Formulation of Filgrastim Influences Protein Dynamics.

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CASSS Washington Discussion Group
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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 (²H,¹³C,¹⁵N) 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)

Aubin et al. Pharm. Res. 2015
# Formulation of Neupogen®

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount /0.5 mL</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCSF</td>
<td>300 µg</td>
<td>32 µM</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.295 mg</td>
<td>10 mM</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>25.0 mg</td>
<td>274 mM</td>
</tr>
<tr>
<td>Polysorbate 80</td>
<td>0.02 mg</td>
<td>0.03 mM</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.0175 mg</td>
<td>1.52 mM</td>
</tr>
<tr>
<td>Water</td>
<td>0.5 mL</td>
<td>---</td>
</tr>
</tbody>
</table>

Resulting pH is 4.0
A bit of NMR Concepts

$^1\text{H}, \quad ^{13}\text{C}, \quad ^{15}\text{N}$ =

$\begin{align*}
\text{N} &\quad \text{S} \\
\text{S} &\quad \text{N}
\end{align*}$
NMR Time Scale of Protein Dynamics

- Residual dipolar couplings
- NOE, $T_1, T_2, T_{1ρ}$
- $J$ couplings, chemical shifts
- $T_2, T_{1ρ}$, H/D exchange

**Time Scale**
- fs (femtoseconds)
- ps (picoseconds)
- ns (nanoseconds)
- μs (microseconds)
- ms (milliseconds)
- s (seconds)

**Motions**
- Vibrational motions
- Overall tumbling
- Enzyme catalysis
- Fast loop motions
- Slow loop motions
- Domain motions
- Side chain rotation/reorientation
- Aromatic ring flips
- Protein folding
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
Lowering pH: Cation-π Interaction
Lowering pH: Cation-$\pi$ Interaction
Lowering pH: Cation-π Interaction

H156 W58 H52

H79 W118
Effects of pH on Filgrastim Stability

GCSF - Thermal Denaturation at Varying pH Values

- pH4
- pH5
- pH6

Temperature (°C)

Fobs (normalized CD)
Effects of pH on Protein Dynamics

- \( T_1 \) in ms
- \( T_2 \) in ms
- hetNOE
Effects of pH on Protein Dynamics

Order Parameter ($S^2$) as a Function of pH

Sequence
Average of Order Parameters for Helices and Loops
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.
Loop AB
Loop CD

Glu-93
Glu-98
Asp-104

Cation-π

Loop AB
Loop CD

pH = 6

pH = 4
Effects of Excipients on Protein Dynamics

Tm for G-CSF with various excipients

Normalized Ellipticity

T / deg Celsius

GCSF pH4
GCSF+Tw80
GCSF+Tw20
GCSF+Sorb
Sorbitol-induced chemical shift changes (spectra acquired at 45 °C) are predominantly affecting Trp\textsuperscript{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
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
Average of Order Parameters for Helices and Loops

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

Ghasriani H, Frahm GE, Johnston MJ and Aubin Y (2020), ACS Omega
From Filgrastim to Therapeutic Monoclonal Antibodies

Fragment antigen binding (Fab):
\[ \text{V}_H \text{ C}_H1 \text{ V}_L \text{ C}_L \]
Fragment crystallisable (Fc):
\[ \text{C}_H2 \text{ C}_H3 \]
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)

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 Ghasrani,c Yves Aubin,c Gregory K. Pierens,d Xinying Jia,d Mehdi Mobli,d Hamish G. Grant,e David W. Keizer,e Kristian Schweimer,f Jonas Stähle,g Göran Widmalm,g Edward R. Zartler,h Chad W. Lawrence,i Patrick N. Readon,† i John R. Cort,i Ping Xu,j Feng Ni,j Saeko Yanaka,k Koichi Kato,k Stuart R. Parnham,k Desiree Tsaol, m Andreas Blomgren,n Torgny Rundlöf,n Nils Trierloff,o Peter Schmieder,o Alfred Ross,p Ken Skidmore,q Kang Chen,r David Keire,r Darón I. Freedberg,s Thea Suter-Stahel,t Gerhard Wider,t Gregor Iic,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. Amezcuta, bb John B. Jordan, cc and Mats Wikströmdd

