Formulation of Filgrastim Influences Protein Dynamics.

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CASSS Washington Discussion Group
19 April 2022

The People

Dr. Houman Ghasriani

From Samples to Structures: NMR data collection and analysis, resonance assignment, structure determination, relaxation parameter extraction and calculations of protein dynamics from NMR data.

Geneviève Gingras

From Genes to Magnet: Molecular biology, construction of expression vectors for the production of labeled proteins (*E. coli* and *Pichia Pastoris*): rhGM-CSF (*E.coli* and *P. Pastoris*)

Sara Ahmadi

Construction of the NIST-mAb-scFv and initial expression-purification protocol development

Derek Hodgson

Protein expression, (Intereron-α2a and α2b; hGH; **Met-G-CSF** and mutants; NIST-mAb-scFv), NMR data collection including all collaborative studies with WHO and USP.

Grant Frahm and Dr. Michael Johnston

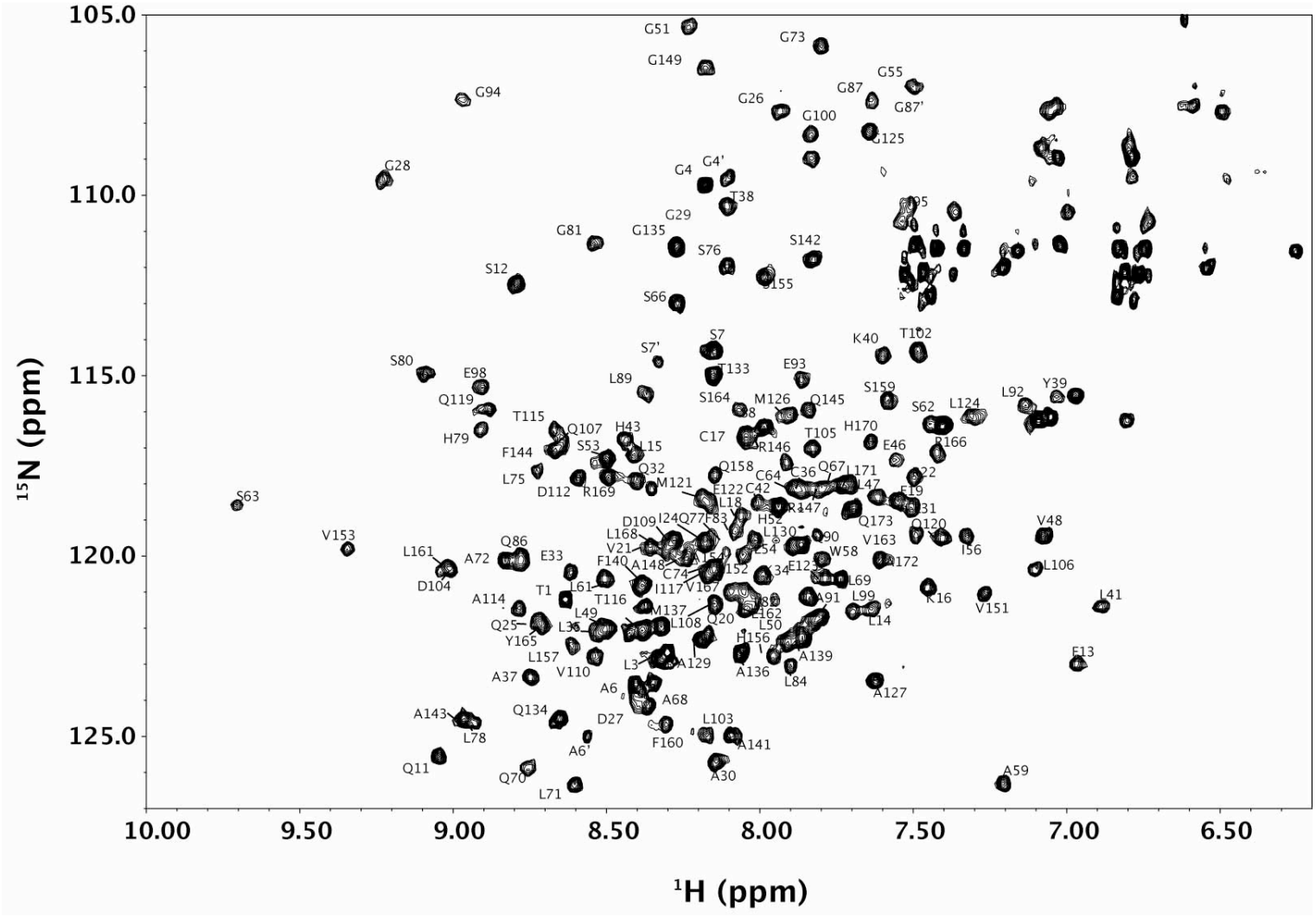
All thermal unfolding studies by CD

Dr. Donald Gagné and Dr. Muzaddid Sarker

Production of isotopically labelled (^{2}H,^{13}C,^{15}N) cleavable single chain Fab fragments of NIST-mAb, adalimumab, bevacizumab, infliximab, rituximab and trastuzumab for the expression in *E. coli*.

Neupogen® (Met-GCSF, filgrastim)

sample: concentration of 20 vials, 1 mM protein (volume: 0.4 mL)



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Aubin et al. Pharm. Res. 2015

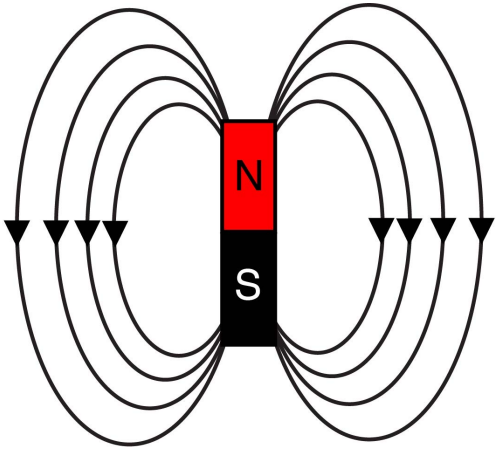
Formulation of Neupogen®

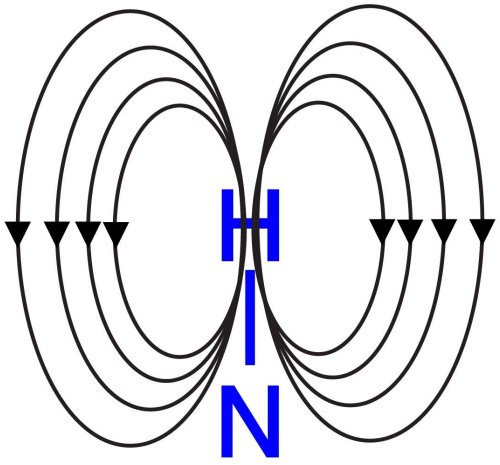
Component	Amount /0.5 mL	Concentration
GCSF	300 µg	32 µM
Acetate	0.295 mg	10 mM
Sorbitol	25.0 mg	274 mM
Polysorbate 80	0.02 mg	0.03 mM
Sodium	0.0175 mg	1.52 mM
Water	0.5 mL	---

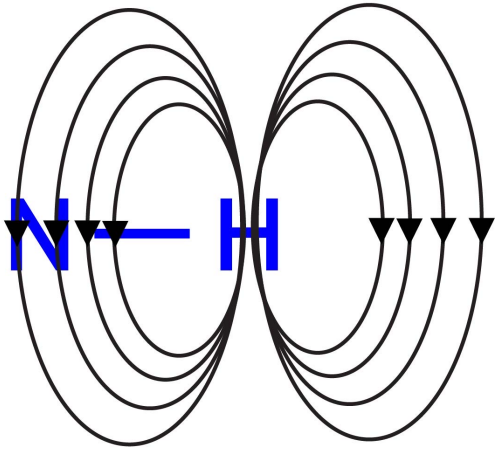
Resulting pH is 4.0

A bit of NMR Concepts

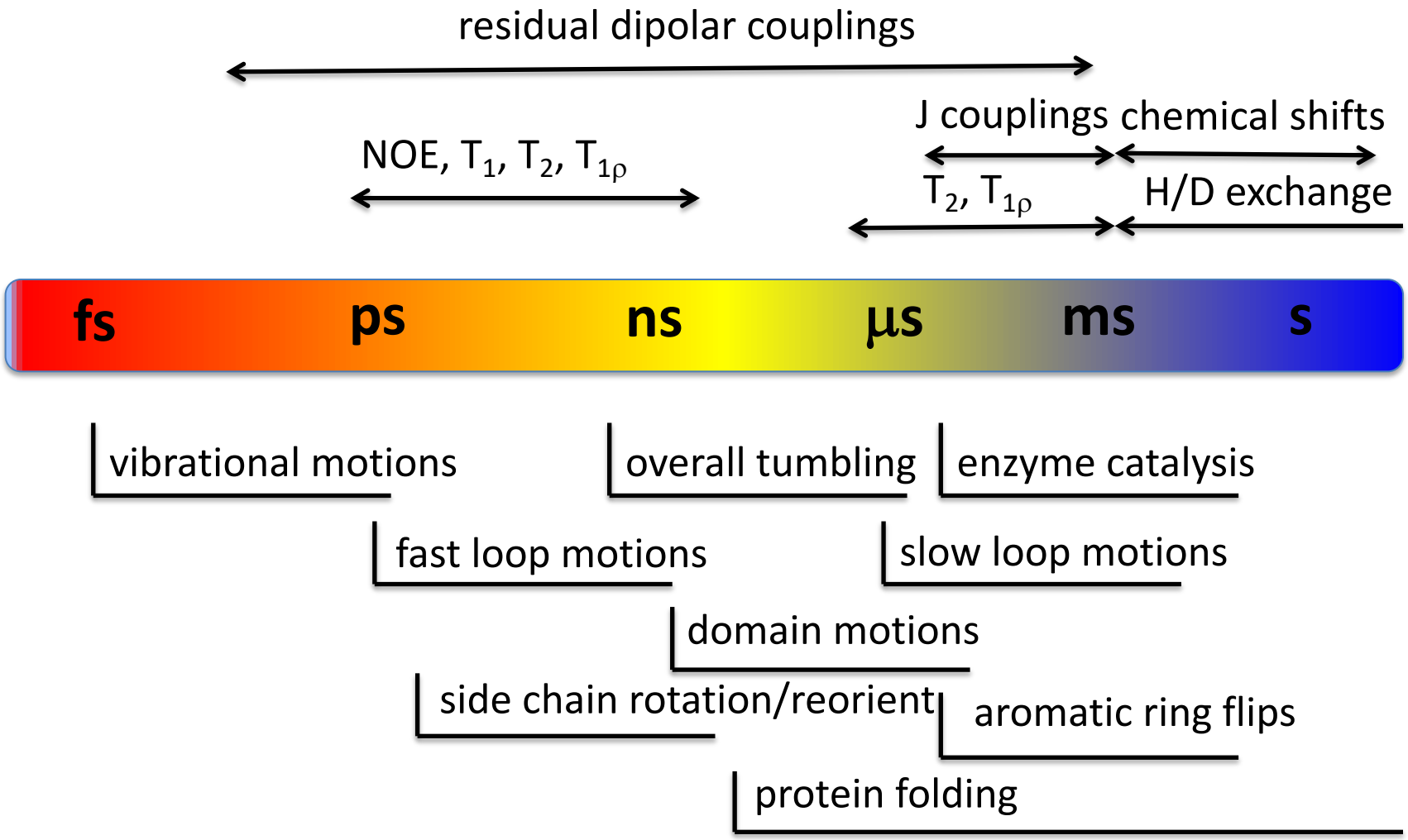
^{1}H, ^{13}C, ^{15}N =







NMR Time Scale of Protein Dynamics



Effects of Solution conditions on the NMR spectra of Filgrastim

pH

Sorbitol

Polysorbate-80 and Polysorbate-20

Thermal unfolding by CD and NMR Measurements carried out on ^{15}N-Filgrastim

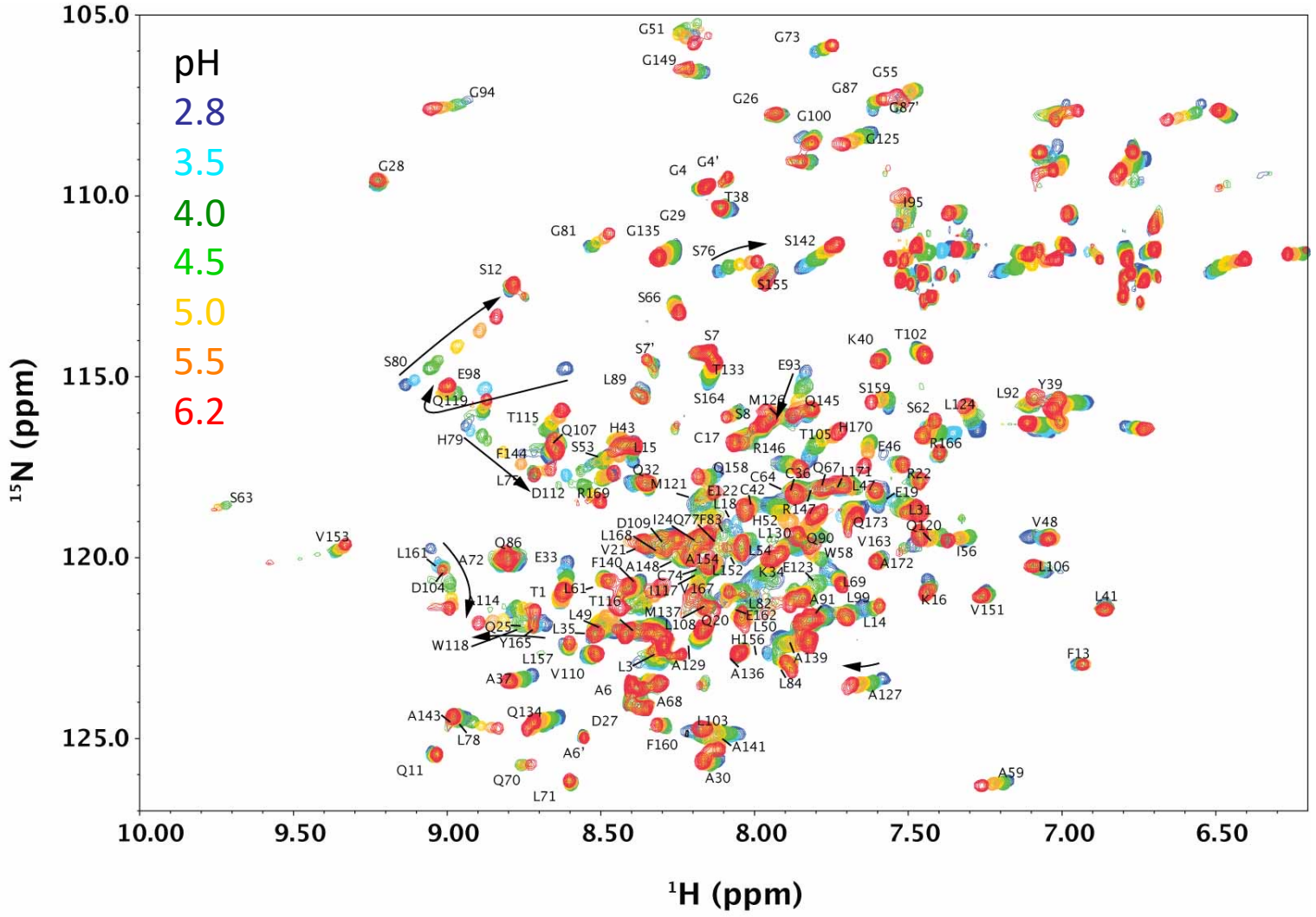
Relaxation parameters T_1, T_2 and het-NOE:

sorbitol (0 mM, 274 mM, 749 mM)
Polysorbate-80 (0, 30, 100, 300 uM)
Polysorbate-20 (0, 30, 100, 300 uM)
pH: 4, 5, 6

Monitoring thermal unfolding by circular dichroism:

sorbitol (0 mM, 274 mM, 749 mM)
Polysorbate-80 (0, 30, 100, 300 uM)
Polysorbate-20 (0, 30, 100, 300 uM)
pH: 4, 5, 6

Effects of pH on chemical shifts



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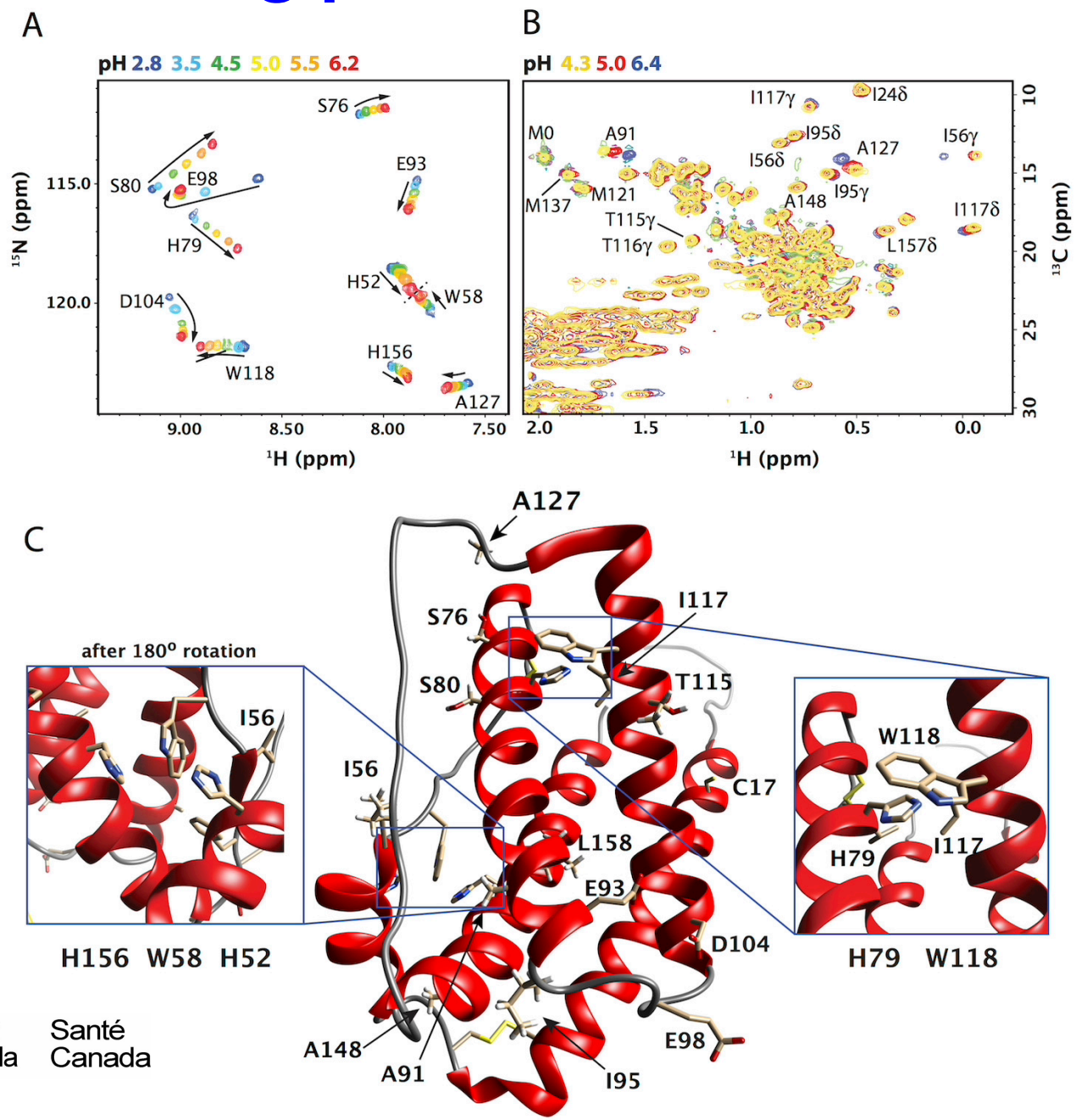
Lowering pH: Cation-π Interaction



