated forms)
- Paramagnetic ions
- Non-homogenous magnetic fields

Two-Site Exchange:
Rotation about a partial double bond in dimethylformamide

Equal Population of Exchange Sites



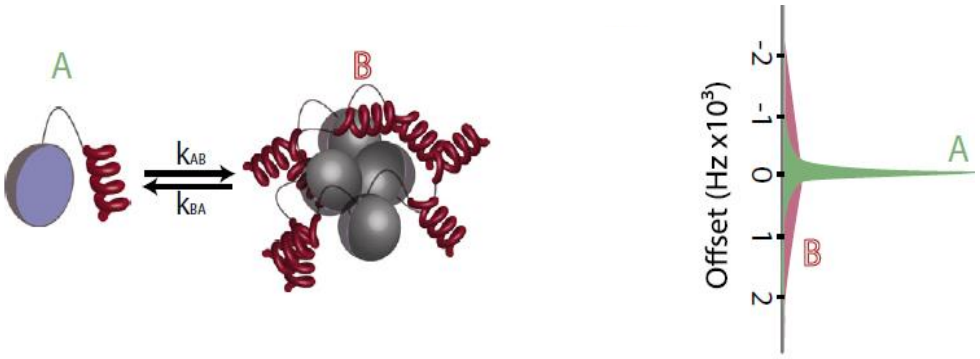
Where exchange occurs between species of unequal population the resulting spectra reflect the population weighted average of those states.



http://www.bioc.aecom.yu.edu/labs/girvlab/nmr/course/COURSE_2012/Lecture_ExchangeDynamics_2012.pdf

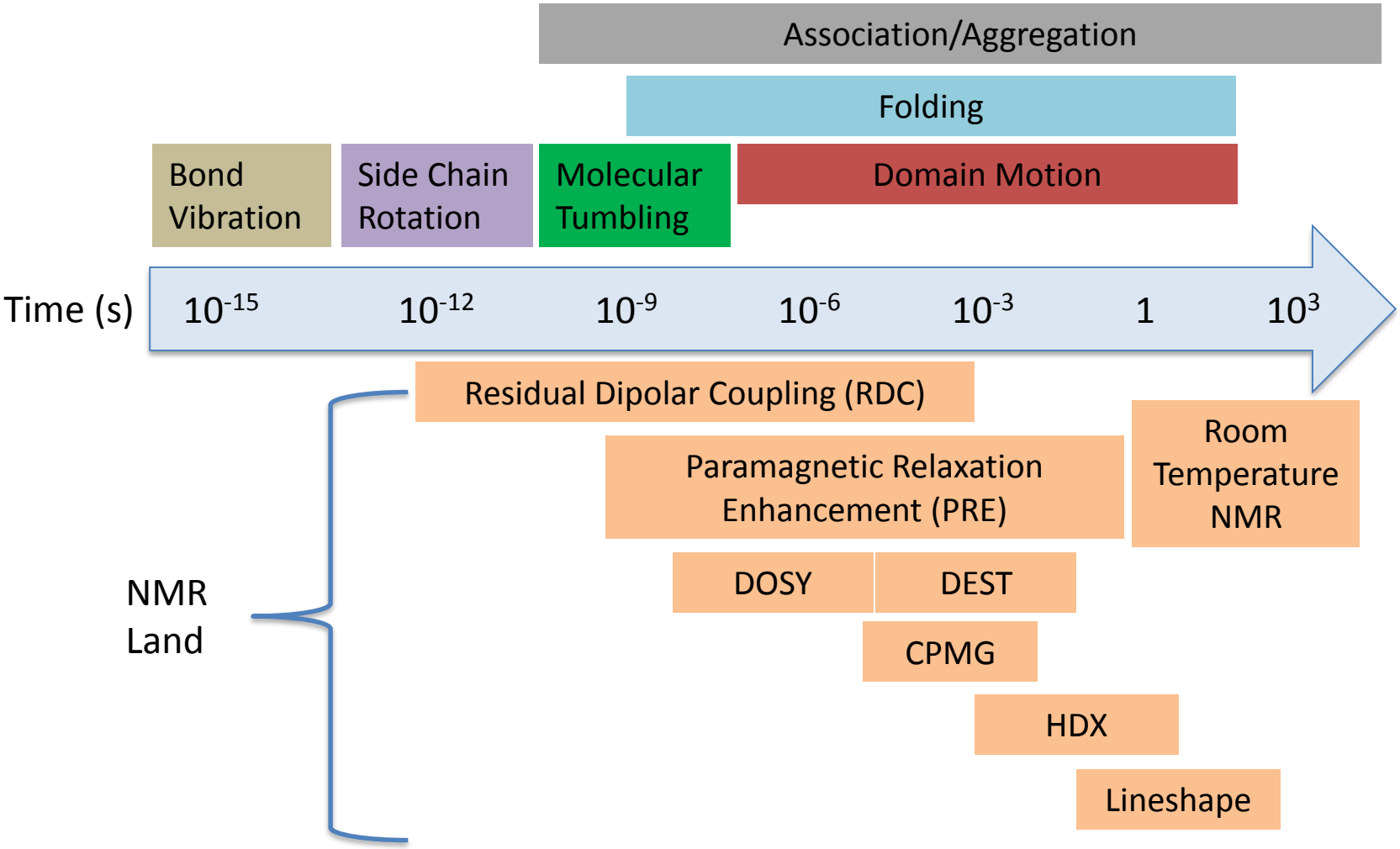
Chemical Exchange for Aggregates

- An “NMR invisible signal” (*e.g.,* a larger aggregated protein form) can impact the measurement of an NMR visible signal (monomer form).
- Probe by dark exchange saturation transfer (DEST) or paramagnetic relaxation experiments (PRE).