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vNMR Centre, National Institute of Chemistry, Ljubljana, Slovenia
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ddHigher Order Structure, Attribute Sciences, Amgen Inc., Thousand Oaks, CA, USA
# Approved Therapeutic mAbs in US or EU
(as of November 2014)

<table>
<thead>
<tr>
<th>Approved mAb</th>
<th>Type of mAb</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abthrax (ruxibumab)</td>
<td>IgG1-lambda</td>
<td>IgG1-lambda</td>
</tr>
<tr>
<td>Actemra (tocilizumab)</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
</tr>
<tr>
<td>Adcetris (brentuximab vedotin)</td>
<td>IgG1-ADC</td>
<td>IgG1- ADC</td>
</tr>
<tr>
<td>AlprollXd (Factor IX Fc fusion protein)</td>
<td>IgG1-Fc + Factor IX</td>
<td>IgG1-Fc + Factor IX</td>
</tr>
<tr>
<td>Arcalystf</td>
<td>IgG1-Fc + IL1R</td>
<td>IgG1-Fc + IL1R</td>
</tr>
<tr>
<td>Arzerra (ofatumumab)</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
</tr>
<tr>
<td>Avastin (bevacizumab)</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
</tr>
<tr>
<td>Benlysta (belimumab)</td>
<td>IgG1-lambda</td>
<td>IgG1-lambda</td>
</tr>
<tr>
<td>Cimziag (certolizumab pegol)</td>
<td>Fab'-PEG2MAL 40K</td>
<td>Fab'-PEG2MAL 40K</td>
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<tr>
<td>Cyramza (ramucirumab)</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
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<tr>
<td>El sectateh (Factor VIII Fc fusion protein)</td>
<td>IgG1-Fc + Factor VIII</td>
<td>IgG1-Fc + Factor VIII</td>
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<tr>
<td>Enbrel (etanercept)</td>
<td>IgG1-Fc + TNFR</td>
<td>IgG1-Fc + TNFR</td>
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<td>Entyvio (vedolizumab)</td>
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<td>Erbitux (cetuximab)</td>
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<td>Eyleaj (afibercept)</td>
<td>IgG1-Fc + VEGF</td>
<td>IgG1-Fc + VEGF</td>
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<td>Gazyva (obinutuzumab)</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
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<tr>
<td>Herceptin (trastuzumab)</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
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<tr>
<td>Humira (adalimumab)</td>
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<td>IgG1-kappa</td>
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<td>Ilaris (canakinumab)</td>
<td>IgG1-kappa</td>
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</tr>
<tr>
<td>Inflectra l (infliximab [biosimilar])</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
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<tr>
<td>Kadrylan (ado-trastuzumab emtansine)</td>
<td>IgG1-kappa + emtansine (ADC)</td>
<td>IgG1-kappa + emtansine (ADC)</td>
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<tr>
<td>Keytruda (pembrolizumab)</td>
<td>IgG4-kappa</td>
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<tr>
<td>Lemtrada (alemtuzumab)</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
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<tr>
<td>Lucentiso (ranibizumab)</td>
<td>Fab from IgG1-kappa</td>
<td>Fab from IgG1-kappa</td>
</tr>
<tr>
<td>Nplatep (romiplostim)</td>
<td>IgG1-Fc + peptide (trombopoietin receptor)</td>
<td>IgG1-Fc + peptide (trombopoietin receptor)</td>
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<tr>
<td>Nulojix (belatacept)</td>
<td>IgG1-Fc + CTLA-4</td>
<td>IgG1-Fc + CTLA-4</td>
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<td>Orencriar (abatacept)</td>
<td>IgG1-Fc + CTLA-4</td>
<td>IgG1-Fc + CTLA-4</td>
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<tr>
<td>Perjeta (pertuzumab)</td>
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<td>IgG1-kappa</td>
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<tr>
<td>Prolias (denosumab)</td>
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<tr>
<td>Remicade (infliximab)</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
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<tr>
<td>Removabt (catumaxomab)</td>
<td>IgG2ab (rat-mouse hybrid)</td>
<td>IgG2ab (rat-mouse hybrid)</td>
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<td>Remsimak l (infliximab [biosimilar])</td>
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<td>IgG1-kappa</td>
</tr>
<tr>
<td>ReoProu (abciximab)</td>
<td>Fab fragment</td>
<td>Fab fragment</td>
</tr>
<tr>
<td>Rituxan (rituximab)</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
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<tr>
<td>Simponi/ Simponi Aria (golimumab)</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
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<tr>
<td>Simulect (basiliximab)</td>
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<td>IgG1-kappa</td>
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<tr>
<td>Soliris (eculizumab)</td>
<td>IgG2/4-kappa</td>
<td>IgG2/4-kappa</td>
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<td>Stelara (ustekinumab)</td>
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<td>IgG1-kappa</td>
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<td>Sylvant (siltuximab)</td>
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<td>Synagis (palivizumab)</td>
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<td>IgG1-kappa</td>
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<td>Ty sabri (natalizumab)</td>
<td>IgG4-kappa</td>
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<tr>
<td>Vectibix (panitumumab)</td>
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<td>Xgevas (denosumab)</td>
<td>IgG2-kappa</td>
<td>IgG2-kappa</td>
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<tr>
<td>Xolair (omalizumab)</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
</tr>
<tr>
<td>Yervoy (ipilimumab)</td>
<td>IgG1-kappa</td>
<td>IgG1-kappa</td>
</tr>
<tr>
<td>Zalm tra p (ziv-afiblerecept)</td>
<td>IgG1-Fc + VEGF</td>
<td>IgG1-Fc + VEGF</td>
</tr>
<tr>
<td>Zevalin (ibritumomab tiuxetan)</td>
<td>IgG1-kappa + linker + Yttrium-90</td>
<td>IgG1-kappa + linker + Yttrium-90</td>
</tr>
</tbody>
</table>

