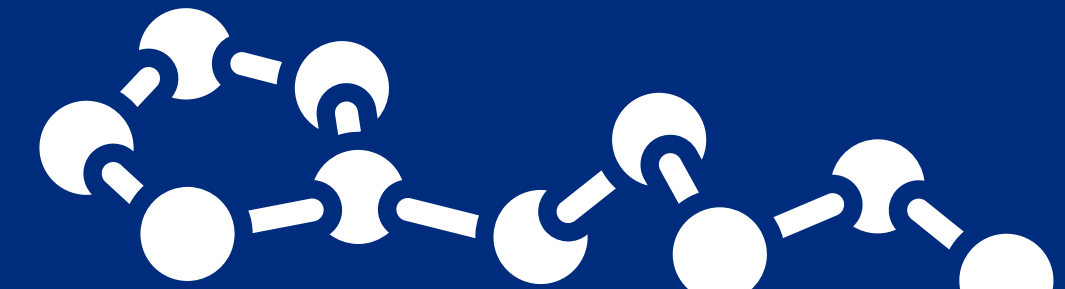


SARomics

Accelerating Discovery Through Structural Insight

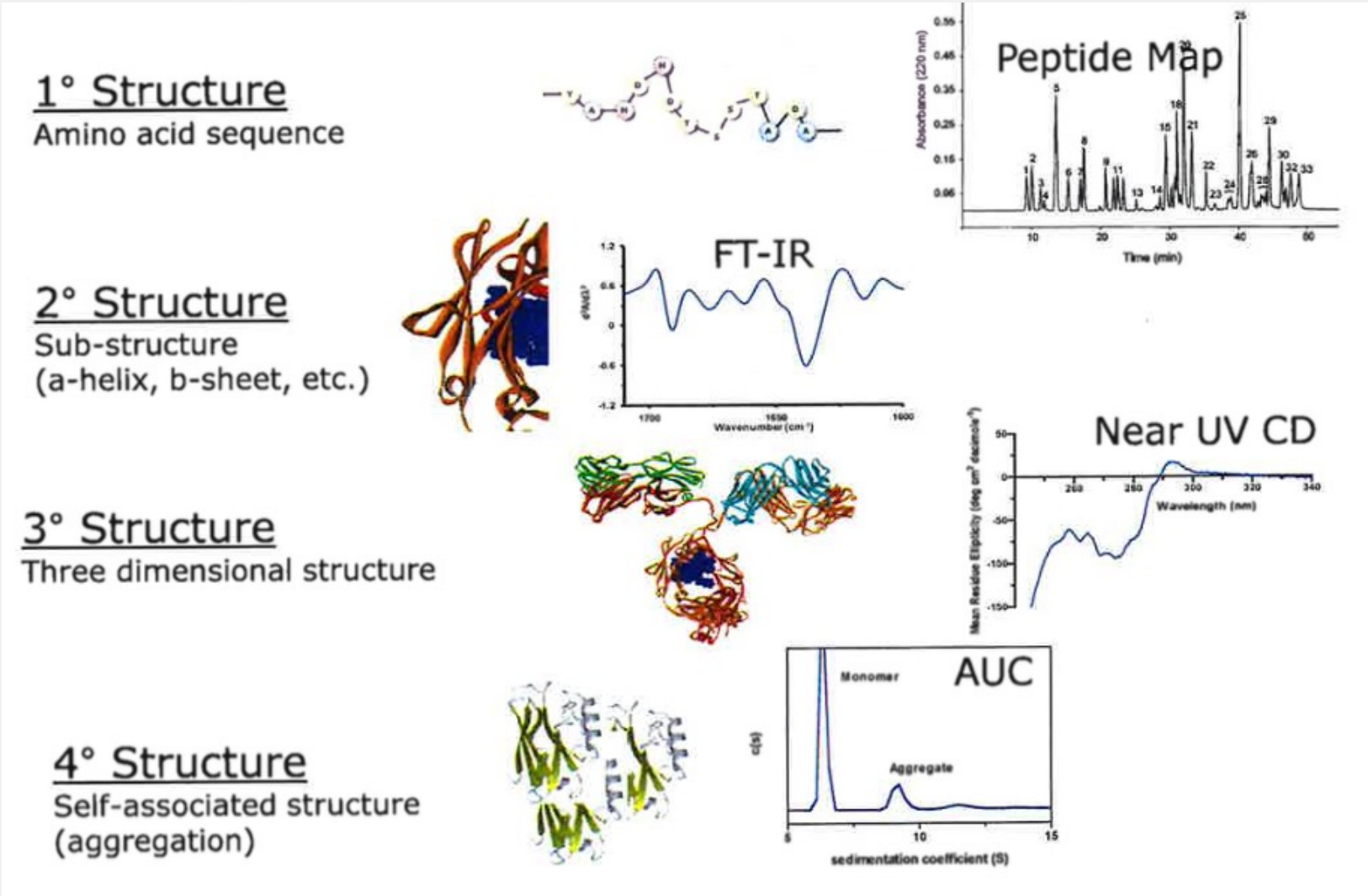
v. 230124

Higher order structure (HOS) assessment, comparability and characterization using NMR spectroscopy