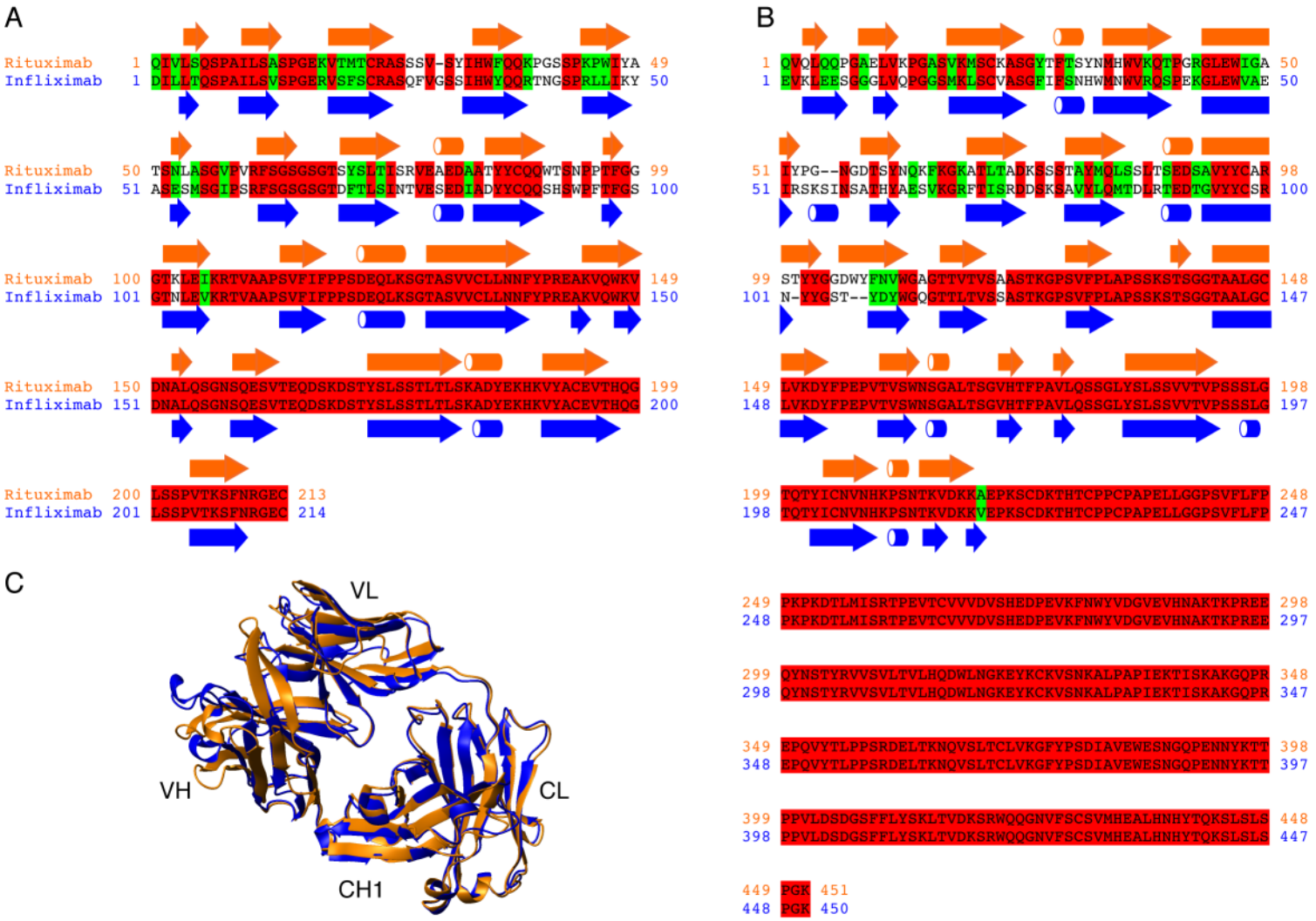
Work coming from the Clore group at NIH e.g., Anthis and Clore, Q. Rev. Biophys., 48(1), 35-116, 2015
Review for beta-amyloid studies Karamanos *et al.,* Prog NMR Spectr., Vol 88-89, 86-104, 2015