Therapeutic mAbs

Avastin® (bevacizumab)
Herceptin® (trastuzumab)
Humira® (adalimumab)
Remicade® (infliximab)
Rituxan® (rituximab)
Enbrel® (etanercept)
Papain cleaves after His between Fab and Fc
Sample Preparation

Rx Mix

#1

#2

Flow Through

Papain → ProteinA

+ CapSelect
2D-NMR of Rituximab-Fab 600 MHz

~0.5 mM Rituximab-Fab (444 a.a.)
50 mM NaOAc-d3, pH 5.77
600 MHz, 50° C,
Total acquisition time 4 days
2D-$^1$H,$^{15}$N-NMR of four Fab 700 MHz

Bevacizumab

Adalimumab

Trastuzumab

Infliximab

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

**Bevacizumab**

**Adalimumab**

**Trastuzumab**

**Infliximab**

<table>
<thead>
<tr>
<th>Current Methods</th>
<th>QA</th>
<th>2D-NMR</th>
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<tbody>
<tr>
<td>Electrospray ionization time-of-flight mass</td>
<td>Primary Sequence</td>
<td>YES</td>
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<tr>
<td>spectroscopy (ESI-TOF-MS)</td>
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<tr>
<td>Reduced and non-reduced tryptic peptide mapping</td>
<td>Primary Sequence</td>
<td>YES</td>
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<tr>
<td>LC-MS</td>
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<tr>
<td>Far-UV and Near-UV CD, FT-IR, Fluorescence etc.</td>
<td>Secondary and Tertiary structure</td>
<td>YES</td>
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<tr>
<td>Bioassays (Binding and Potency)</td>
<td>3D structure</td>
<td>YES</td>
</tr>
</tbody>
</table>
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 methylotrophic *Pichia Pastoris* Polypeptide is secreted in the culture media after removal of the signal peptide.

**Signal Peptide-EKRE1EA –N-ter(Heavy [V_H-C_H1])**

**Signal Peptide-EKRE1EA –N-ter(Light [V_L-C_L])**

**Fc**

**Signal Peptide-EKRE1EA –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*

NIST-Fab Pichia

NIST-Fab papain
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- Side chain rotation/reorientation
- Aromatic ring flips
- Protein folding
$^{13}$C, $^{15}$N-NISTmAb-scFv
$^{15}$N-Adalimumab-Fab prepared from E. coli
Humira®-Fab over $^{15}$N-Adalimumab-Fab
$^{15}$N-Rituximab-Fab prepared from E.coli
Rituxan®-Fab over $^{15}$N-Rituximab-Fab
Herceptin®-Fab over $^{15}\text{N}-\text{Trastuzumab-Fab}$
NISTmAb-Fab (900) over $^{15}\text{N}$-NISTmAb-Fab
Thank you