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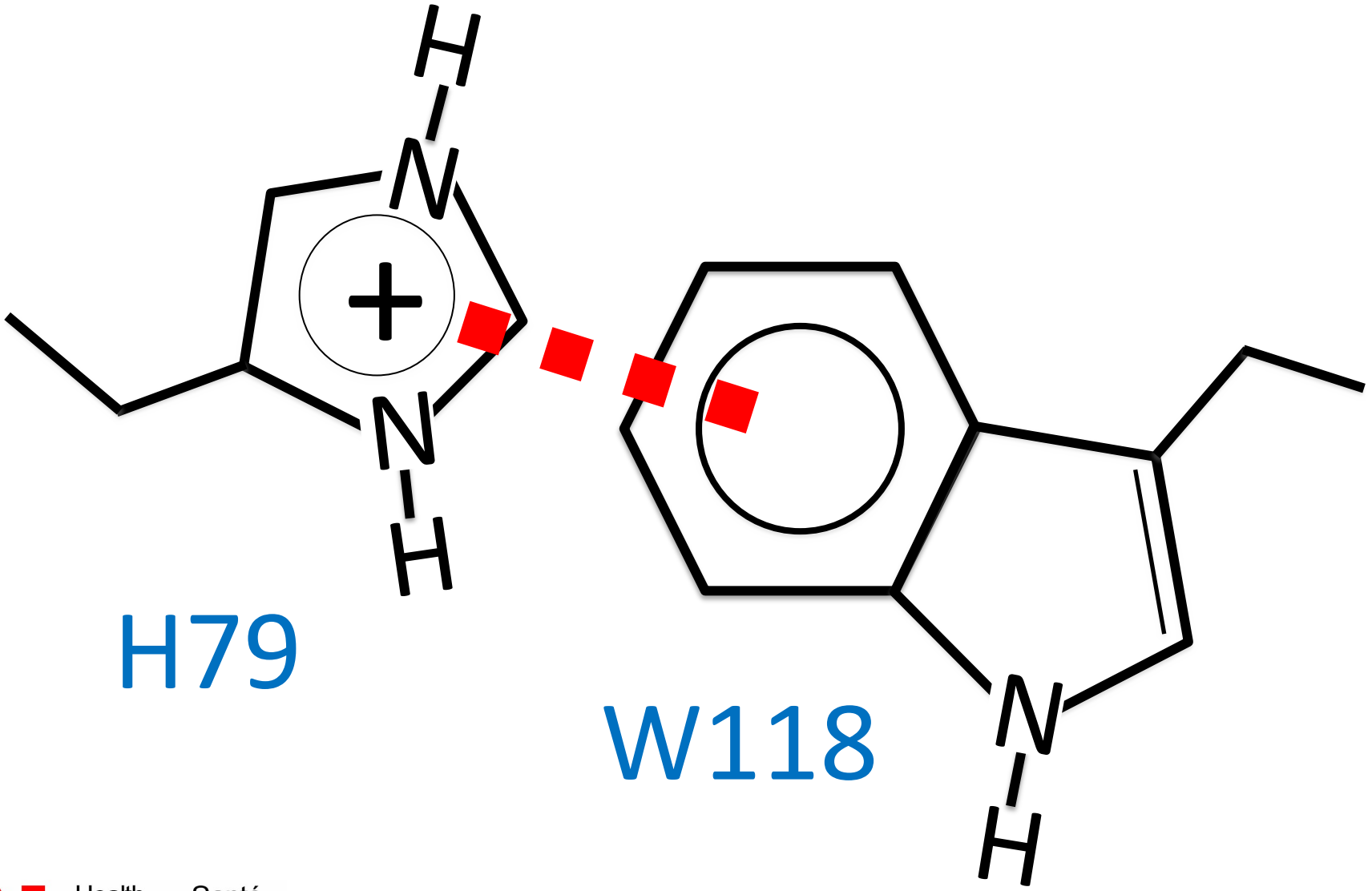
Lowering pH: Cation-π Interaction



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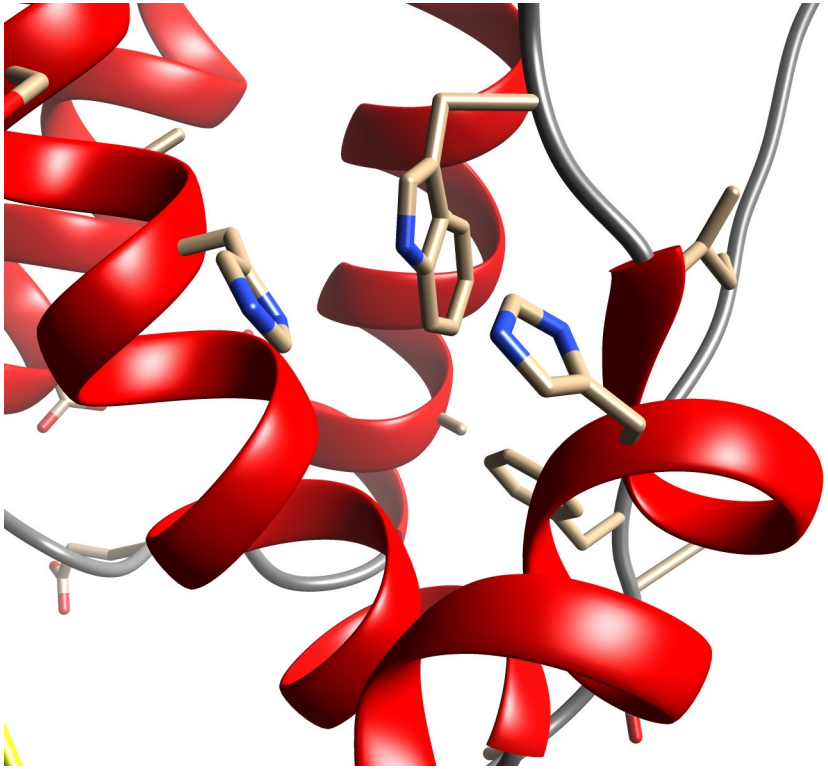
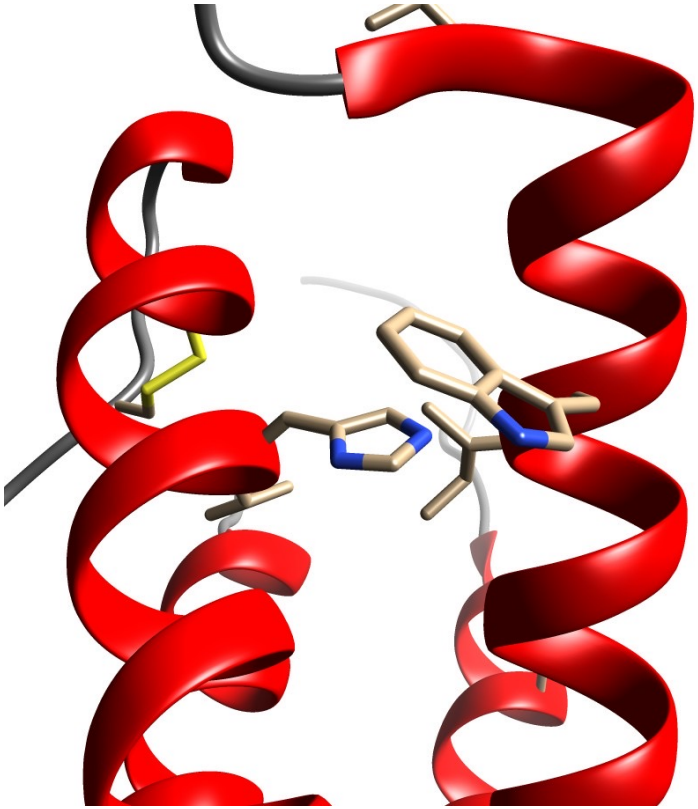
Lowering pH: Cation-π Interaction



H156 W58 H52



H79 W118

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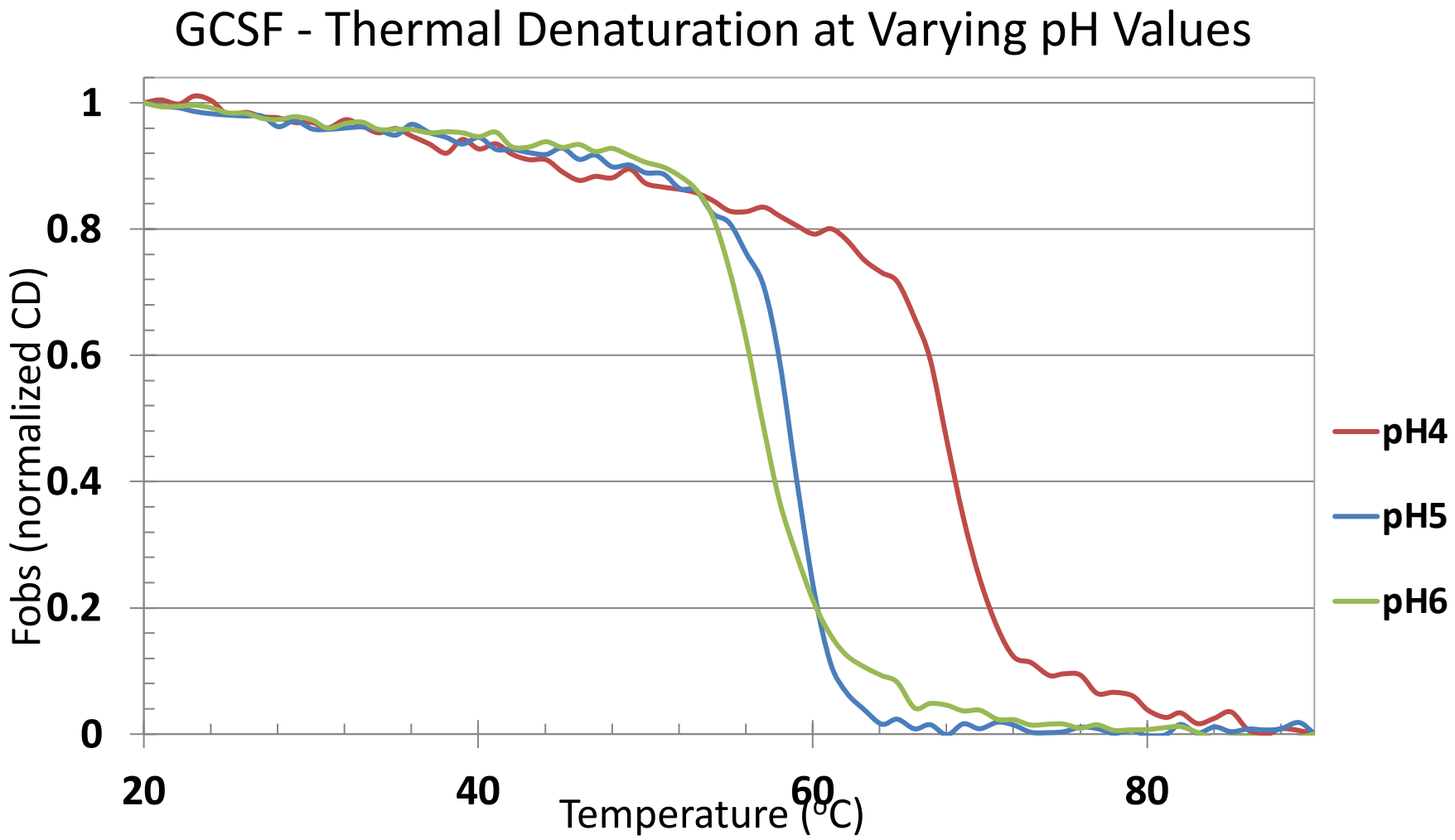
Effects of pH on Filgrastim Stability



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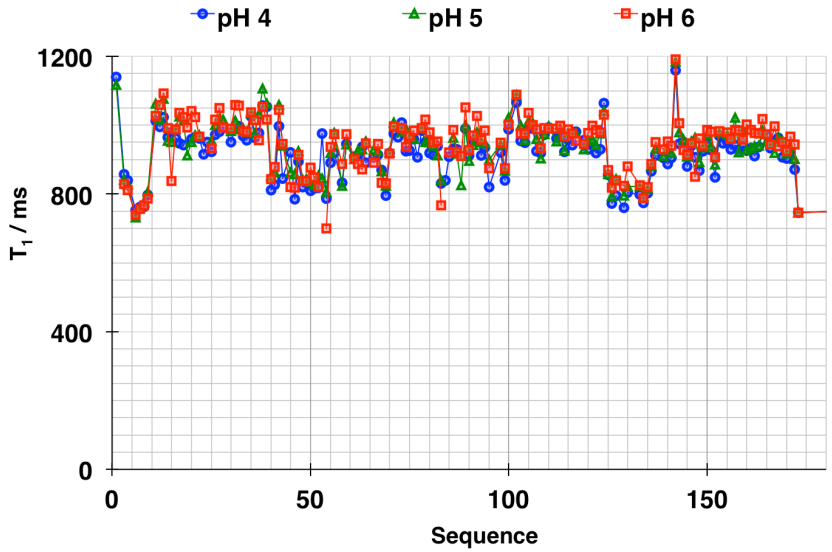
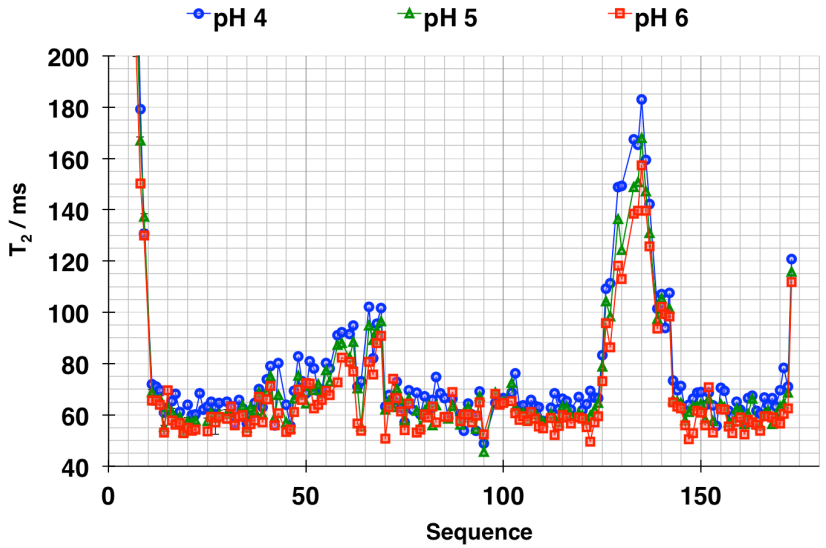
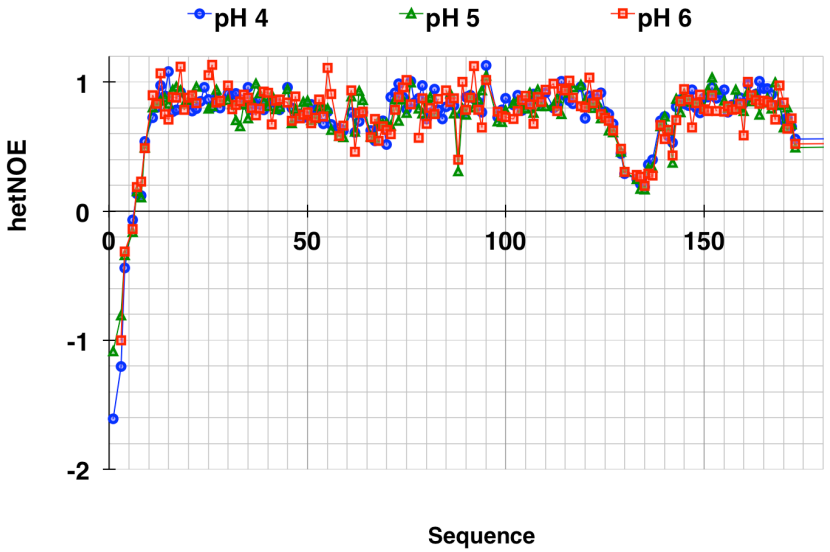
Effects of pH on Protein Dynamics