Protein Timescales



Rituximab vs Infliximab

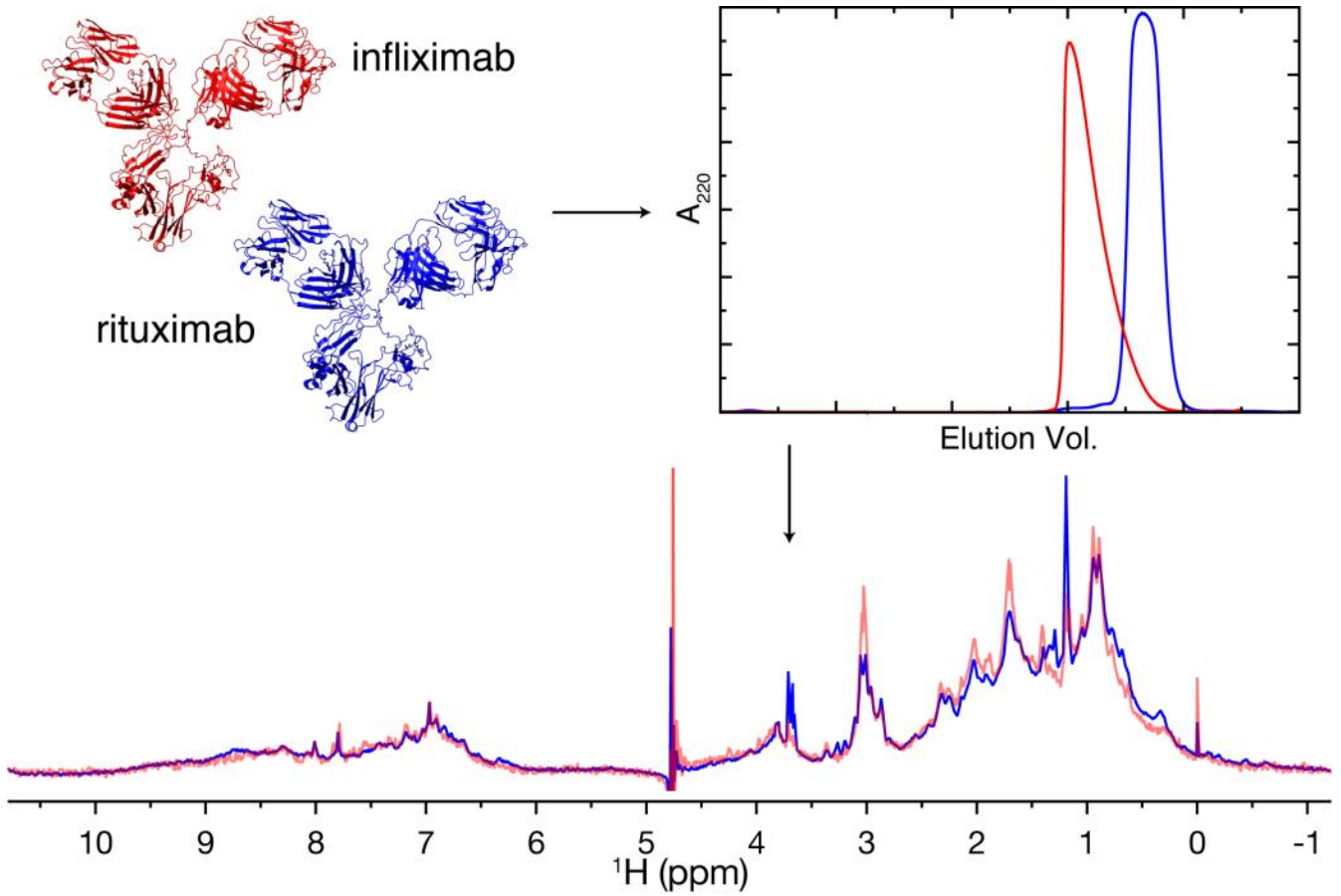
83% Identity
91% Similarity



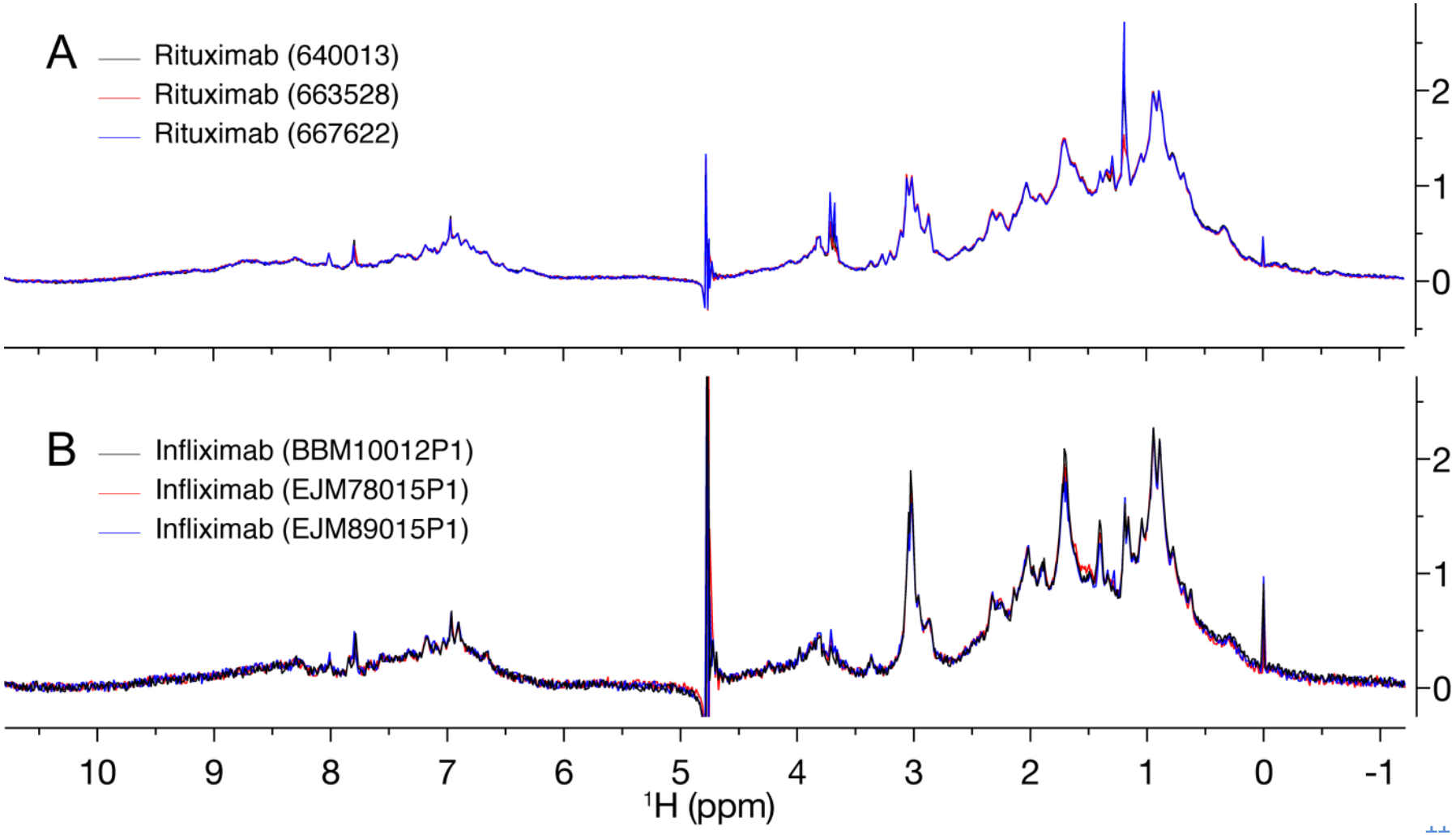
Fab RMSDs
BB 1.6 Å
SC 2.2 Å

Chen *et al.,* JPBA, 128, 398-407, 2016

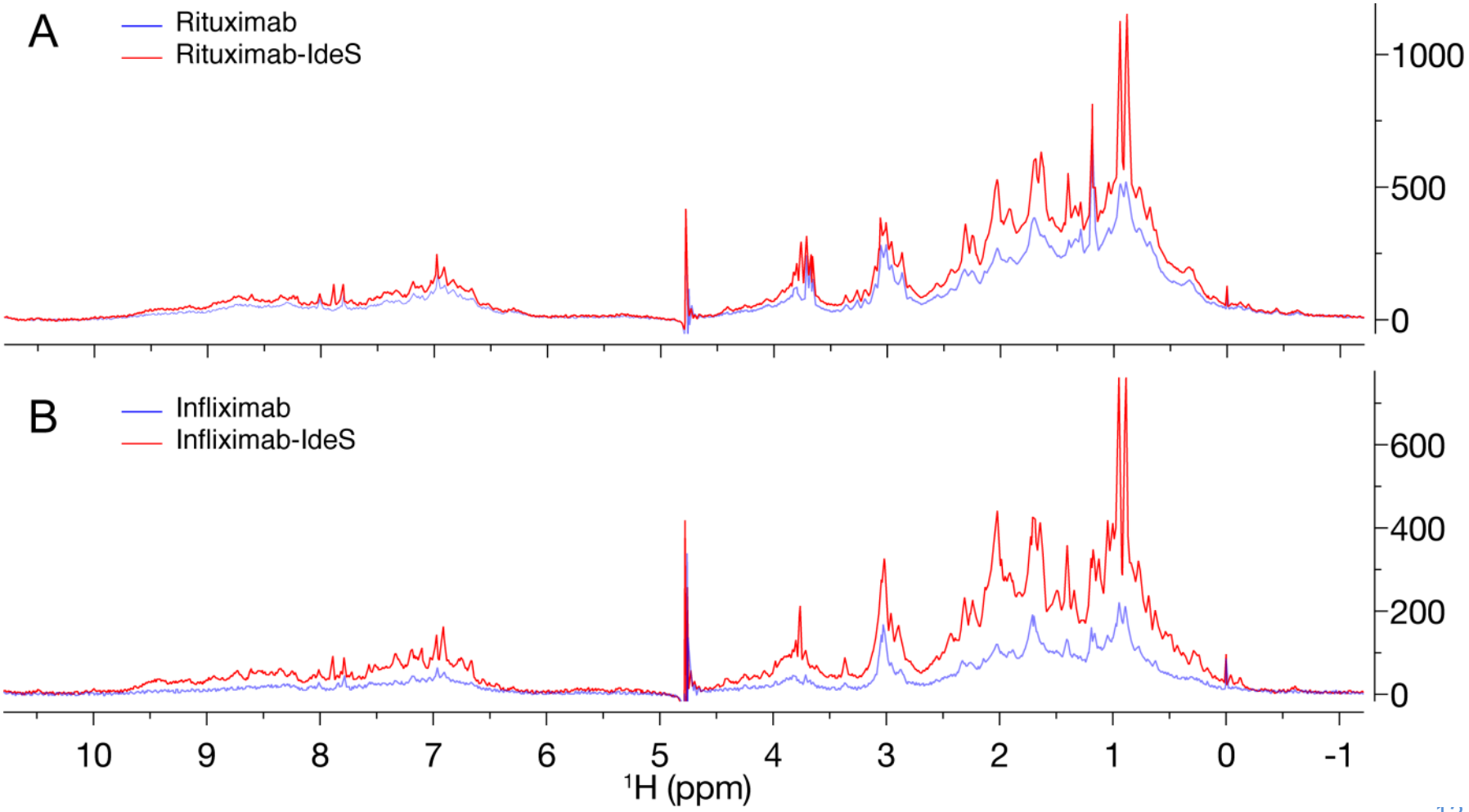
1D Proton NMR at 600 MHz



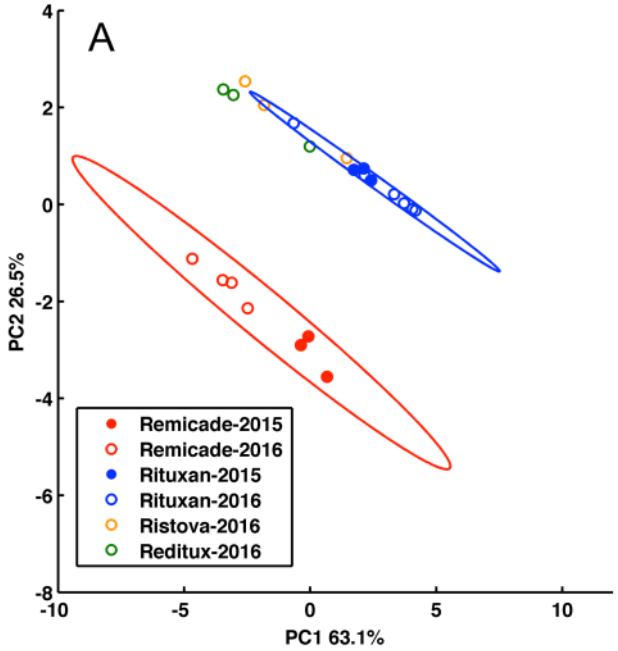
Reproducibility

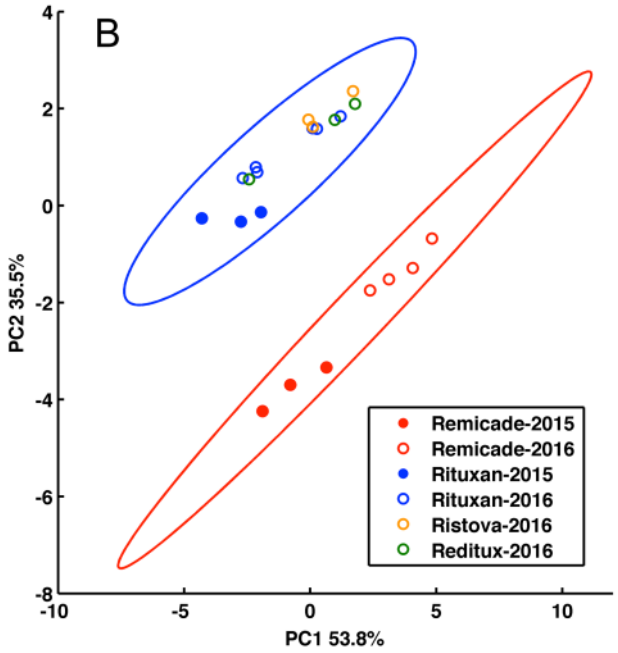


Size matters



PCA

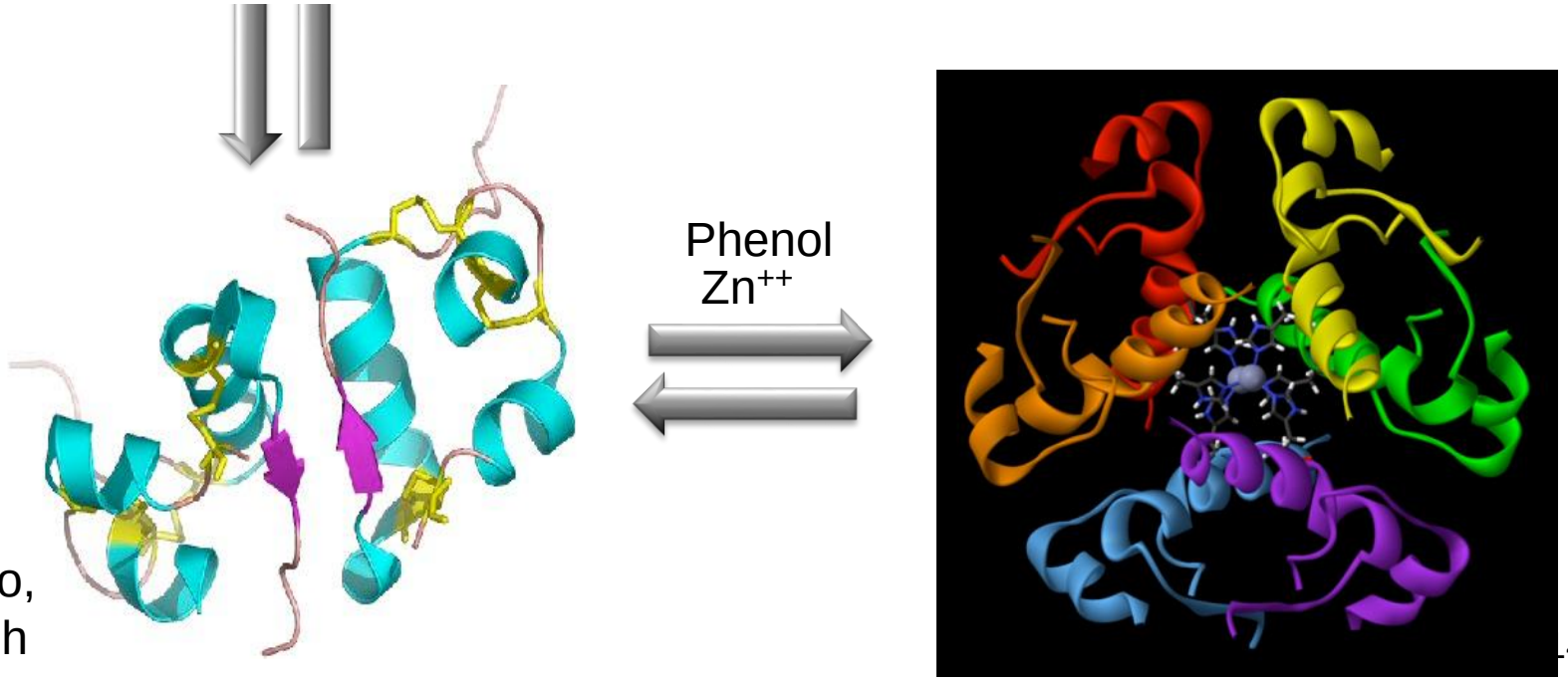

A
PC2 26.5%
PC1 63.1%
Remicade-2015
Remicade-2016
Rituxan-2015
Rituxan-2016
Ristova-2016
Reditux-2016


B
PC2 35.5%
PC1 53.8%
Remicade-2015
Remicade-2016
Rituxan-2015
Rituxan-2016
Ristova-2016
Reditux-2016

Insulin Structure and Equilibrium

Spectra peak position: sequence, 2º and 3º structure.

Peak line-width and intensity: 4º structure, concentration, equilibrium and kinetics.