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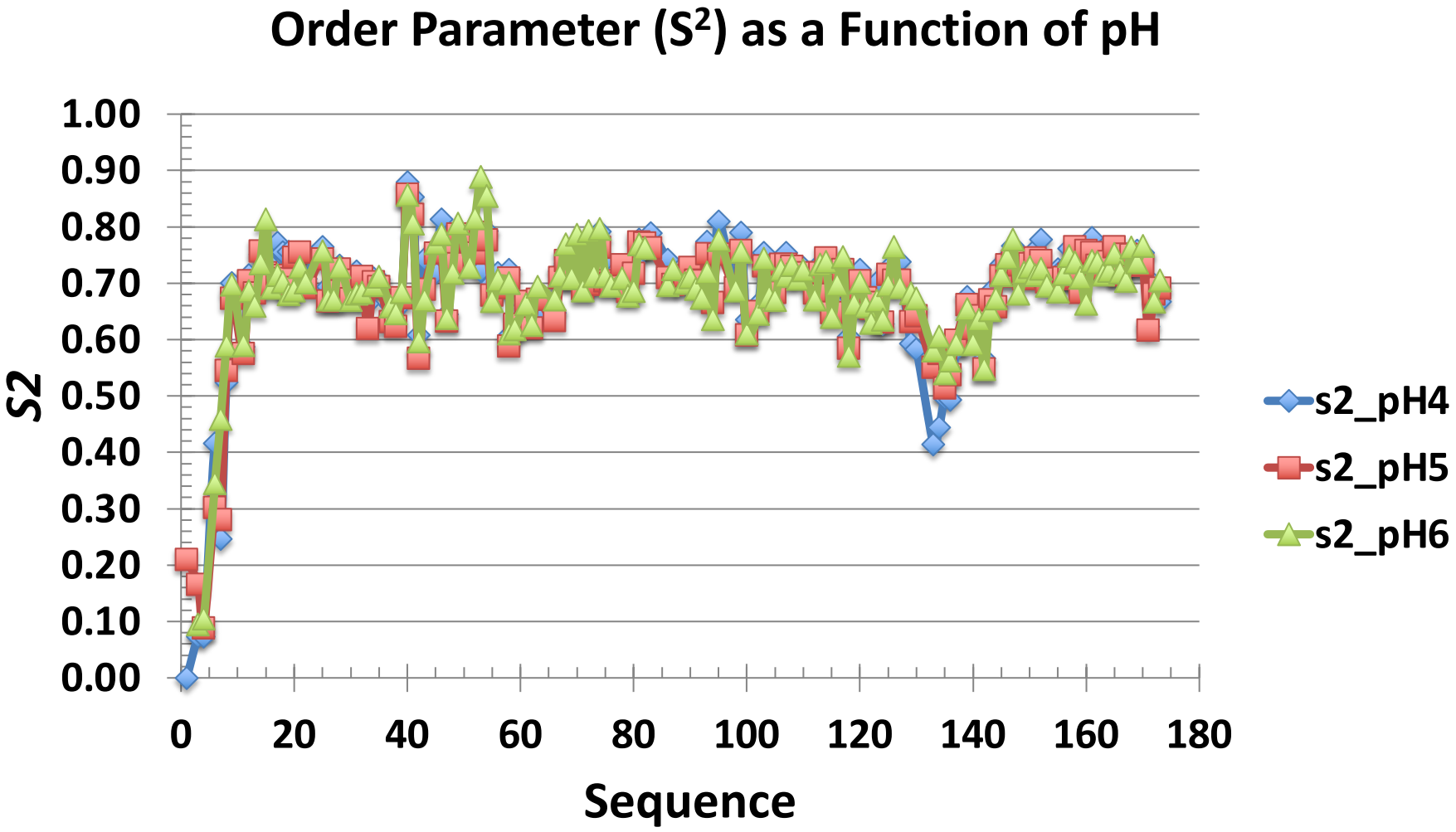
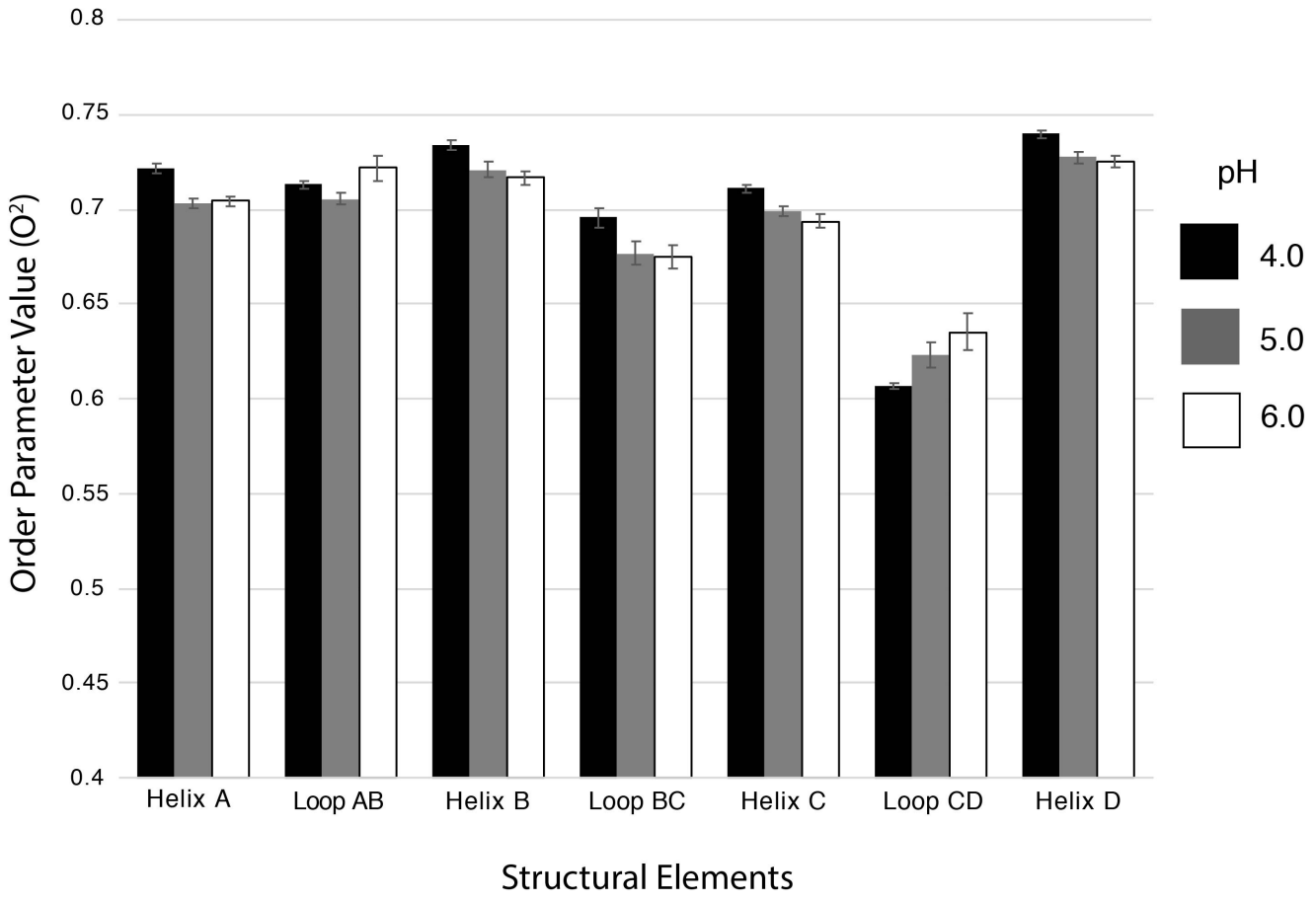
Effects of pH on Protein Dynamics



Average of Order Parameters for Helices and Loops



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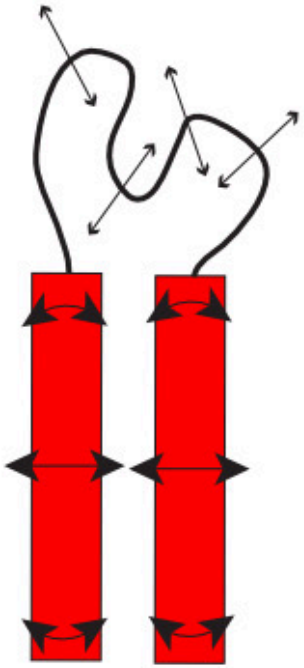
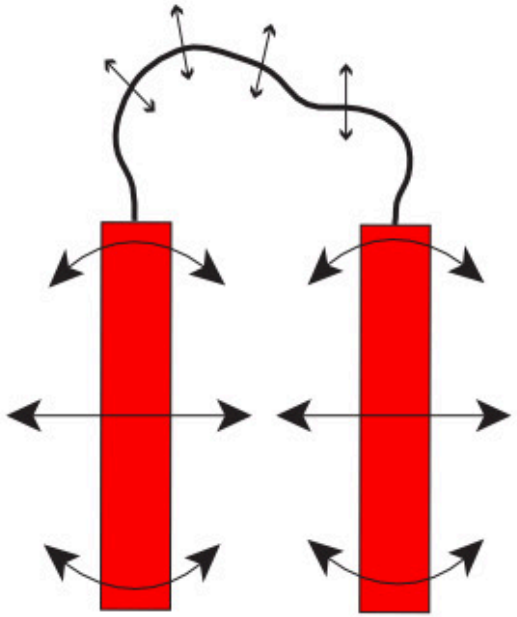
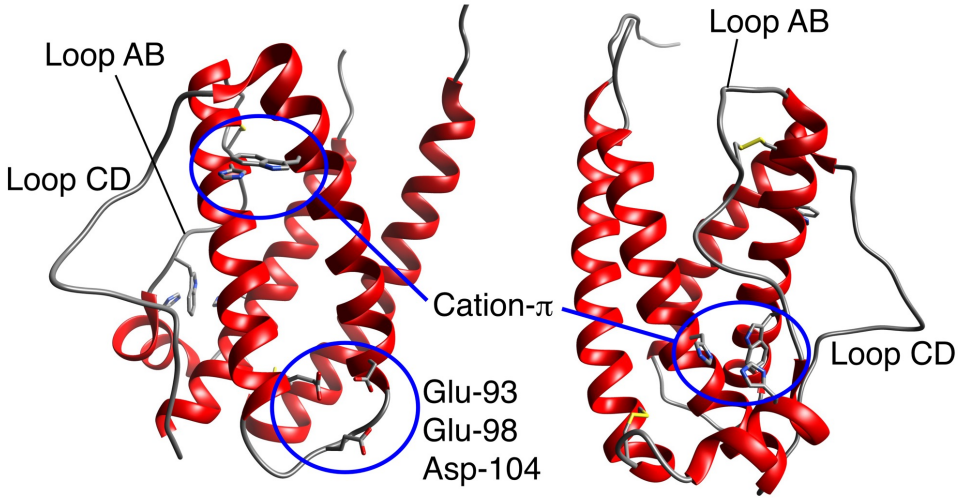
Amplitudes of Motions of Helices and Loops

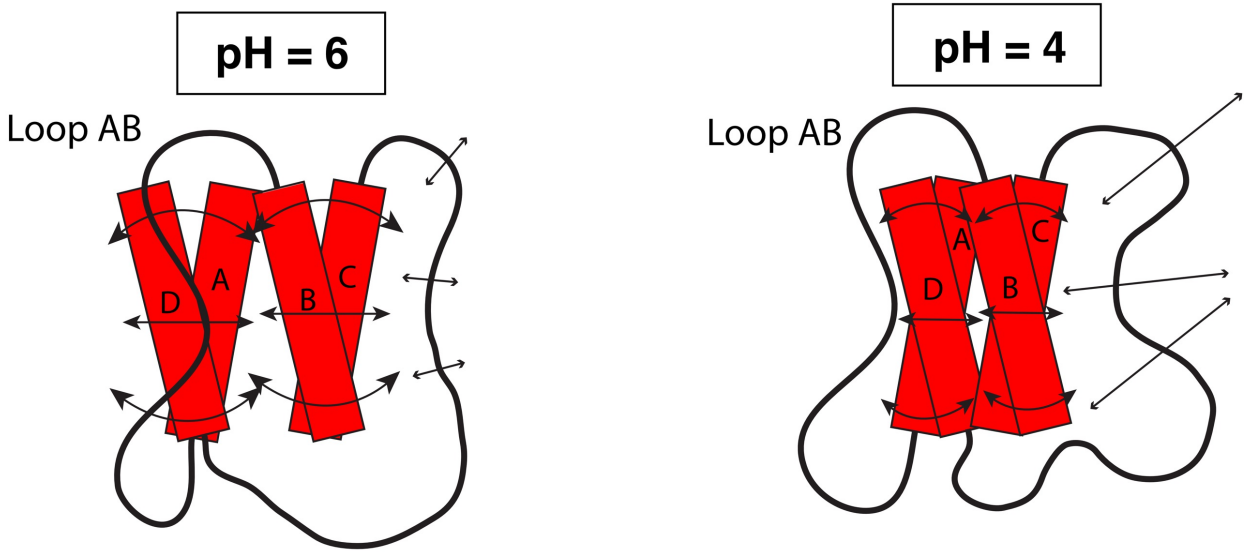


Helices with low amplitudes of motions allow loop motions with larger amplitudes



Helices with large amplitudes of motions restrict loop motions; lower motion amplitudes





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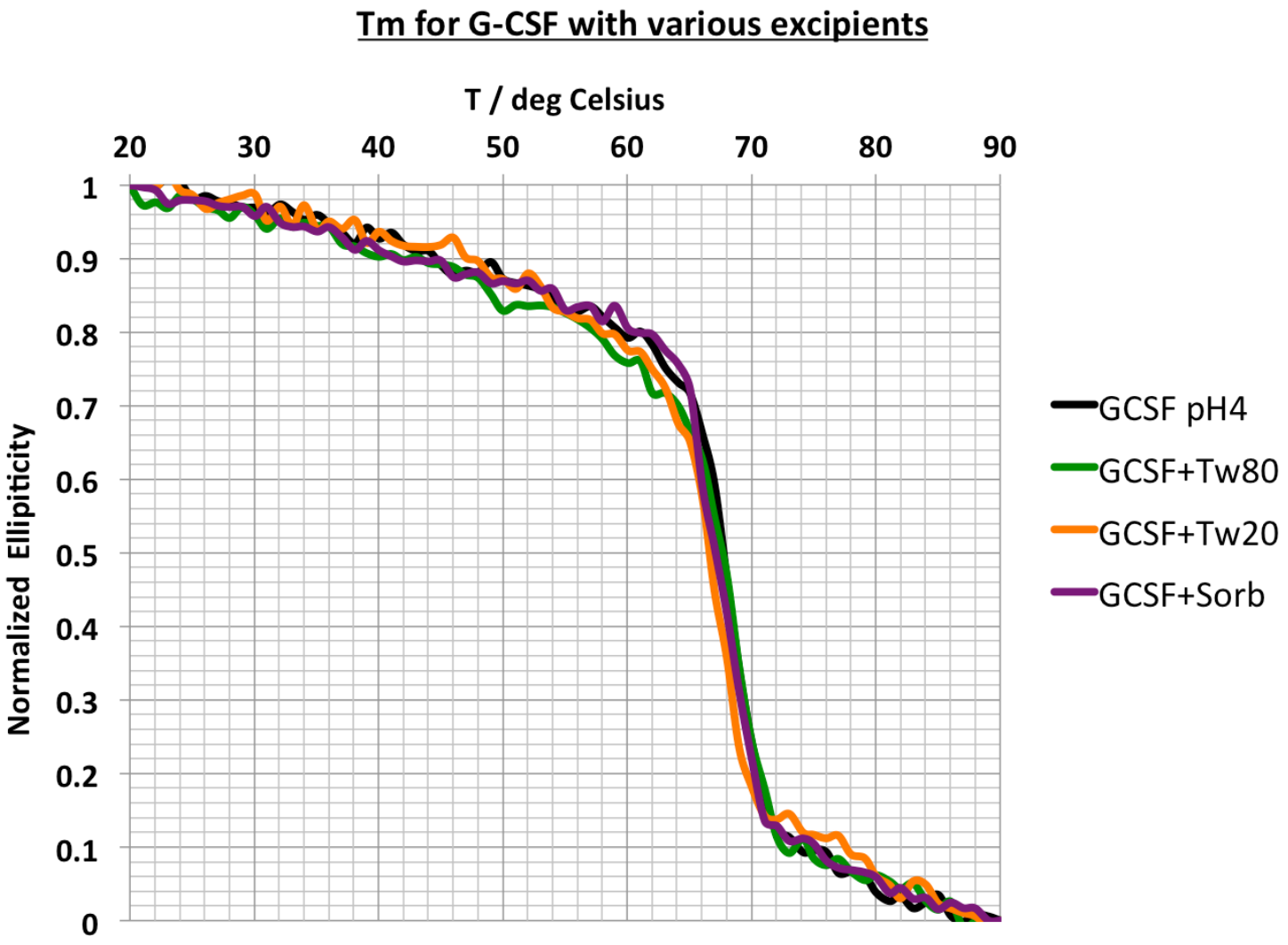
Effects of Excipients on Protein Dynamics



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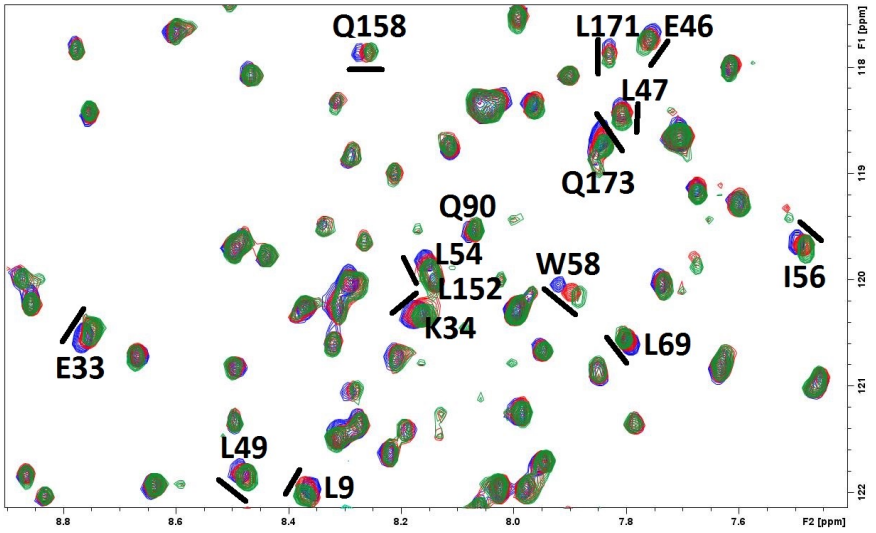
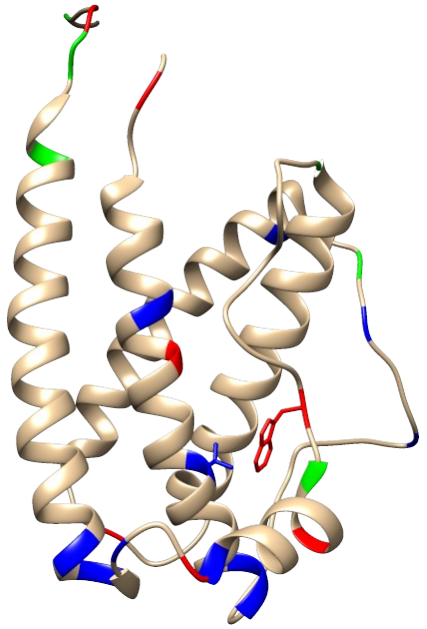
Effects of sorbitol on Protein Dynamics