Pdb:1mso, 1ev6, 1lph

Journal of Molecular Biology [2002, 318(2):479-490]

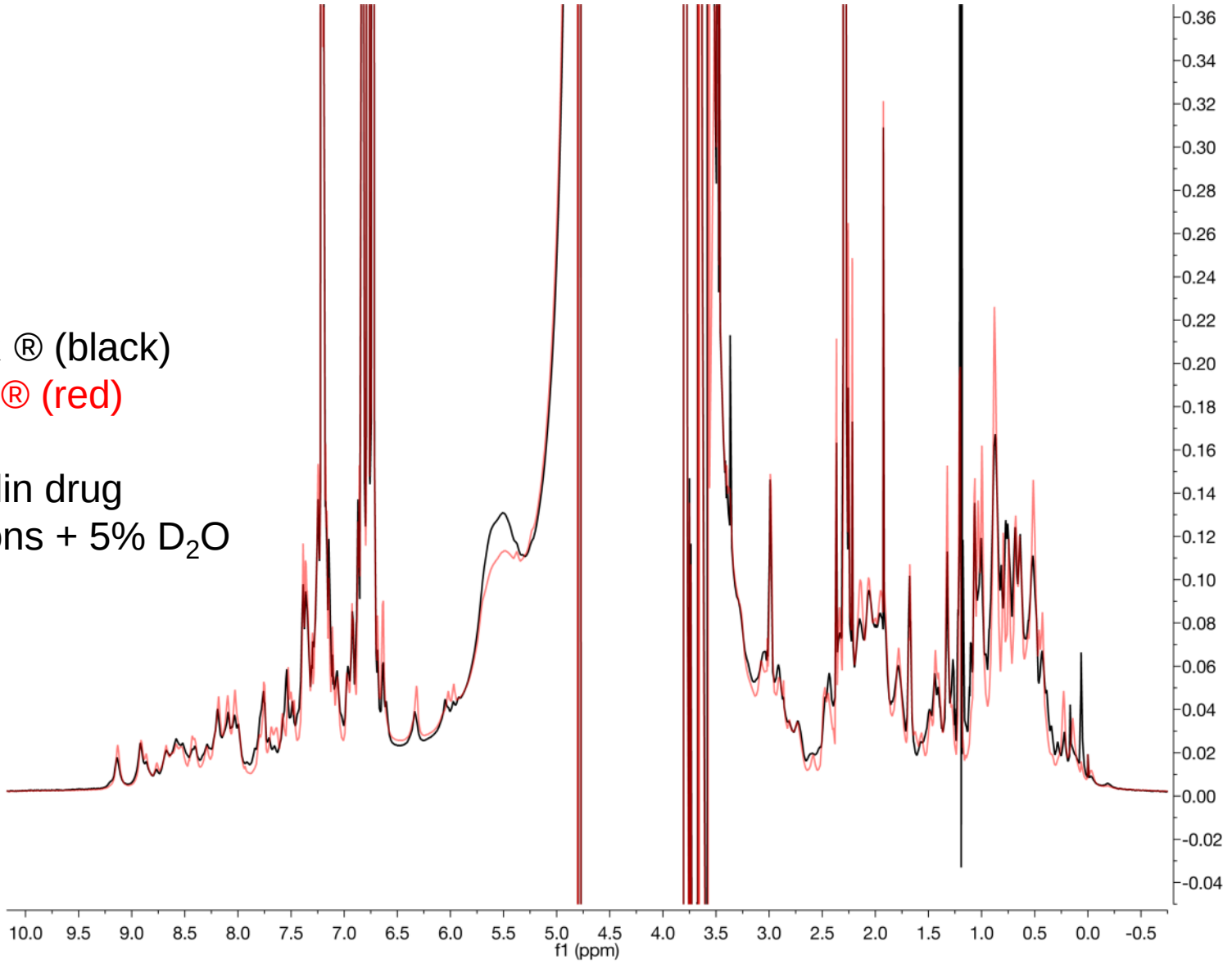
Rapid Insulins in US market

Drug Name	Pharma. Company	API Conc. (0.6 mM)	M.W. (Da)	Organic Excipients	Conc. (mM)
Novolin R	Novo Nordisk	Insulin Regular	5808	glycerol metacresol	173.7 27.74
Humulin R	Lilly	Insulin Regular	5808	glycerin metacresol	173.7 23.12
Apidra	Sanofi-Aventis	Glulisine B3: Asn->Lys B29: Lys->Glu	5823	Metacresol tromethamine polysorbate-20	29.13 49.52 8.15
Novolog	Novo Nordisk	Aspart B28: Pro->Asp	5826	glycerol Phenol metacresol	173.7 15.94 15.91
Humalog	Lilly	Lispro B28: Pro->Lys; B29: Lys->Pro.	5808	glycerin metacresol	173.7 29.13

1D ^{1}H spectra on insulin drug product

HumulinR ® (black)
NovolinR ® (red)