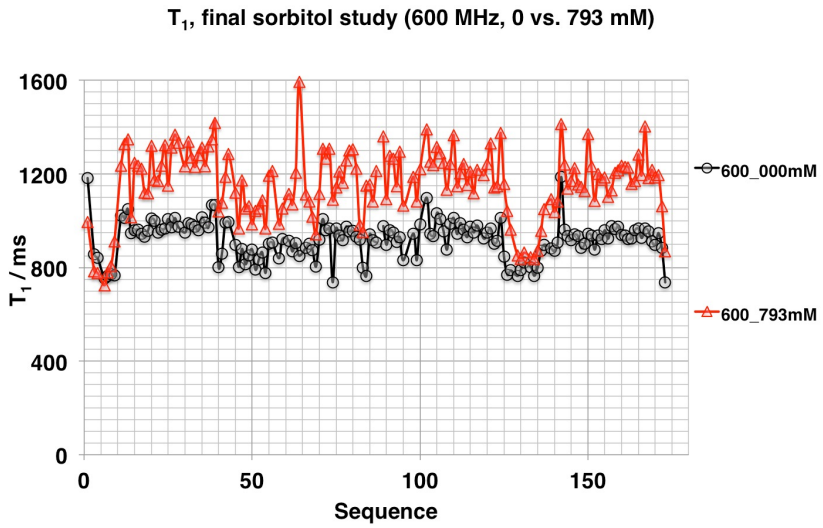


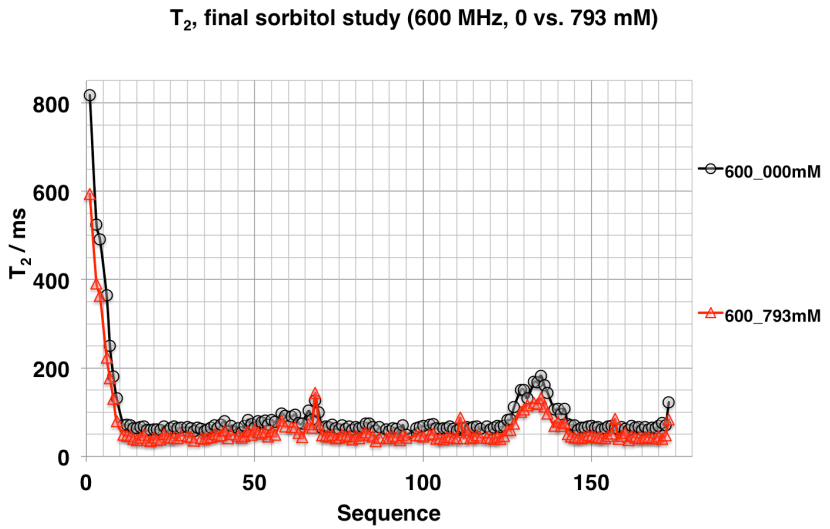
Sorbitol-induced chemical shift changes (spectra acquired at 45 °C) are predominantly affecting Trp^{58}. Sorbitol concentrations were at 0, 274, or 749 mM. CCSDs of shifting resonances were between 8-20ppb for 274mM sorbitol, and 20-50ppb for 749mM sorbitol. G-CSF concentration was kept at ~30uM

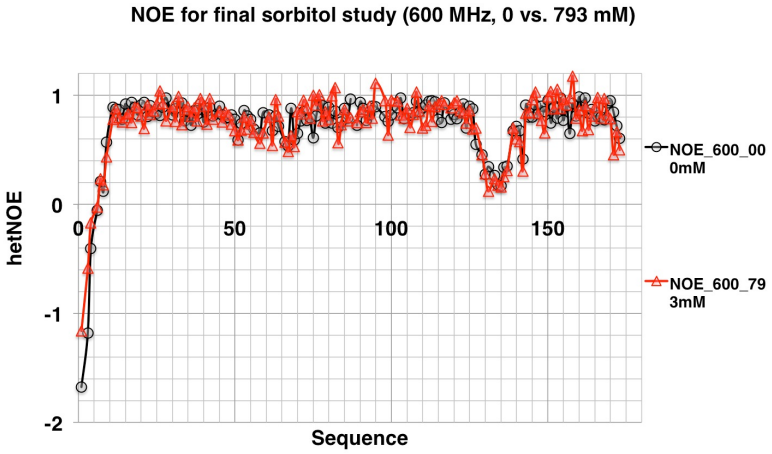


Sorbitol-induced chemical shift changes color-coded onto G-CSF, where red represents large, green moderate, and blue small chemical shift changes. Sorbitol concentration was 749 mM

Effects of sorbitol on Protein Dynamics







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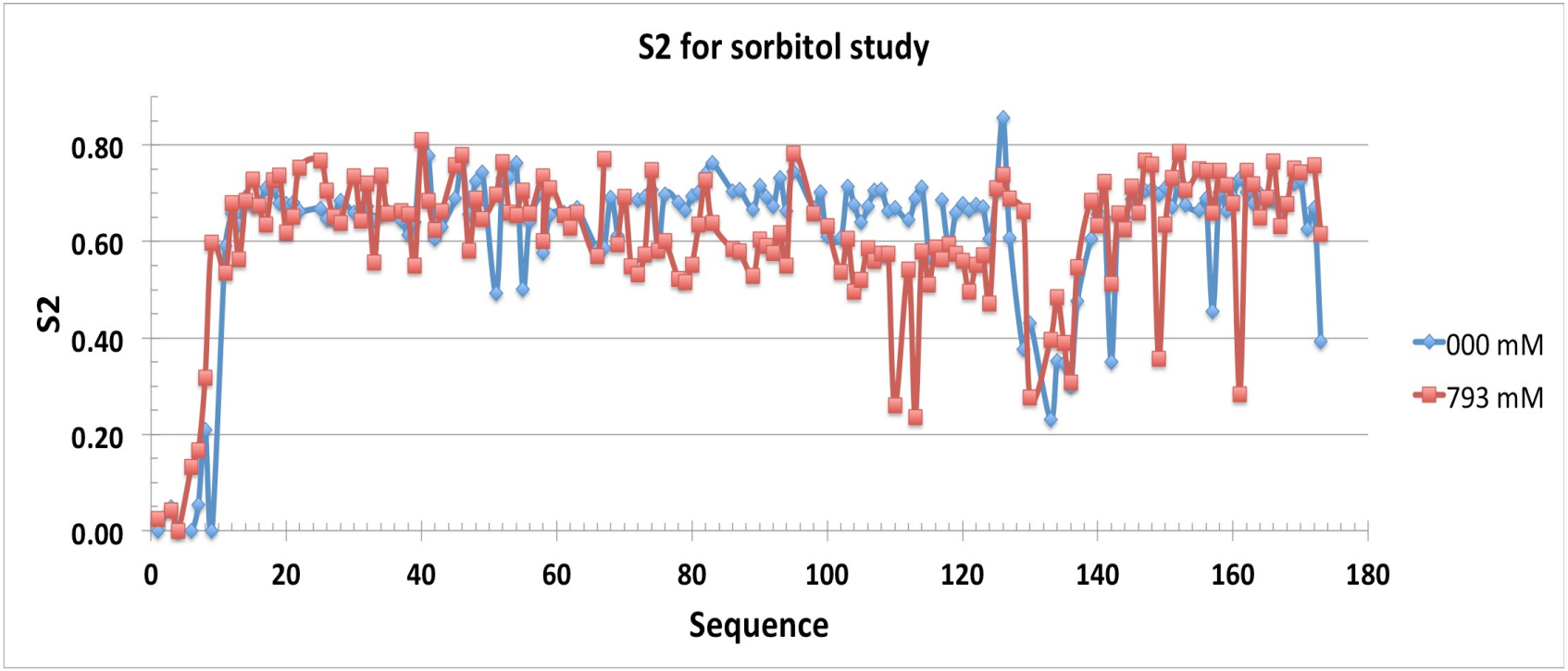
Effects of Excipients (pH) on Filgrastim Dynamics



Effect of sorbitol on S^2. S^2 for G-CSF in presence and absence of sorbitol as a function of residue number, using ellipsoid diffusion tensor and a model that accounts for exchange dynamics in model-free analysis. High sorbitol concentration (793 mM) results in enhanced flexibility (increased entropy, decreased S^2), for helix B, loop BC, and helix C.

Ghasriani H, Frahm GE, Johnston MJ and ***Aubin Y*** *(2020), ACS Omega*

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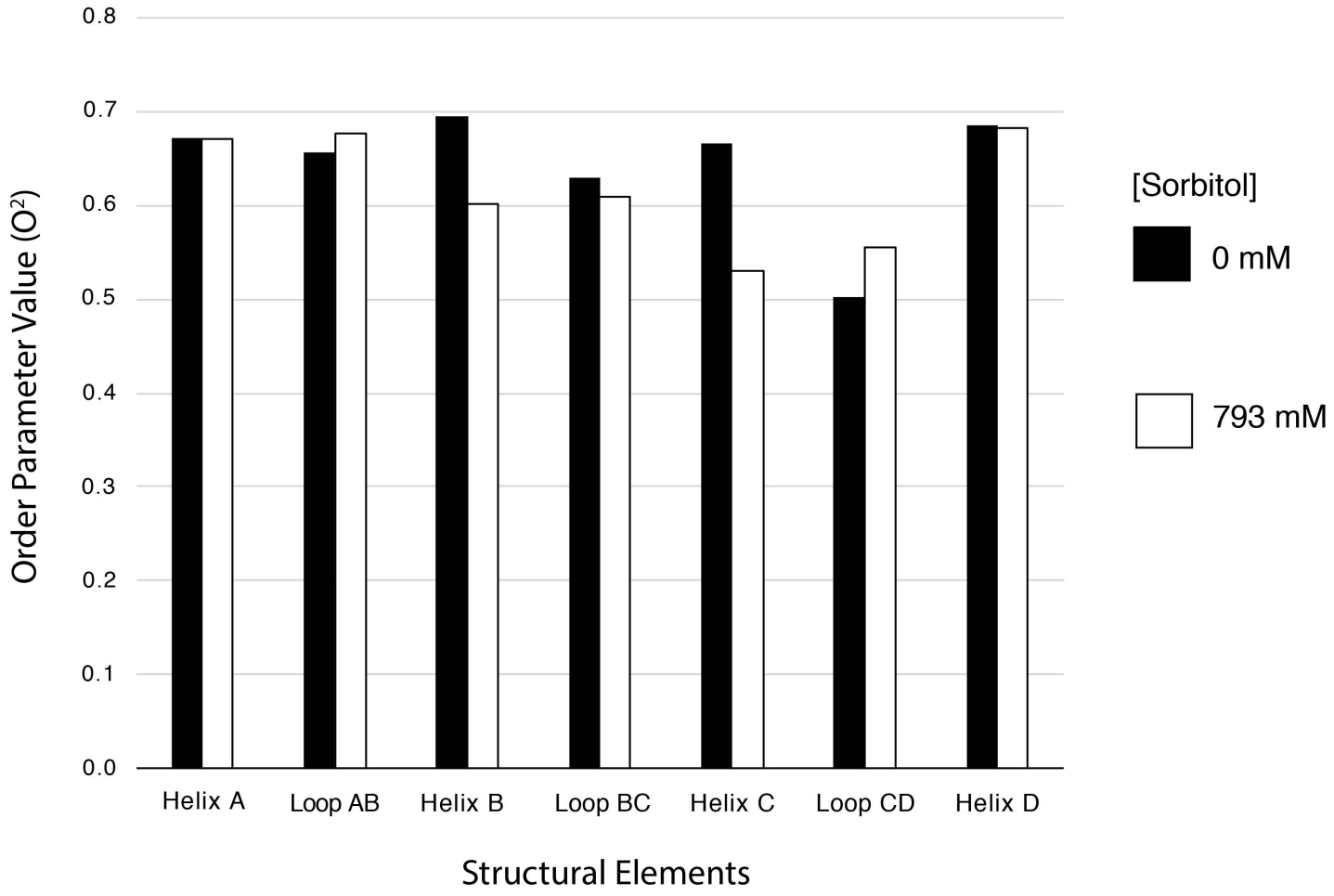
Average of Order Parameters for Helices and Loops



*Ghasriani H, Frahm GE, Johnston MJ and **Aubin Y** (2020), ACS Omega*

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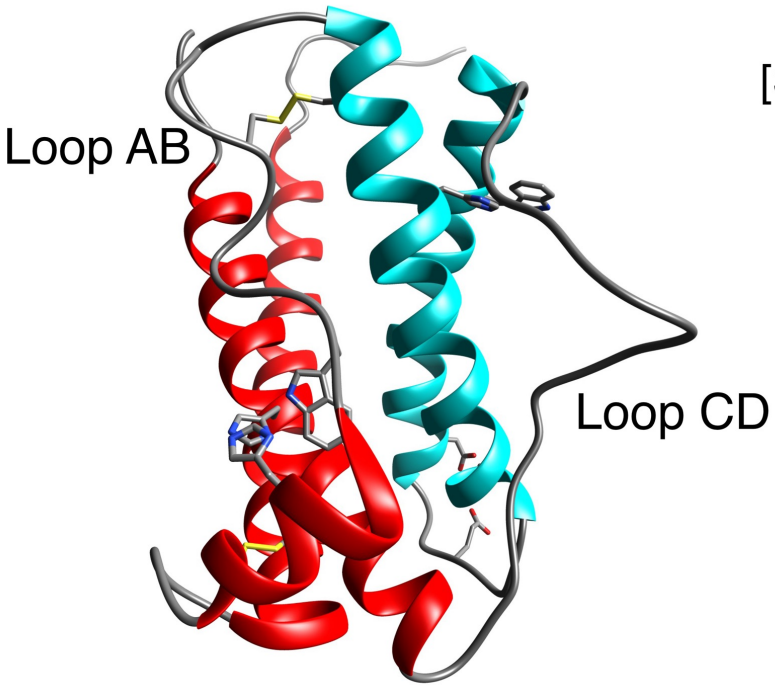
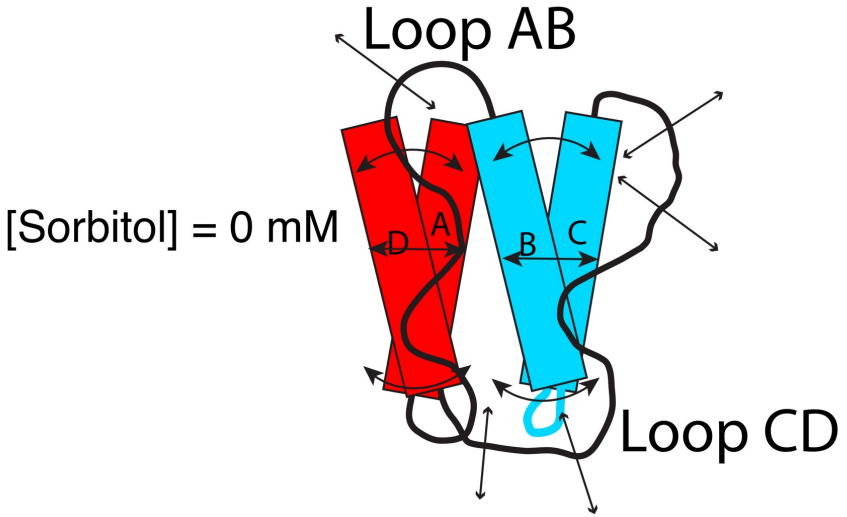
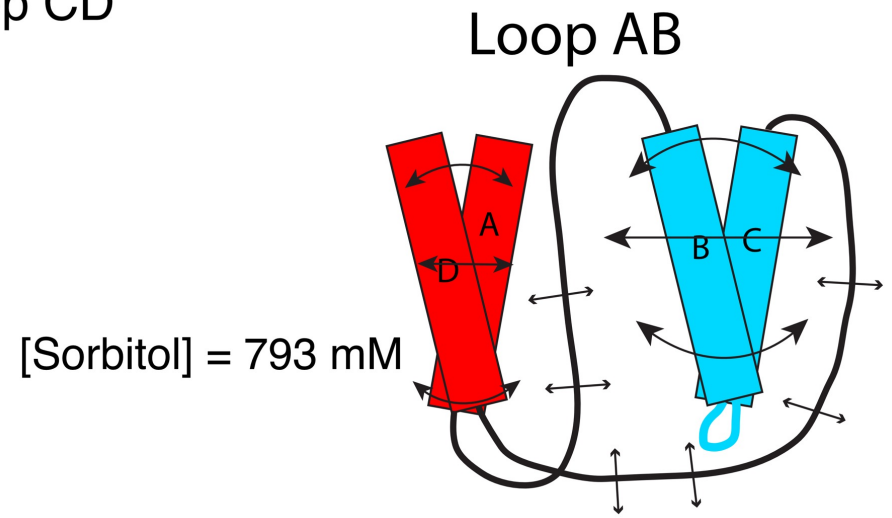
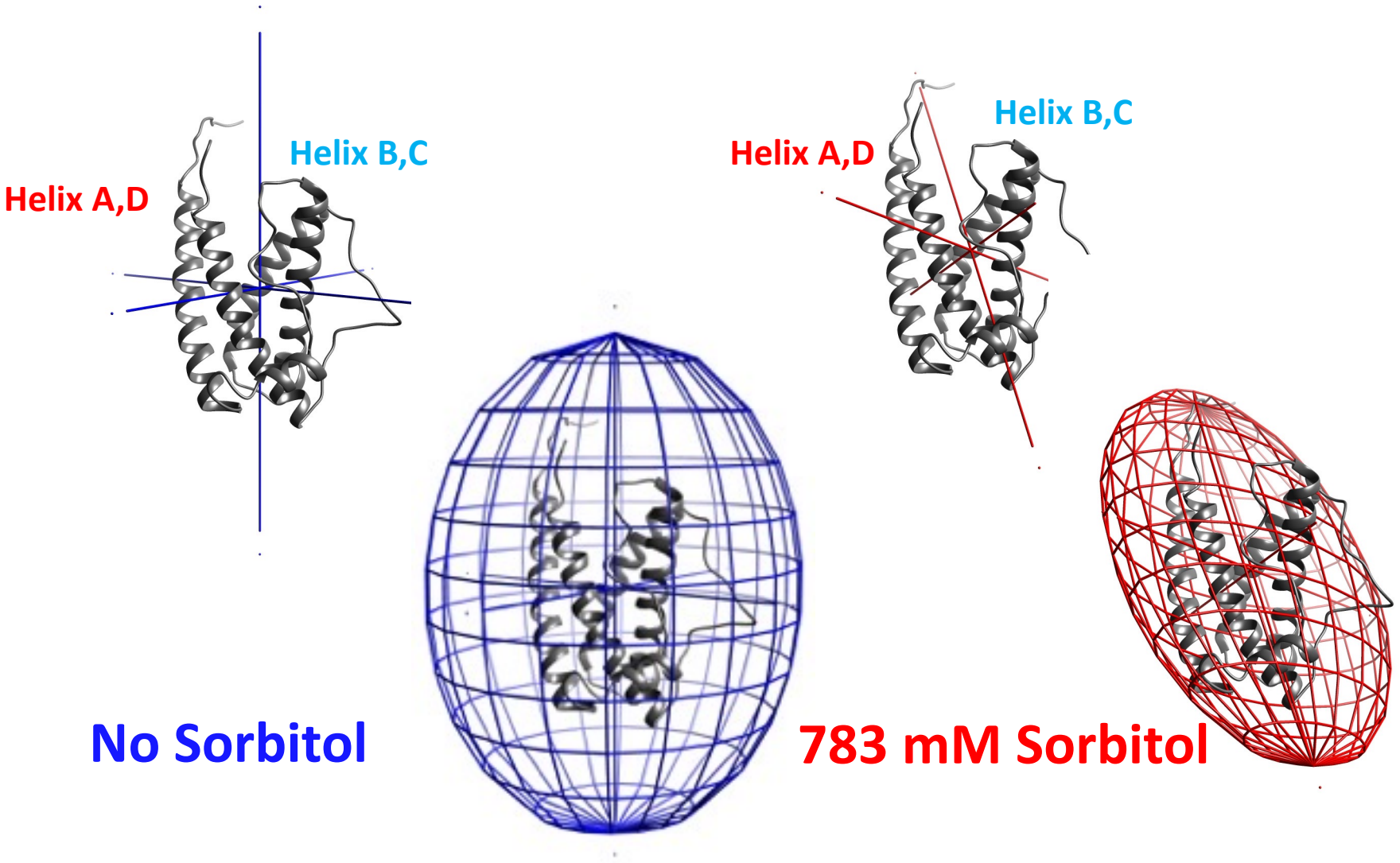






*Ghasriani H, Frahm GE, Johnston MJ and **Aubin Y** (2020), ACS Omega*

Diffusion Tensors Reflect a Change of Protein Shape in the Presence of Sorbitol



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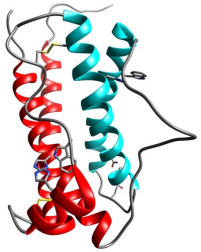
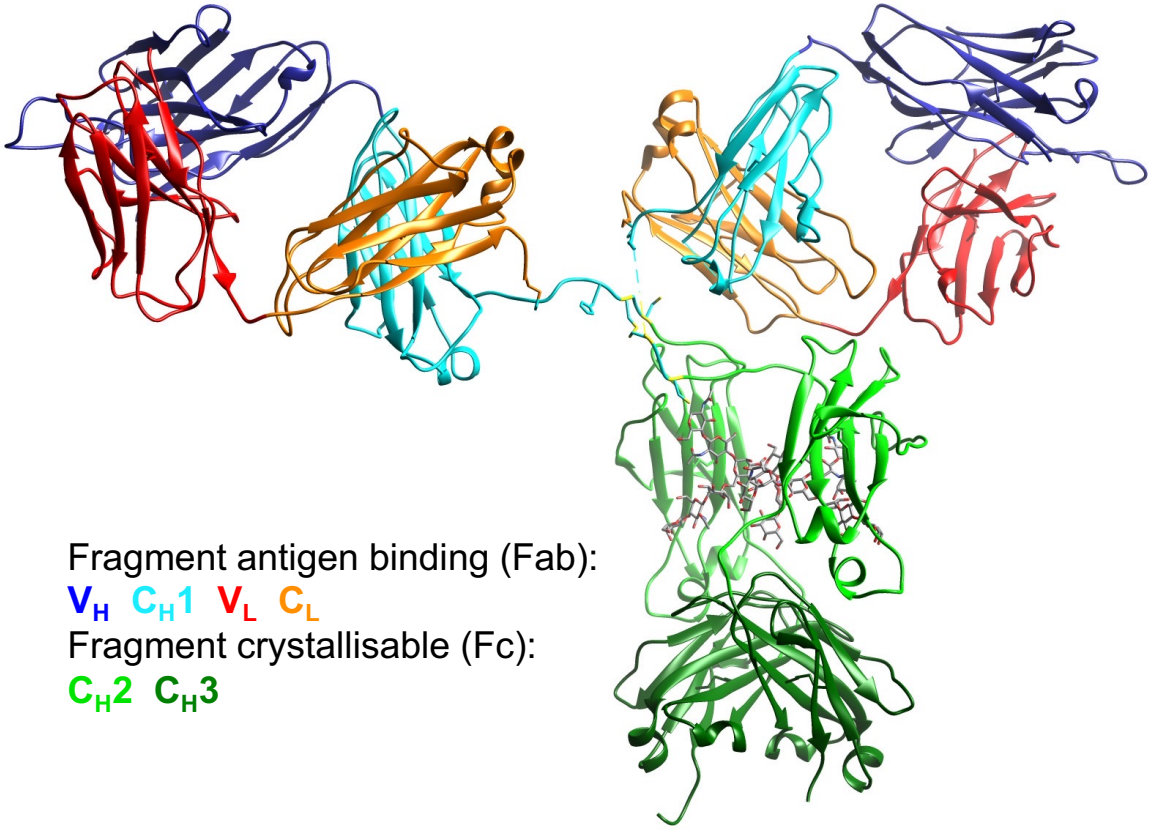
Ghasriani H, Frahm GE, Johnston MJ and ***Aubin Y*** *(2020), ACS Omega*

From Filgrastim to Therapeutic Monoclonal Antibodies





Fragment antigen binding (Fab):
V_H C_H1 V_L C_L
Fragment crystallisable (Fc):
C_H2 C_H3

Pushing the Limit: NMR of Therapeutic mAbs

Arbogast, Brinson and Marino demonstrated the application of NMR spectroscopy to obtain high-resolution 2D spectra of the NIST-mAb fragments (Fab and Fc)

Arbogast, LW, Marino, JP, Brinson, RG (2015) *Analytical Chemistry*, *87*: 3556-3561

Arbogast, LW, Brinson, RG, Formolo, T, Hoopes JT, Marino, JP (2016) *Pharmaceutical Research*, 33:462-475

Enabling adoption of 2D-NMR for the higher order structure assessment of monoclonal antibody therapeutics, MABS (2019) 11: 94–105

Robert G. Brinson,a John P. Marino,a Frank Delaglio,a Luke W. Arbogast,a Ryan M. Evans,b Anthony Kearsley,b Geneviève Gingras,c Houman Ghasriani,c Yves Aubin,c Gregory K. Pierens,d Xinying Jia,d Mehdi Mobli,d Hamish G. Grant,eDavid W. Keizer,e Kristian Schweimer,f Jonas Ståhle,g Göran Widmalm,gEdward R. Zartler,h Chad W. Lawrence,i Patrick N. Reardon,†,i John R. Cort,iPing Xu,j Feng Ni,j Saeko Yanaka,k Koichi Kato,k Stuart R. Parnham,l Desiree Tsao,m Andreas Blomgren,n Torgny Rundlöf,n Nils Trieloff,o Peter Schmieder,oAlfred Ross,p Ken Skidmore,q Kang Chen,r David Keire,r Darón I. Freedberg,sThea Suter-Stahel,t Gerhard Wider,t Gregor Ilc,u,v Janez Plavec,u,v Scott A. Bradley,w Donna M. Baldisseri,x Mauricio Luis Sforça,y Ana Carolina de Mattos Zeri,z Julie Yu Wei,aa Christina M. Szabo,bb Carlos A. Amezcua,bb John B. Jordan,cc and Mats Wikströmdd

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bApplied and Computational Mathematics Division, National Institute of Standards and Technology, Gaithersburg, MD, USA
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gDepartment of Organic Chemistry, Arrhenius Laboratory, Stockholm University, Stockholm, Sweden
hAnalytical R&D, Pfizer Essential Health, Lake Forest, IL, USA
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nLaboratory Unit, Swedish Medical Products Agency, Laboratory, Uppsala, Sweden
oNMR-supported Structural Biology, Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), Berlin, Germany
pRoche Pharmaceutical Research & Early Development, Pre-Clinical CMC, Roche Innovation Center Basel, Basel, Switzerland
qAnalytical Operations, Genentech, South San Francisco, CA, USA
rCenter for Drug Evaluation and Research, Food and Drug Administration, Maryland, USA
sCenter for Biologics Evaluation and Research, Food and Drug Administration, Maryland, USA
tDepartment of Biology, Institute of Molecular Biology and Biophysics, ETH Zurich, Zurich, Switzerland
uNMR Centre, EN-FIST Centre of Excellence, Ljubljana, Slovenia
vNMR Centre, National Institute of Chemistry, Ljubljana, Slovenia
wEli Lilly and Company, Lilly Corporate Center, Indianapolis, IN, USA
xMRS – Application Science, Bruker BioSpin Corporation, Billerica, MA, USA
yBrazilian Biosciences National Laboratory (LNBio), Brazilian Center for Research in Energy and Materials (CNPEM), Campinas, Brazil
zBrazilian Synchrotron Light Laboratory (LNLS), Brazilian Center for Research in Energy and Materials (CNPEM), Campinas, Brazil, CEP
aaProtein Product Development, Biogen Inc., Cambridge, MA, USA
bbBaxter Pharmaceuticals R&D, Baxter Healthcare, Round Lake, IL, USA
ccGlobal Regulatory and R&D Policy, Amgen Inc., Thousand Oaks, CA, USA
ddHigher Order Structure, Attribute Sciences, Amgen Inc., Thousand Oaks, CA, USA

Approved Therapeutic mAbs in US or EU
(as of November 2014)

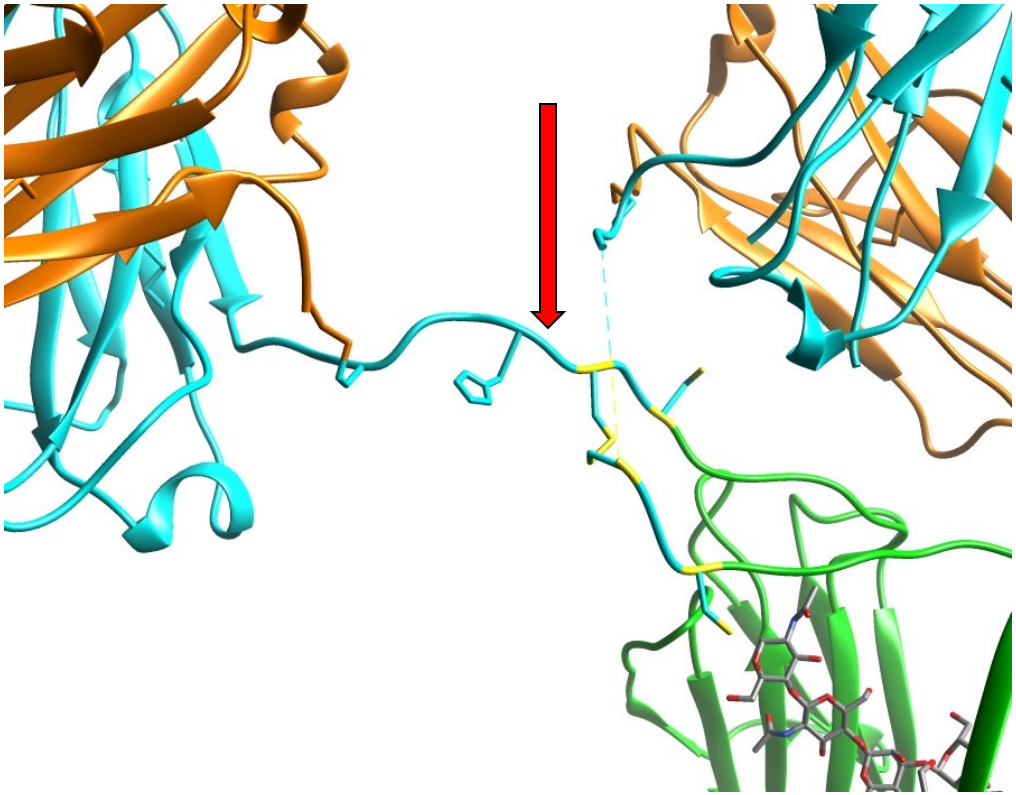
1. Abthrax (raxibacumab)	IgG1-lambda
2. Actemra (tocilizumab)	IgG1-kappa
3. Adcetrisc (brentuximab vedotin)	IgG1- ADC
4. AlprollXd (Factor IX Fc fusion protein)	IgG1-Fc + Factor IX
5. Arcalystf (rilonacept)	IgG1-Fc + IL1R
6. Arzerra (ofatumumab)	IgG1-kappa
7. Avastin (bevacizumab)	IgG1-kappa
8. Benlysta (belimumab)	IgG1-lambda
9. Cimziag (certolizumab pegol)	Fab'-PEG2MAL 40K
10. Cyramza (ramucirumab)	IgG1-kappa
11. Eloctateh (Factor VIII Fc fusion protein)	IgG1-Fc + Factor VIII
12. Enbrel (etanercept)	IgG1-Fc + TNFR
13. Entyvio (vedolizumab)	IgG1-kappa
14. Erbitux (cetuximab)	IgG1-kappa
15. Eyleaj (aflibercept)	IgG1-Fc + VEGF
16. Gazyva (obinutuzumab)	IgG1-kappa
17. Herceptin (trastuzumab)	IgG1-kappa
18. Humira (adalimumab)	IgG1-kappa
19. Ilaris (canakinumab)	IgG1-kappa
20. Inflectrak l (infliximab [biosimilar])	IgG1-kappa
21. Kadcylan (ado-trastuzumab emtansine)	IgG1-kappa + emtansine (ADC)
22. Keytruda (pembrolizumab)	IgG4-kappa
23. Lemtrada (alemtuzumab)	IgG1-kappa
24. Lucentiso (ranibizumab)	Fab from IgG1-kappa
25. Nplatep (romiplostim)	IgG1-Fc + peptide (trombopoeitin receptor)
26. Nulojixq (belatacept)	IgG1-Fc + CTLA-4
27. Orenciar (abatacept)	IgG1-Fc + CTLA-4
28. Perjeta (pertuzumab)	IgG1-kappa
29. Prolias (denosumab)	IgG2-kappa
30. Remicade (infliximab)	IgG1-kappa
31. Removabt (catumaxomab)	IgG2ab (rat-mouse hybrid)
32. Remsimak l (infliximab [biosimilar])	IgG1-kappa
33. ReoProu (abciximab)	Fab fragment
34. Rituxan (rituximab)	IgG1-kappa
35. Simponi/ Simponi Aria (golimumab)	IgG1-kappa
36. Simulect (basiliximab)	IgG1-kappa
37. Soliris (eculizumab)	IgG2/4-kappa
38. Stelara (ustekinumab)	IgG1-kappa
39. Sylvant (siltuximab)	IgG1-kappa
40. Synagis (palivizumab)	IgG1-kappa
41. Tysabri (natalizumab)	IgG4-kappa
42. Vectibix (panitumumab)	IgG2-kappa
43. Xgevas (denosumab)	IgG2-kappa
44. Xolair (omalizumab)	IgG1-kappa
45. Yervoy (ipilimumab)	IgG1-kappa
46. Zaltrapw (ziv-aflibercept)	IgG1-Fc + VEGF
47. Zevalinx (ibritumomab tiuxetan)	IgG1-kappa + linker + Yttrium-90

Dawn M Ecker, Susan Dana Jones, and Howard L Levine (2015) *The therapeutic monoclonal antibody market*, mAbs 7:1, 9—14