95% Insulin drug formulations + 5% D_2O



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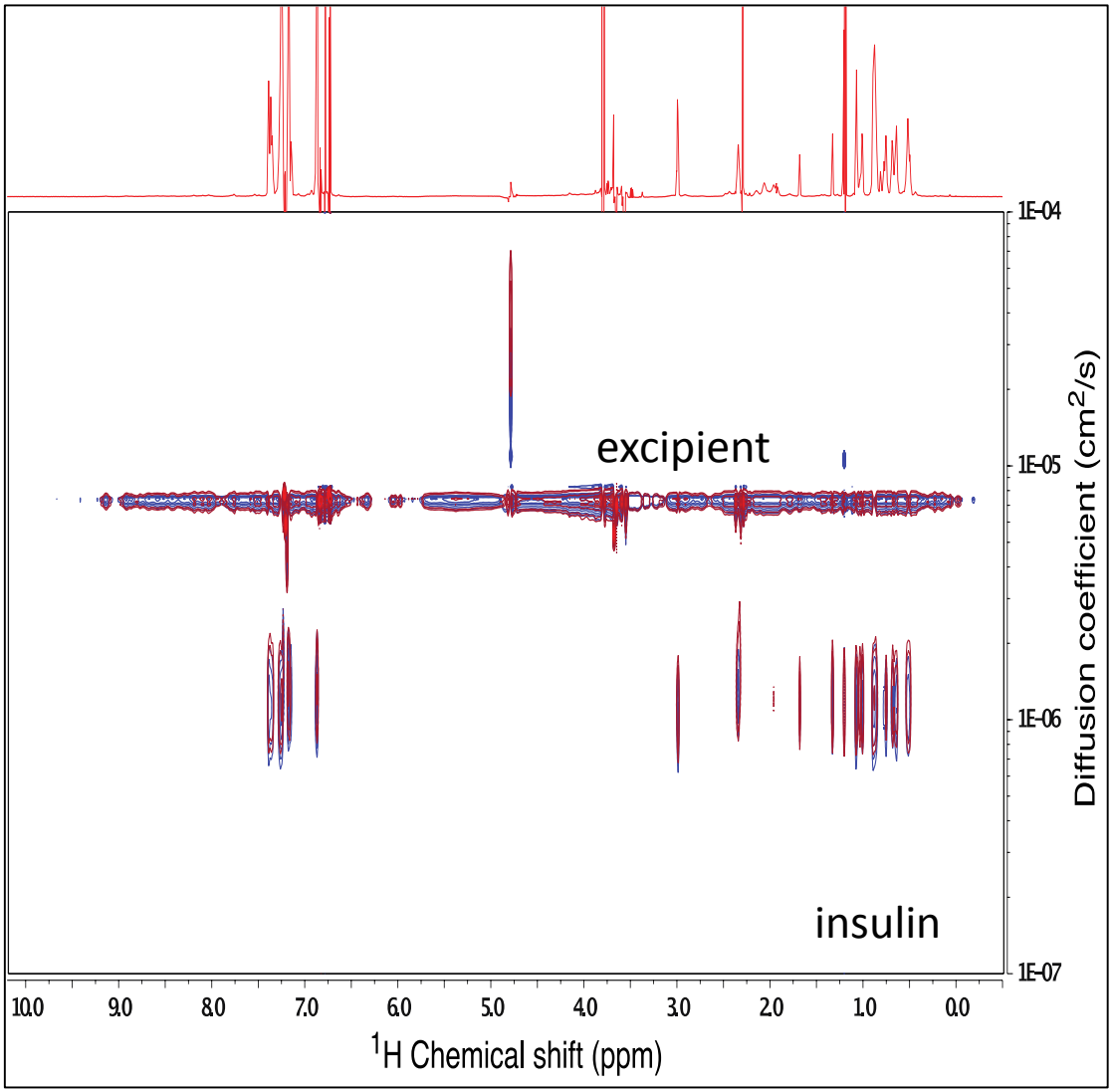
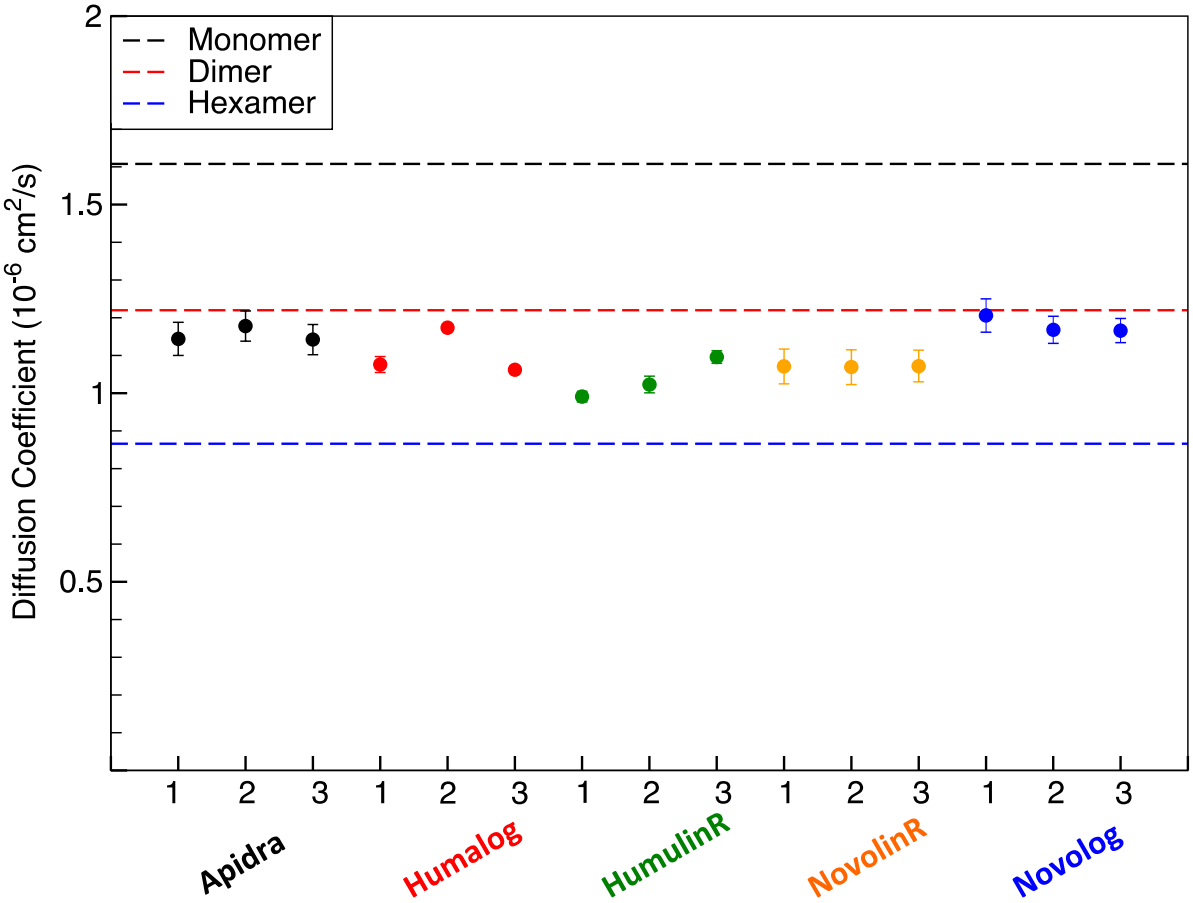
2D DOSY Spectra



Novolin R (Red)
Humulin R (Blue)

Diffusion coefficient for insulin

3 lots for each product, dashed line are computer simulation results of diffusion coefficients



Inter-lot STD	1.8%	5.5%	5.2%	0.13%	1.9%

Summary

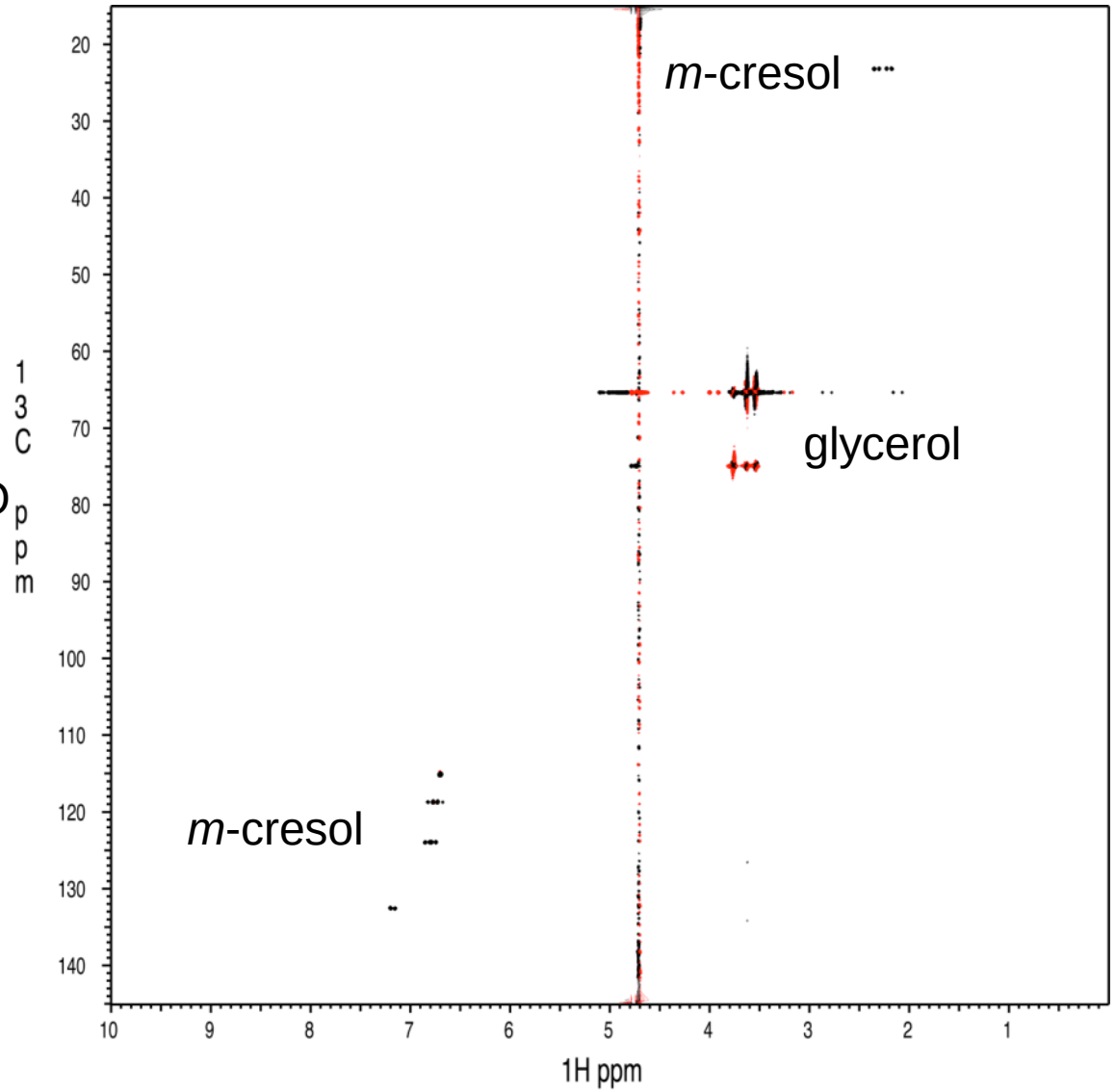
- 2D DOSY separates API signal from excipient and provides data for protein quaternary and quinary structure data.