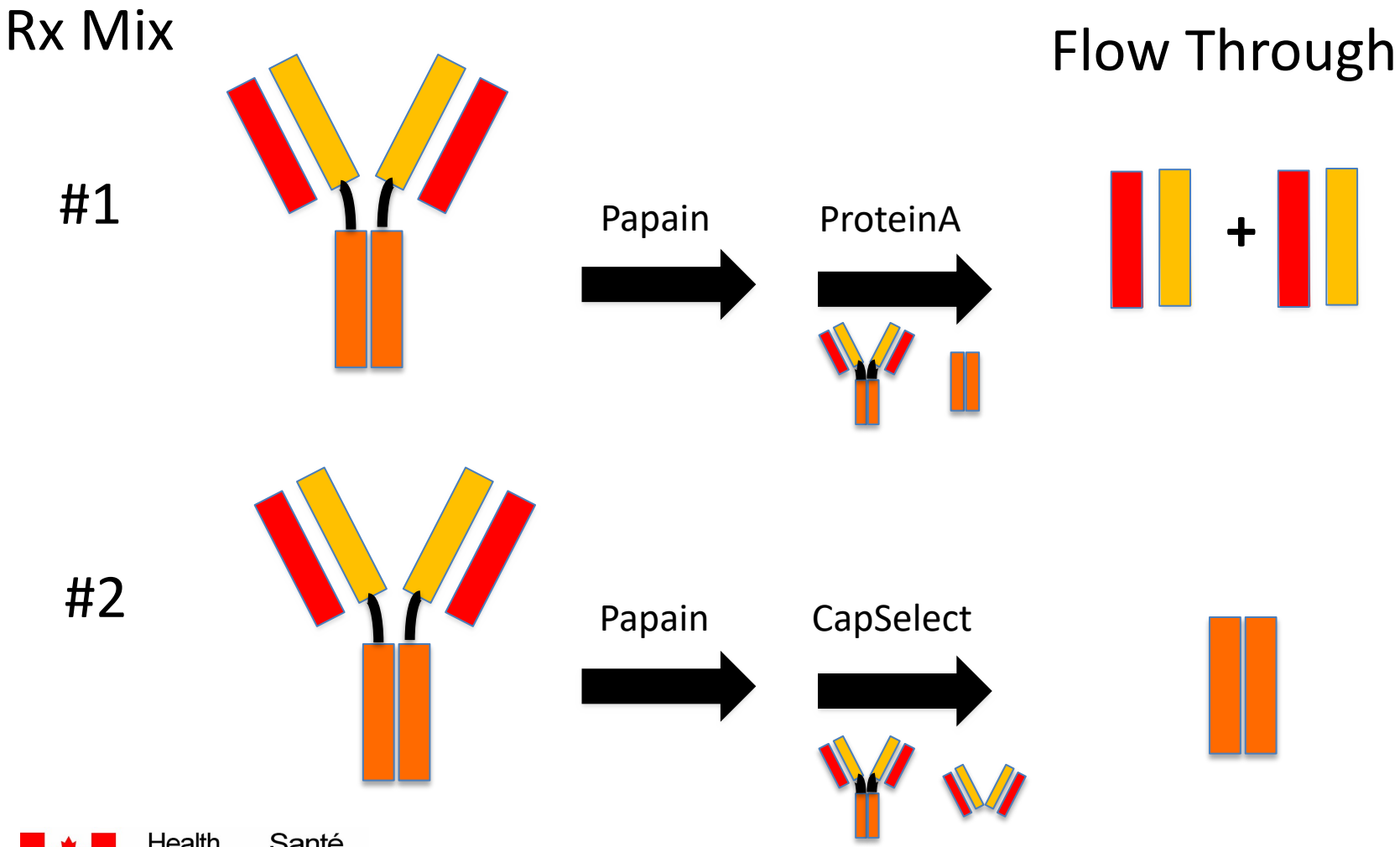
Therapeutic mAbs

Avastin® (bevacizumab)	IgG1-kappa
Herceptin® (trastuzumab)	
Humira® (adalimumab)	
Remicade® (infliximab)	
Rituxan® (rituximab)	
Enbrel® (etanercept)	IgG1-Fc + TNFR

Papain cleaves after His between Fab and Fc



Sample Preparation



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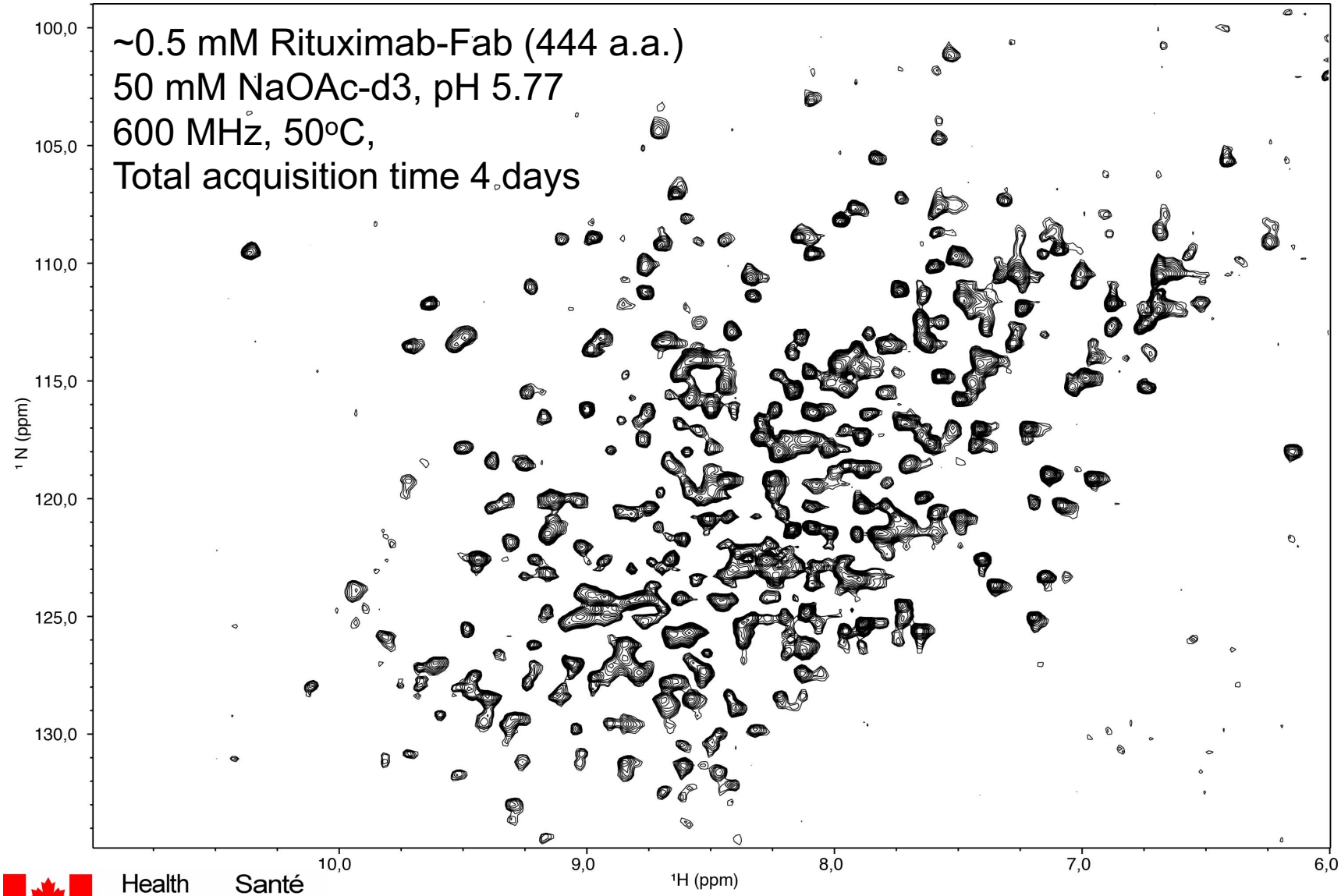
2D-NMR of Rituximab-Fab 600 MHz



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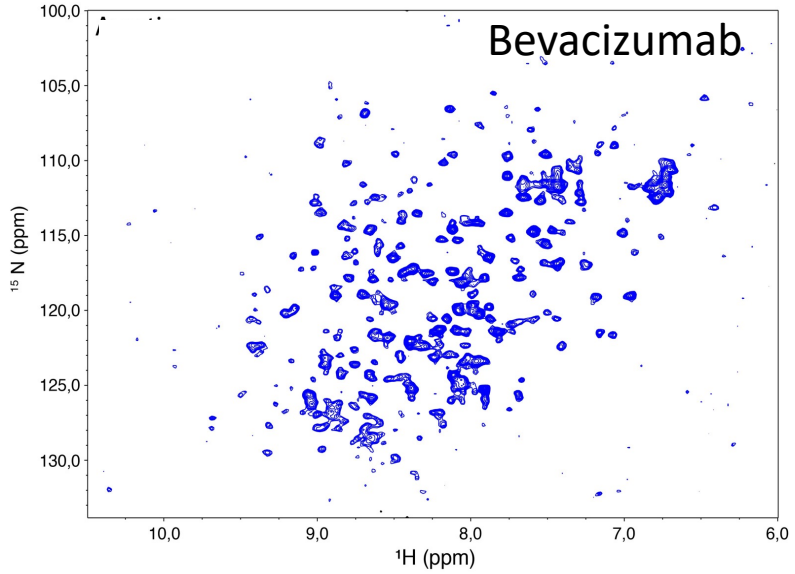
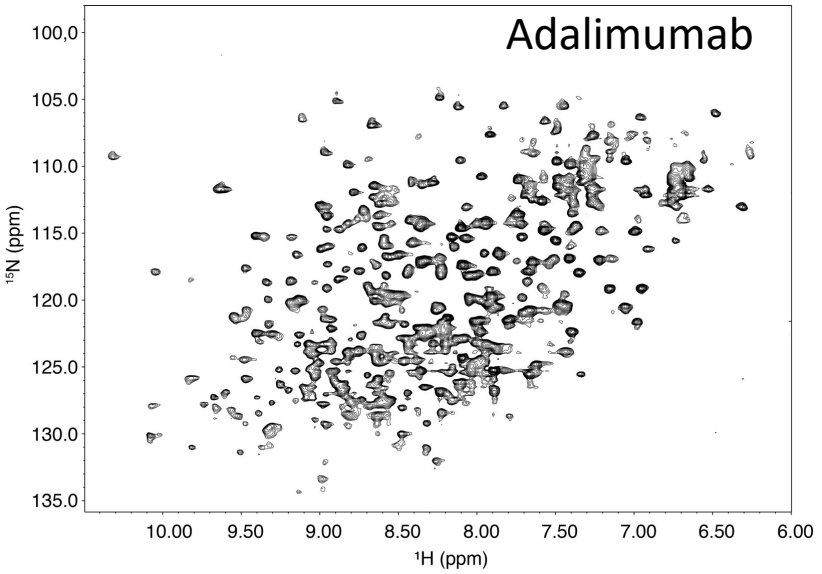
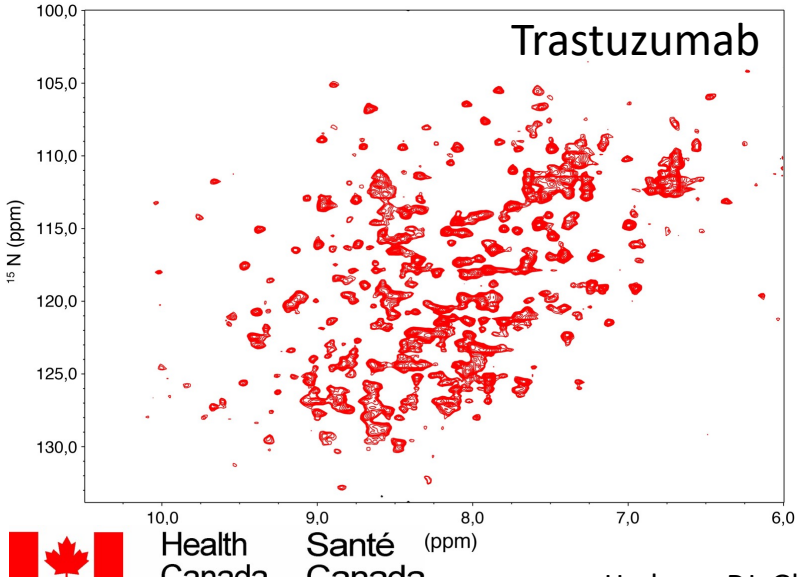
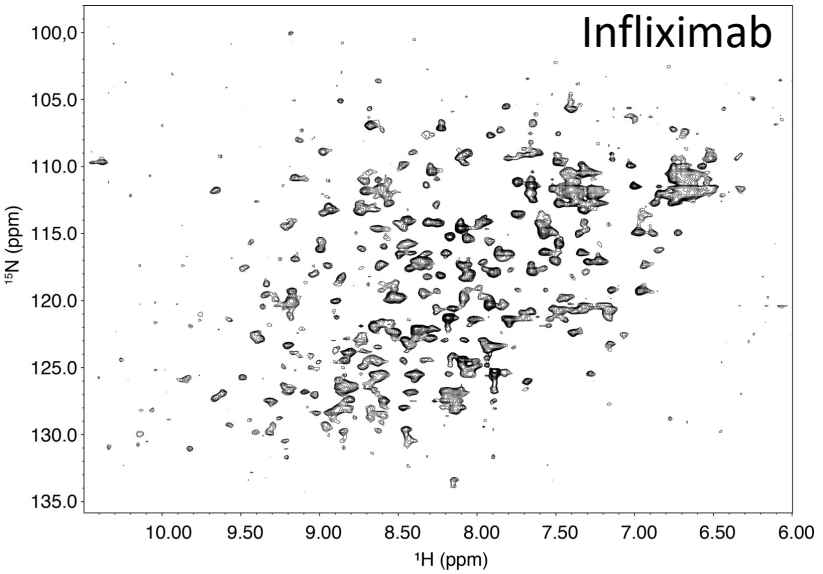
Hodgson DJ, Ghasriani H, Aubin Y. J Pharm Biomed Anal. 2018 Oct 1;163:144-152

2D-^{1}H,^{15}N-NMR of four Fab 700 MHz









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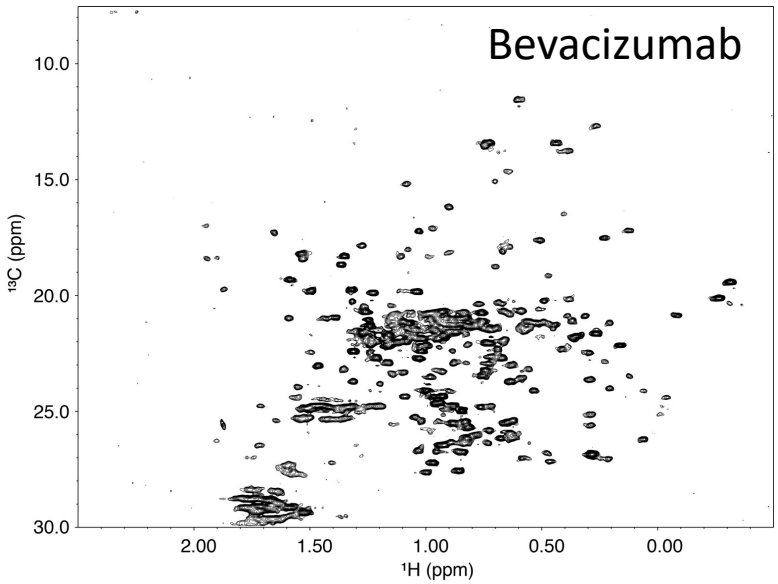
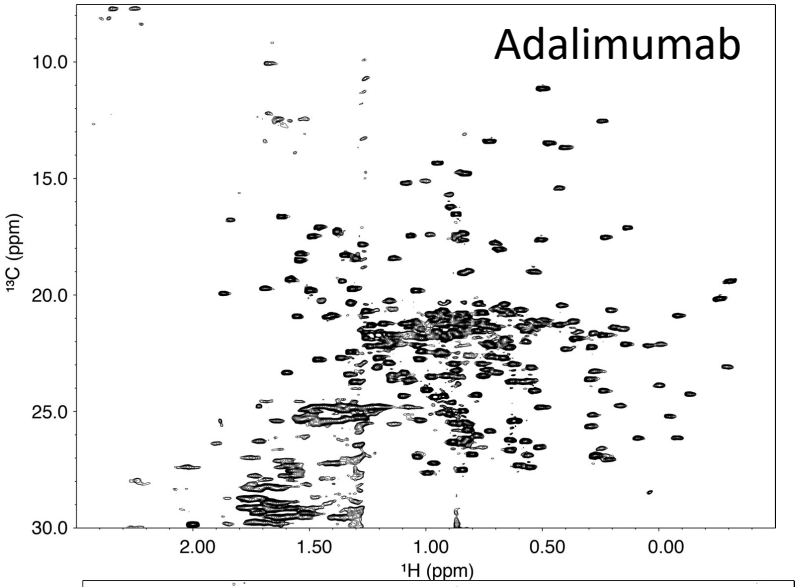
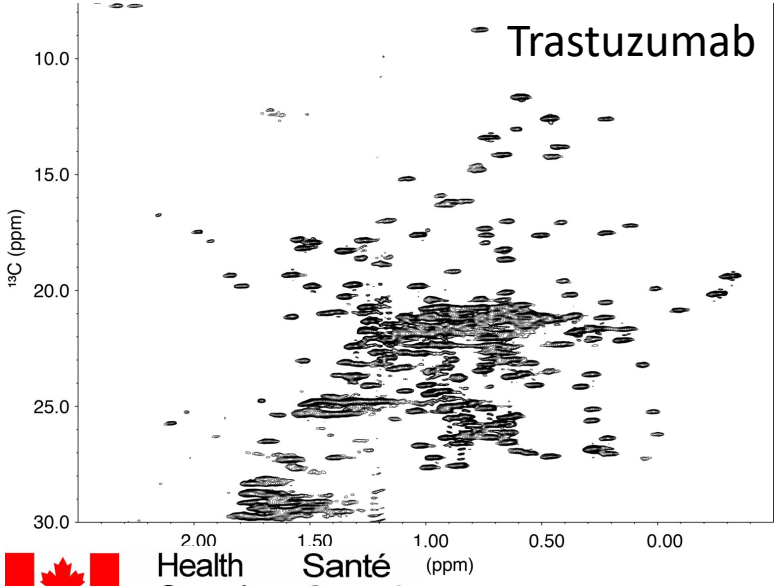
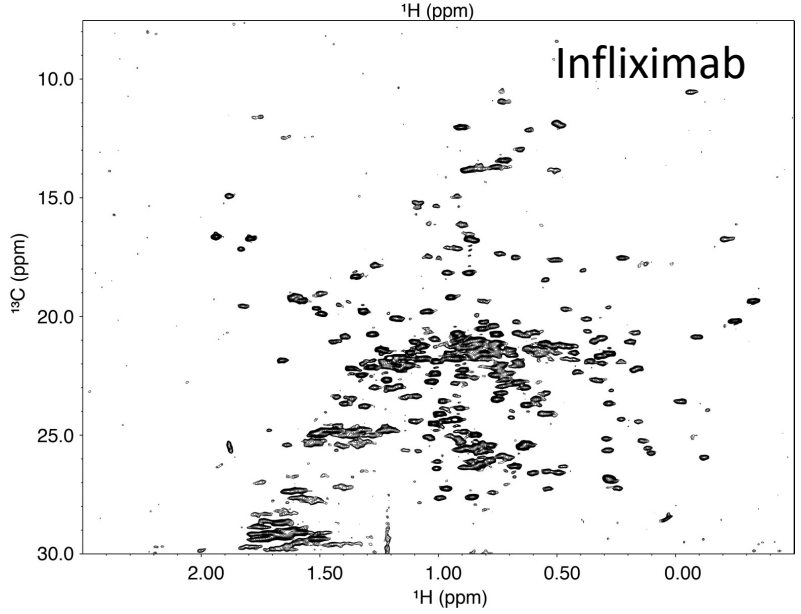
Hodgson DJ, Ghasriani H, Aubin Y. J Pharm Biomed Anal. 2018 Oct 1;163:144-152

2D-$^{1}H^{13}C$-NMR of four Fab 700 MHz









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Current Methods QA 2D-NMR

Electrospray ionization time-of-flight mass spectroscopy (ESI-TOF-MS)	Primary Sequence	YES
Reduced and non-reduced tryptic peptide mapping LC-MS	Primary Sequence	YES
Far-UV and Near-UV CD, FT-IR, Fluorescence etc.	Secondary and Tertiary structure	YES
Bioassays (Binding and Potency)	3D structure	YES

A Deeper Understanding via Assignment of NMR Resonances

A complete or near complete assignment can be:

A powerful tool to understand the significance (or lack of) of spectral changes with regard to the conformation or the dynamics of the drug substance.