- The observed insulin NMR signal represents exchange average between dimer and hexamer. Monomer does not exist in formulation.

- The DOSY method was highly reproducible and demonstrated 0.13% variation in repeated measurements D_0 for Novolin R products.

2D ^{1}H-^{13}C HSQC on insulin drug product

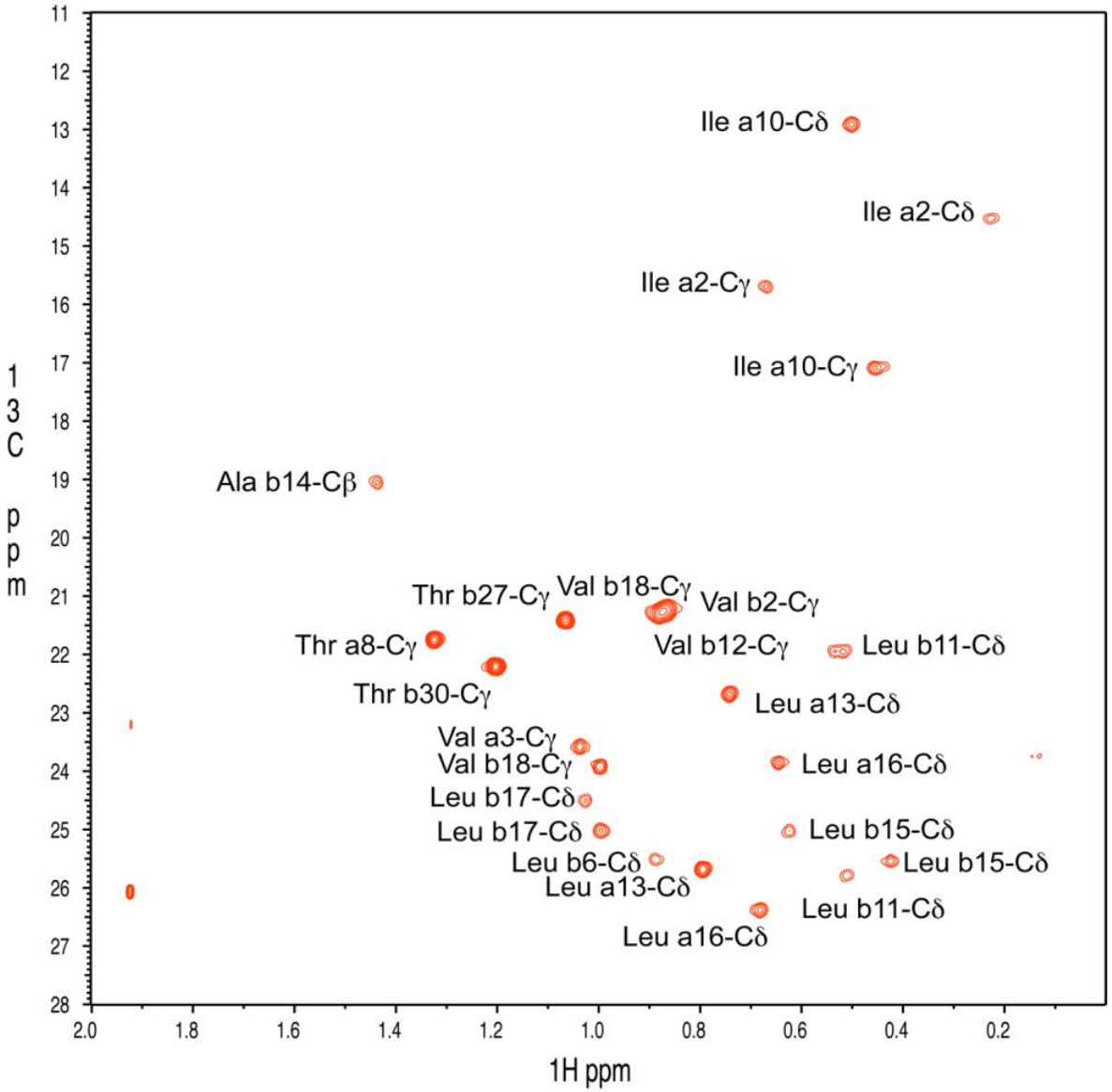
95% Insulin drug formulations + 5% D_2O



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

NovolinR ®

Exp. time = 12 Hr



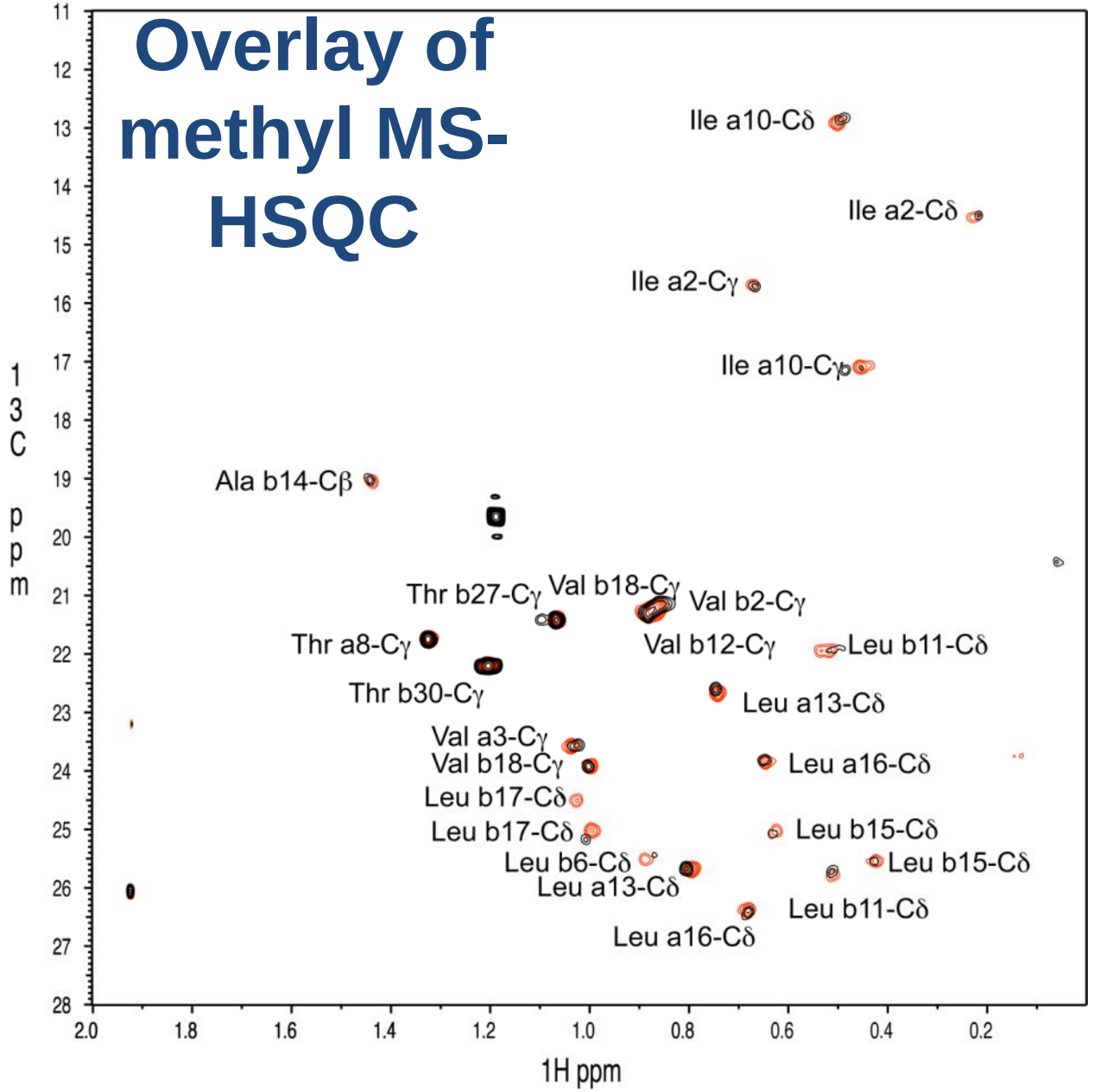
Chen, Freedberg, Keire. JMR, 2015 (251) 65-70.

BMRB

Xiaoquing Chang et.al., Biochemistry, 1997, 36(31), 9409-9422.

Kang Chen et al., JMR, 251, 65-70, 2015

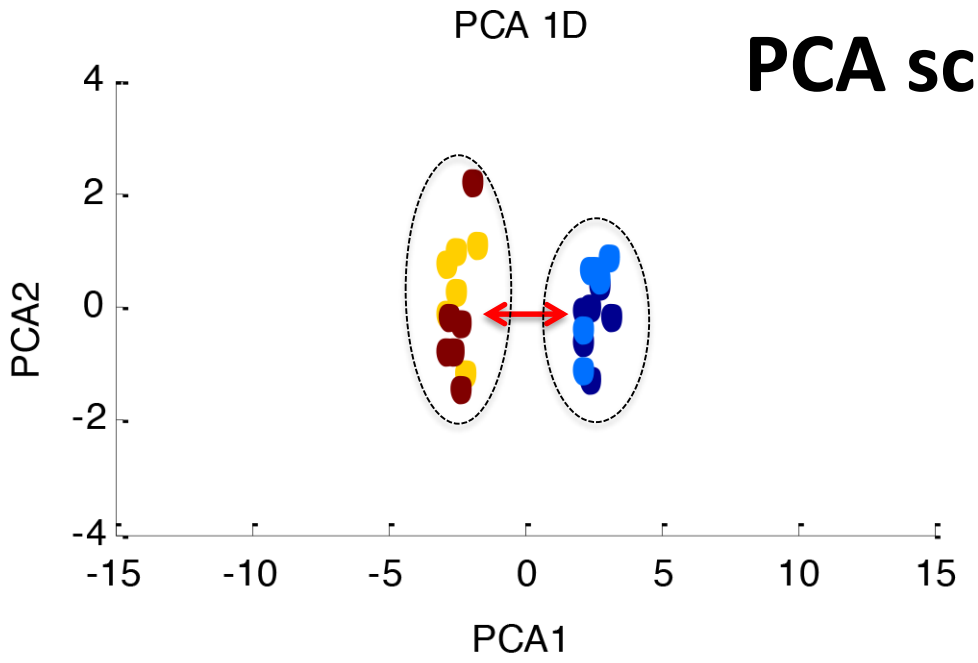