A mean to monitor various perturbations (pH, solution conditions, excipients) at the amino acid level.

Isotopic labeling of NISTmAb Fragments in *Pichia Pastoris*

Fab

Construction of a bis-cistronic vector inserted in the methylotropic *Pichia Pastoris*
Polypeptide is secreted in the culture media after removal of the signal peptide.

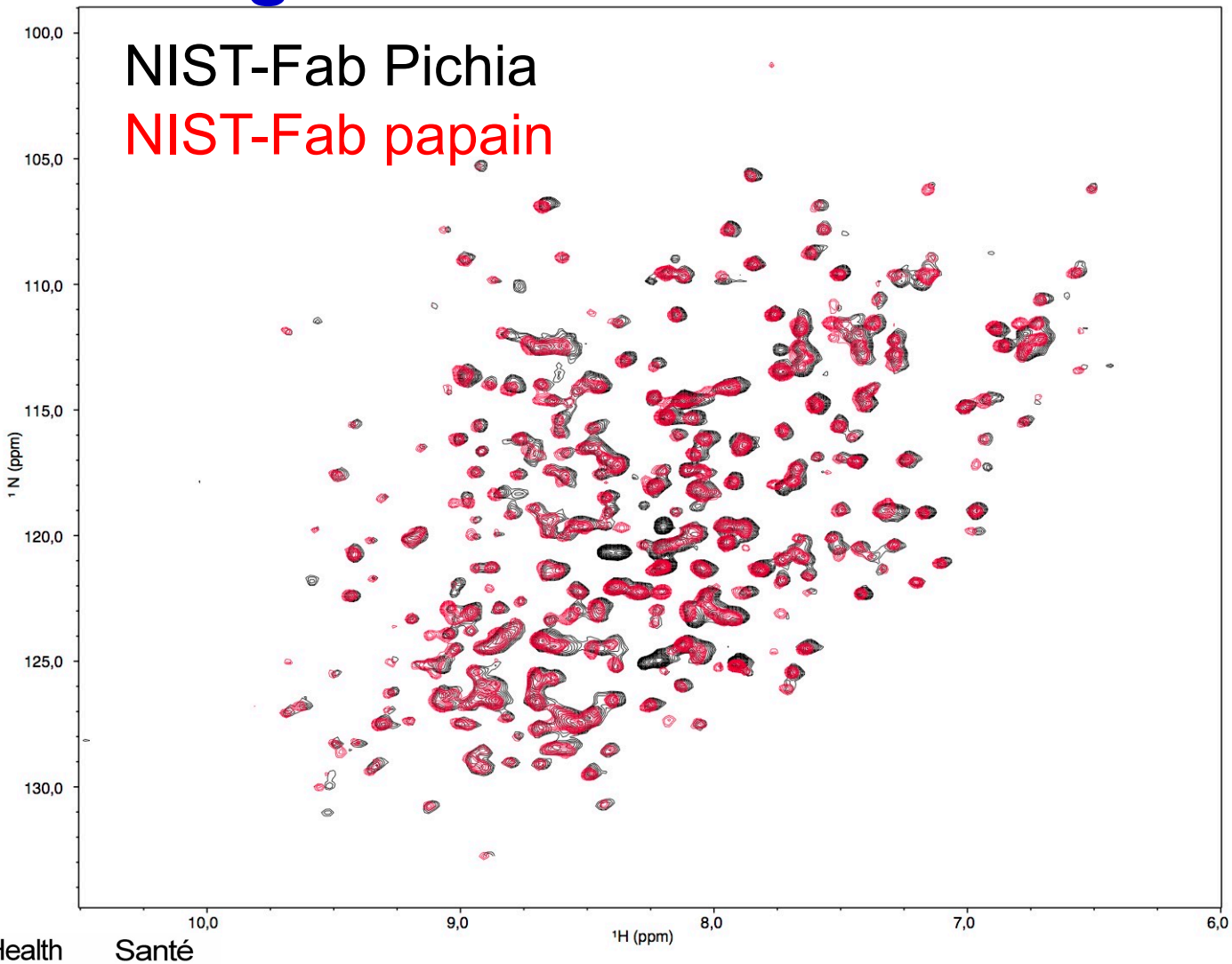
Signal Peptide-EKREAEA –N-ter(Heavy [V_H-C_H1])

Signal Peptide-EKREAEA –N-ter(Light [V_L-C_L])

Fc

Signal Peptide-EKREAEA –N-ter(Heavy [C_H2-C_H3])
The final polypeptide is glycosylated with a high-mannose glycan that is further hyper mannosylated (Glycan MW is ~5000 Da by SDS-PAGE).

Isotopic labeling of NISTmAb Fragments in *Pichia Pastoris*



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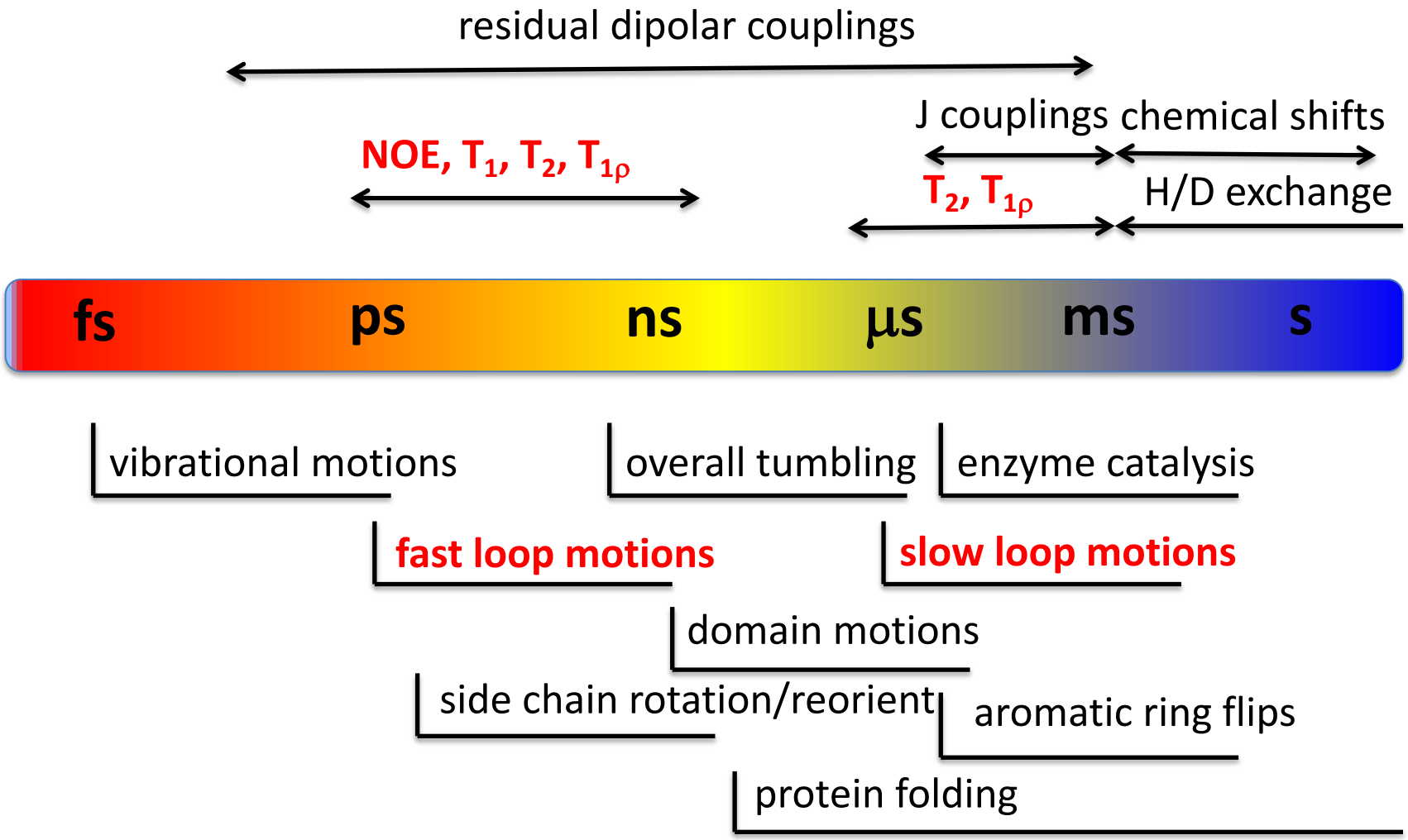
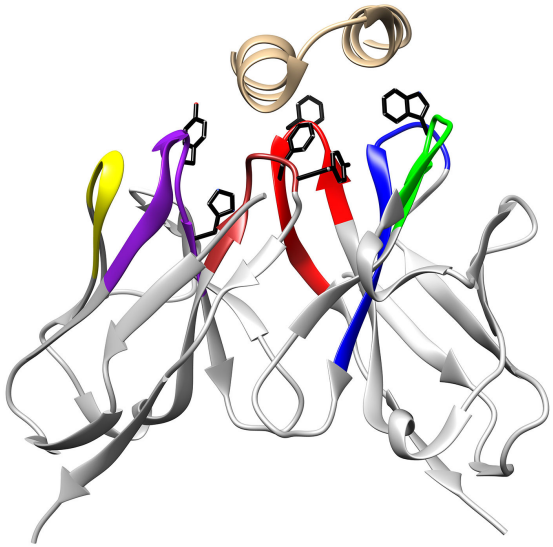
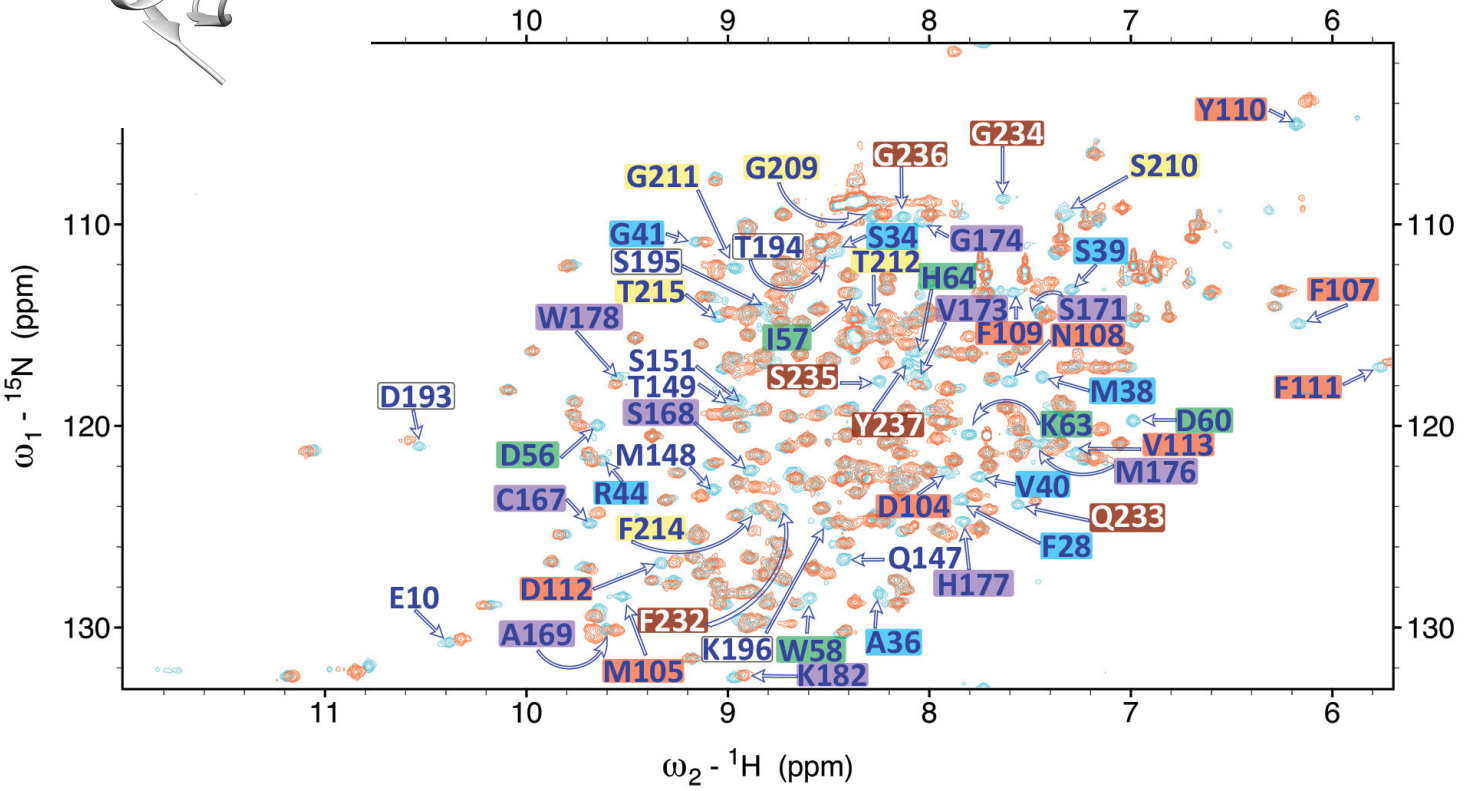
NMR Time Scale of Protein Dynamics



$^{13}C,^{15}N$-NISTmAb-scFv





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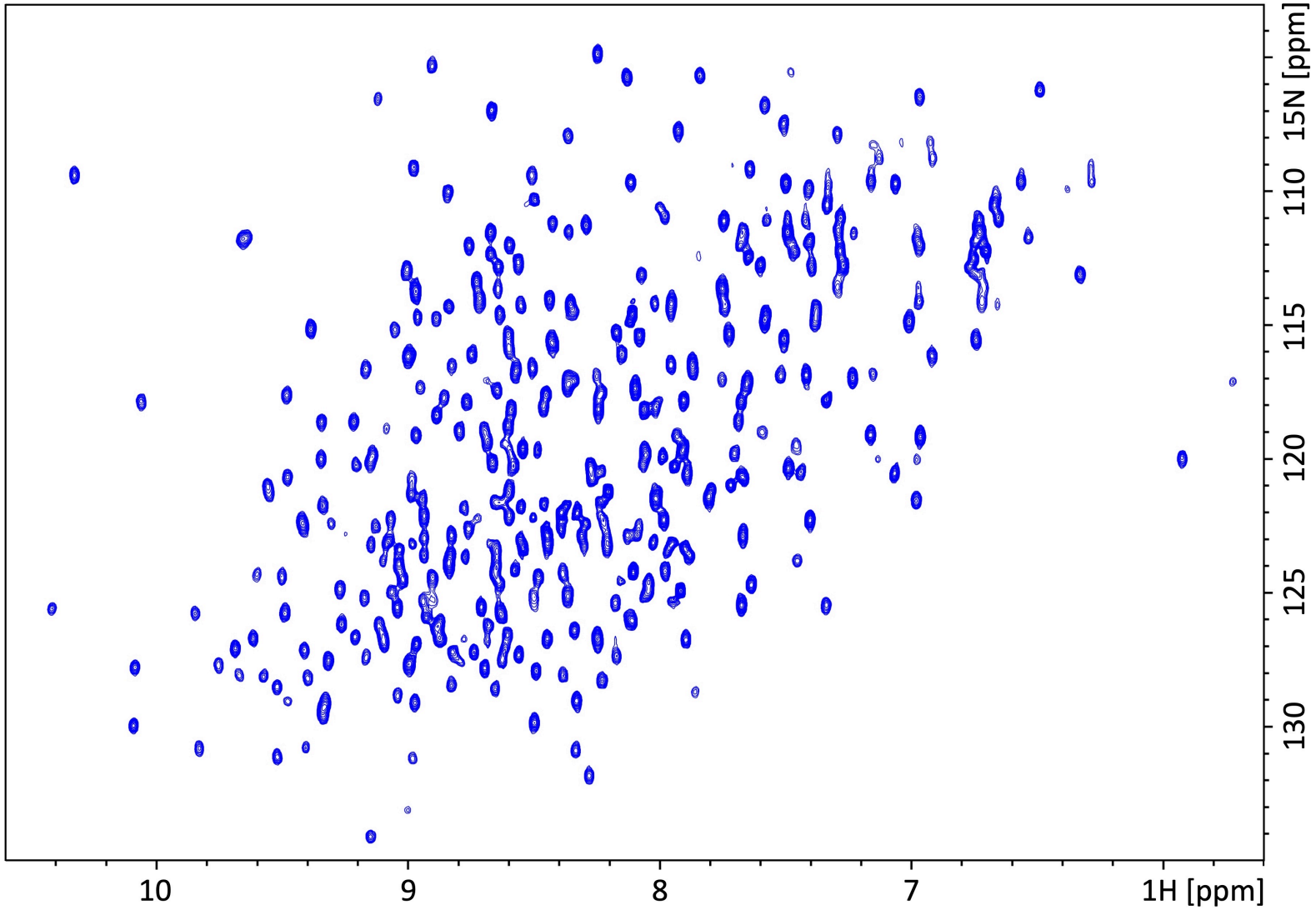
^{15}N-Adalimumab-Fab prepared from E.coli



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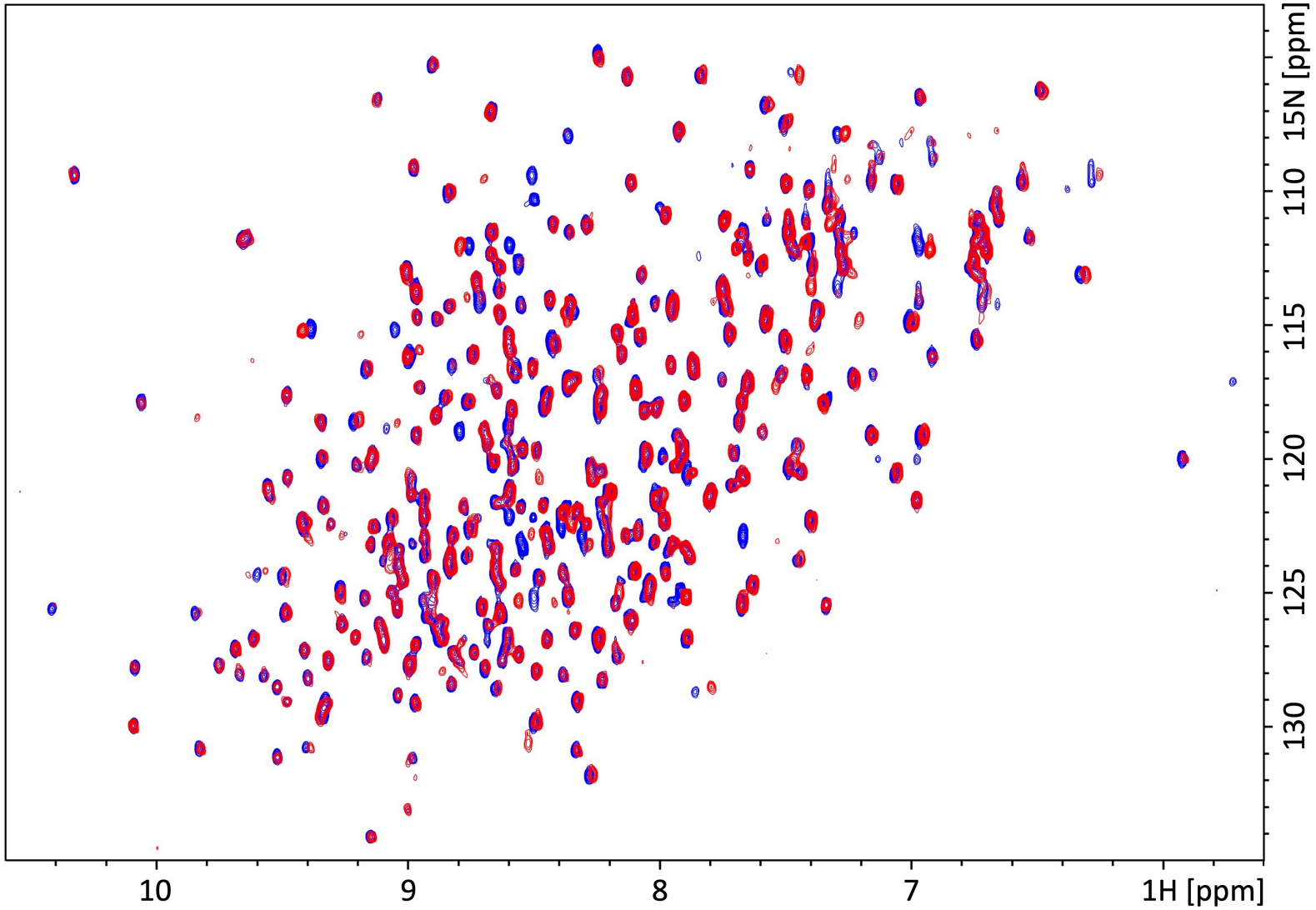
Humira®-Fab over ^{15}N-Adalimumab-Fab



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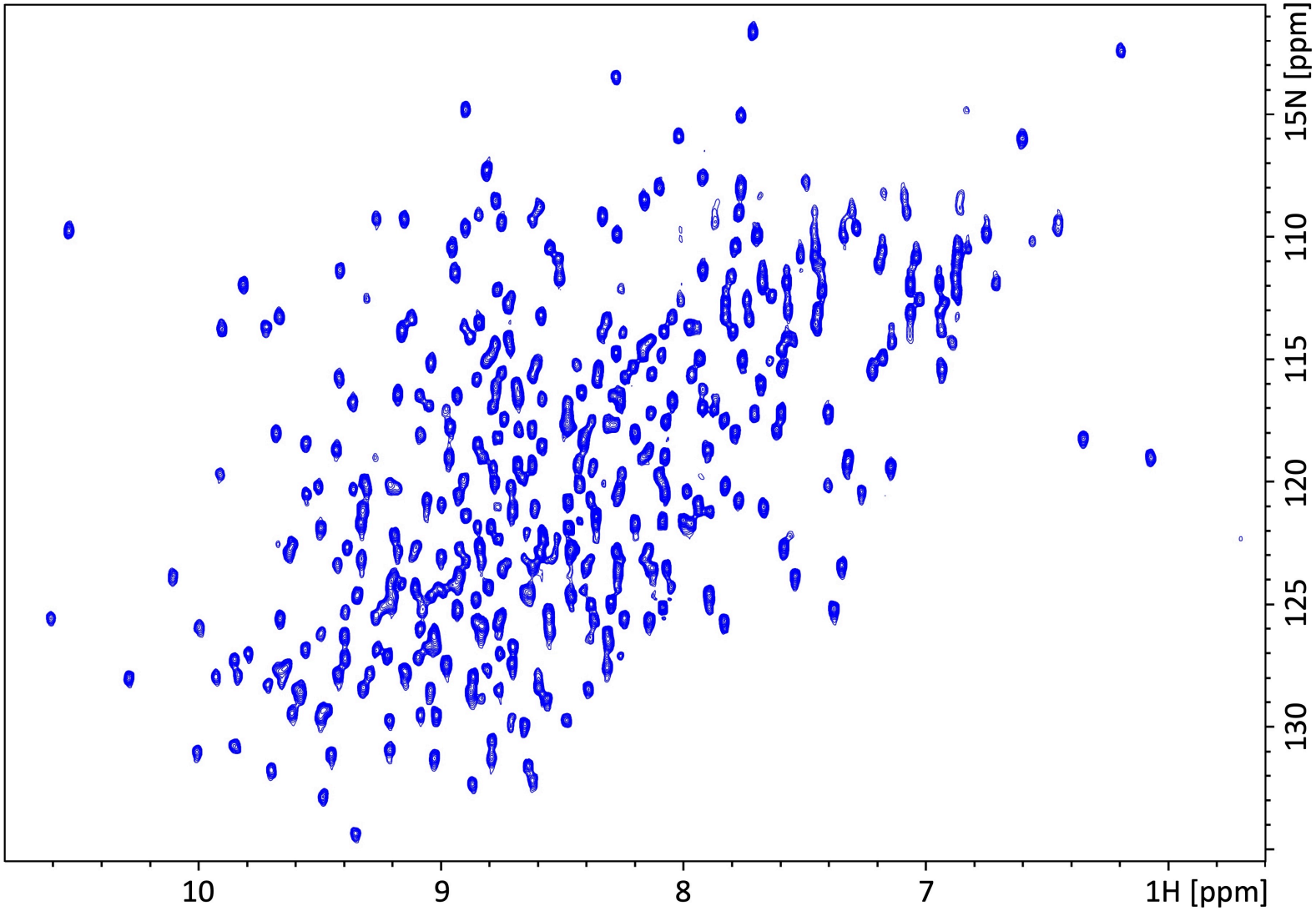
^{15}N-Rituximab-Fab prepared from E.coli



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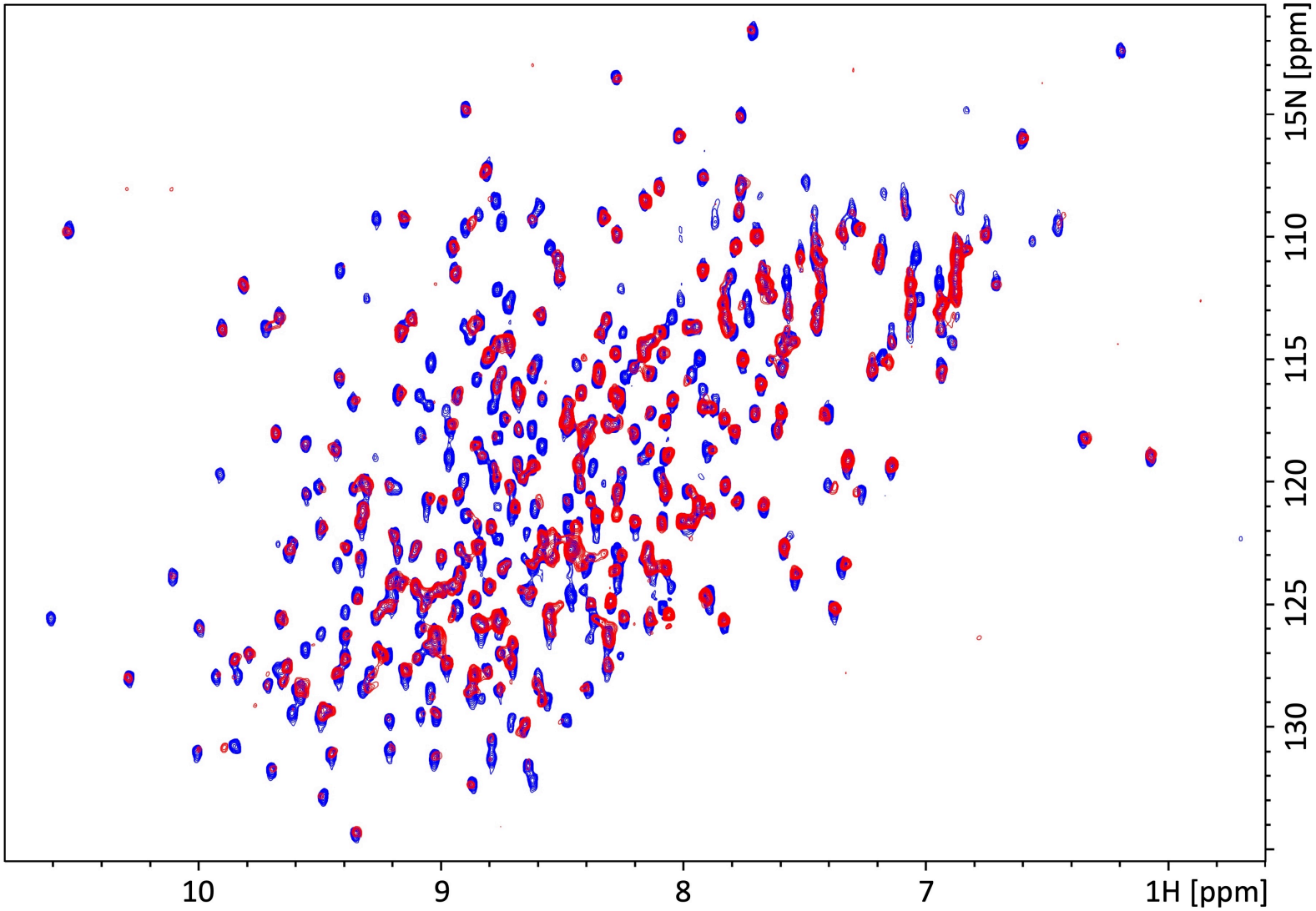
Rituxan®-Fab over ^{15}N-Rituximab-Fab



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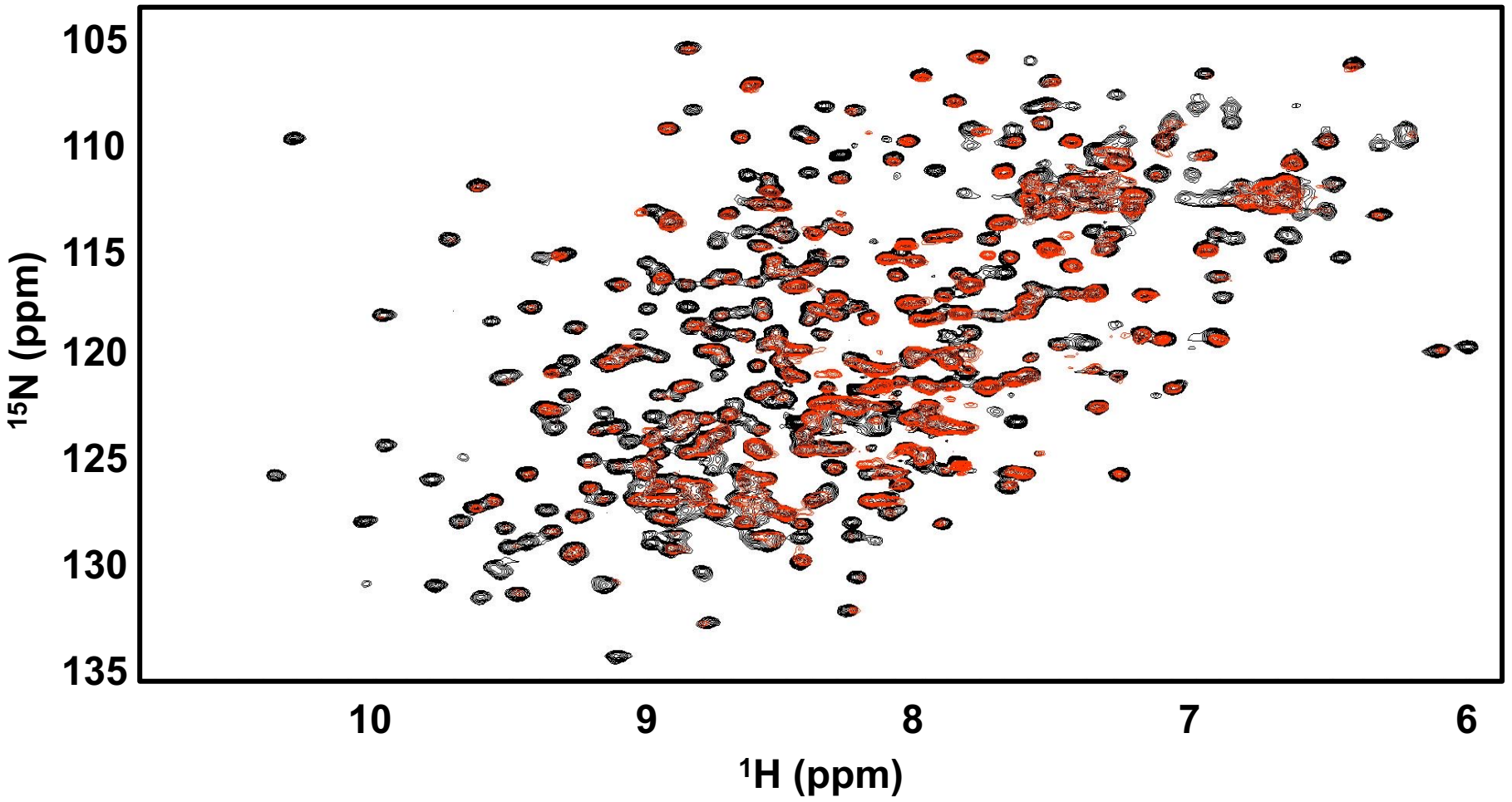
Herceptin®-Fab over ^{15}N-Trastuzumab-Fab



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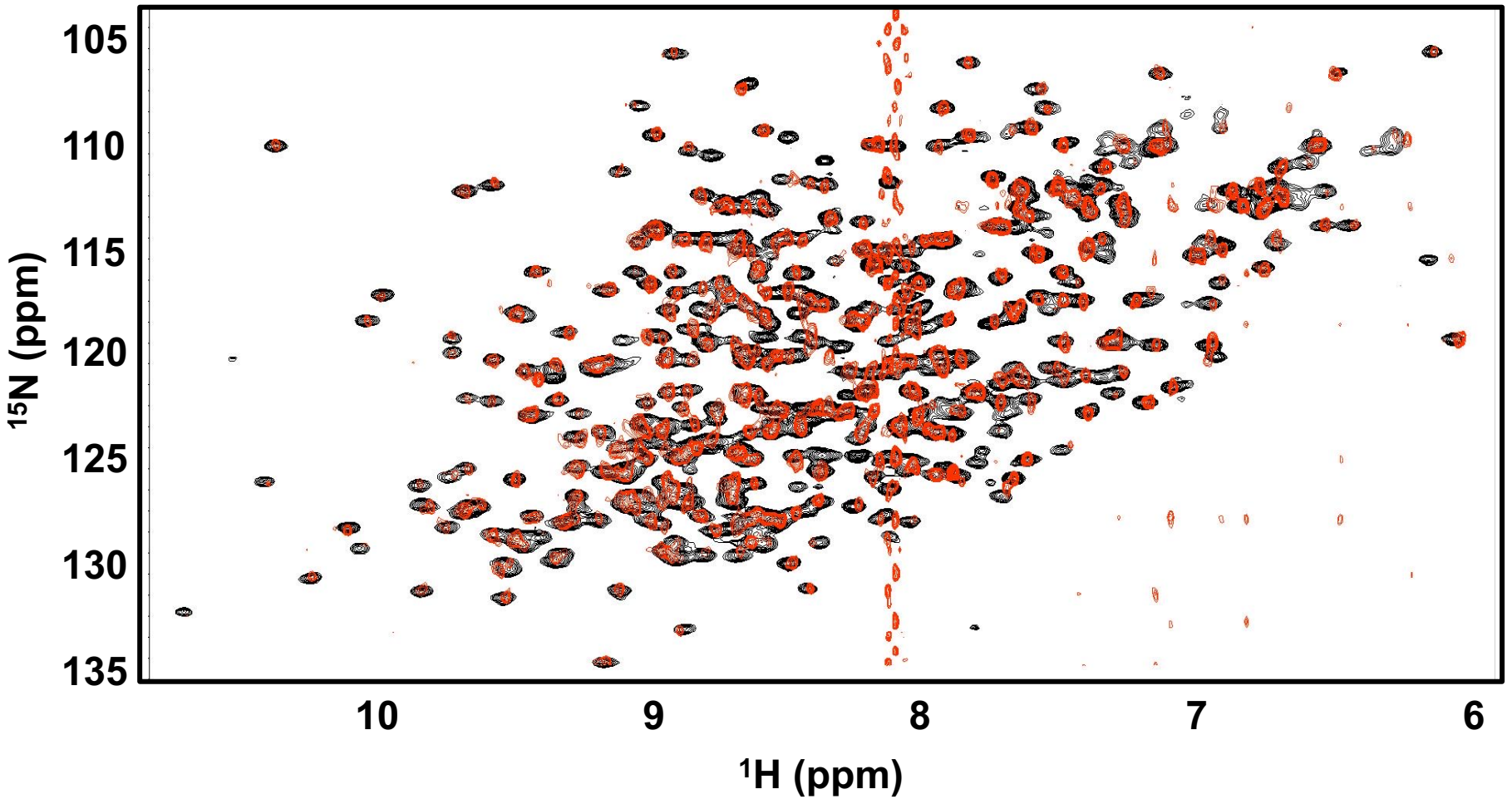
NISTmAb-Fab (900) over ^{15}N-NISTmAb-Fab



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Thank you