Overlay of methyl MS-HSQC



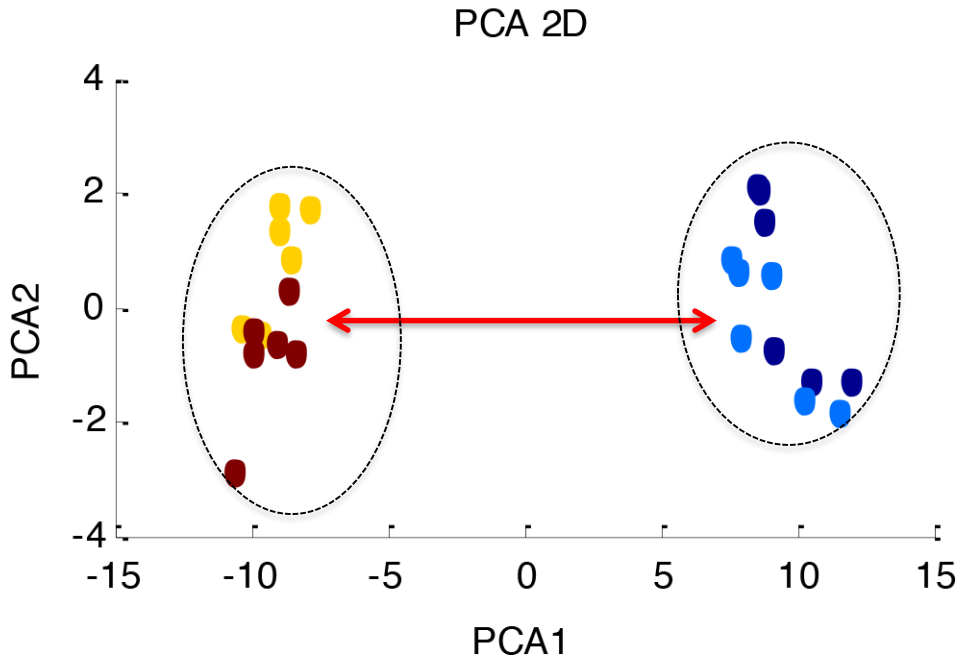
NovolinR®

HumulinR®

PCA score plot on 1D/2D spectra



Intra-brand



Inter-brand

2D PCA method: Ghasriani *et al.* NBT Vol 34(2) Feb 2016

Conclusion

- 1D NMR can inform upon the state of a protein in solution including self-association and aggregation.
  - ➢ Excipients can sometimes interfere
  - ➢ Calibration is needed for quantification.
- 2D NMR spectra, both HSQC and DOSY, can be applied to drug formulations directly.
- PCA is a valuable tool to identify differences between the complex spectra of comparators.

Acknowledgements:

Kang Chen

Sharadrao Patil

Houman Ghasriani

Diana Long

Michael Karfunkle

FDA U.S. FOOD & DRUG ADMINISTRATION