Carl Diehl
2024-01-25

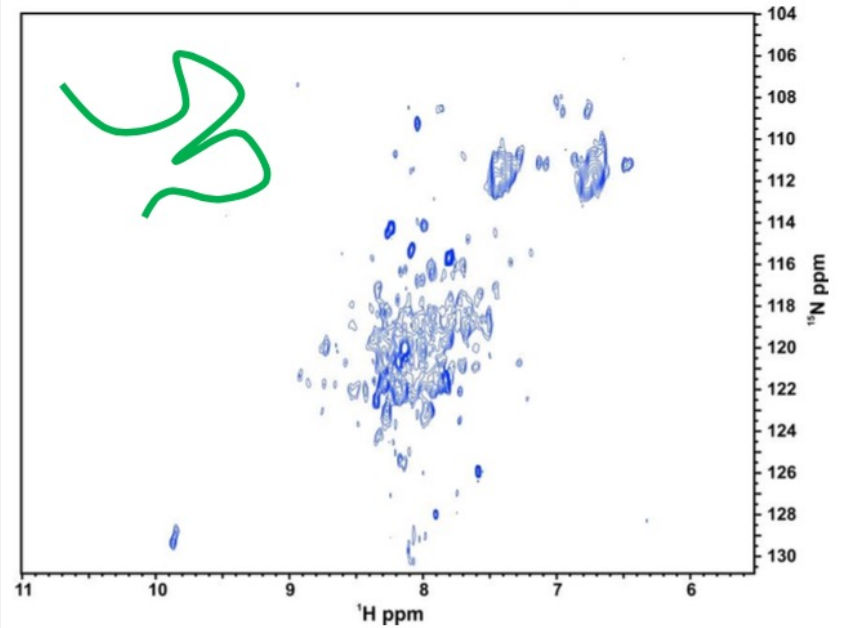
Proteins are complex molecules that form multiple levels of structure

Traditionally higher order structure (HOS) analysis has been performed using indirect methods like: MS, FT-IR, circular dichroism, dynamic light scattering etc.



NMR chemical shifts are sensitive to HOS

Filgrastim: amide fingerprints



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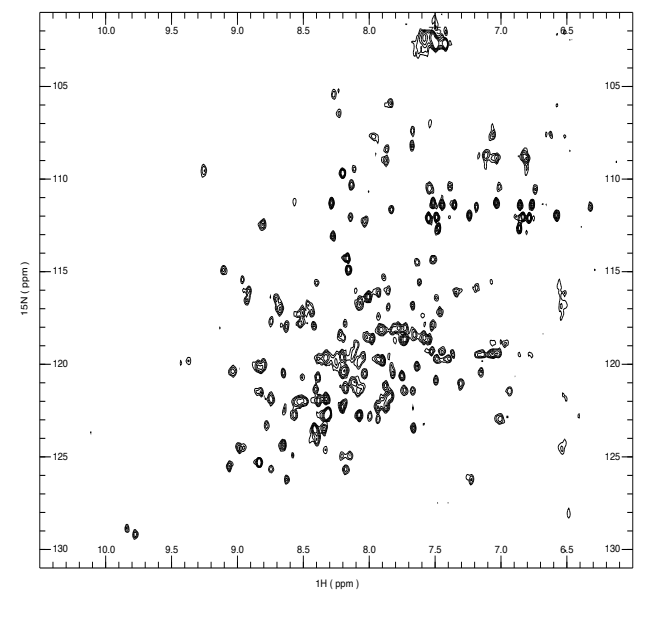

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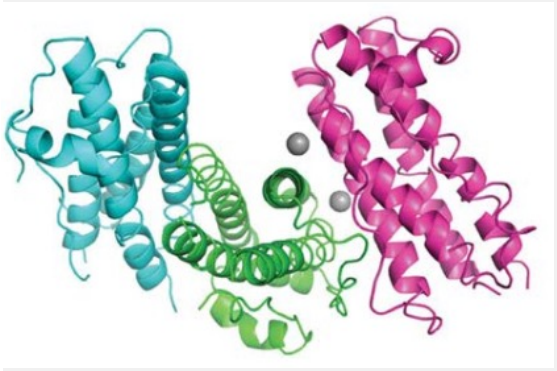
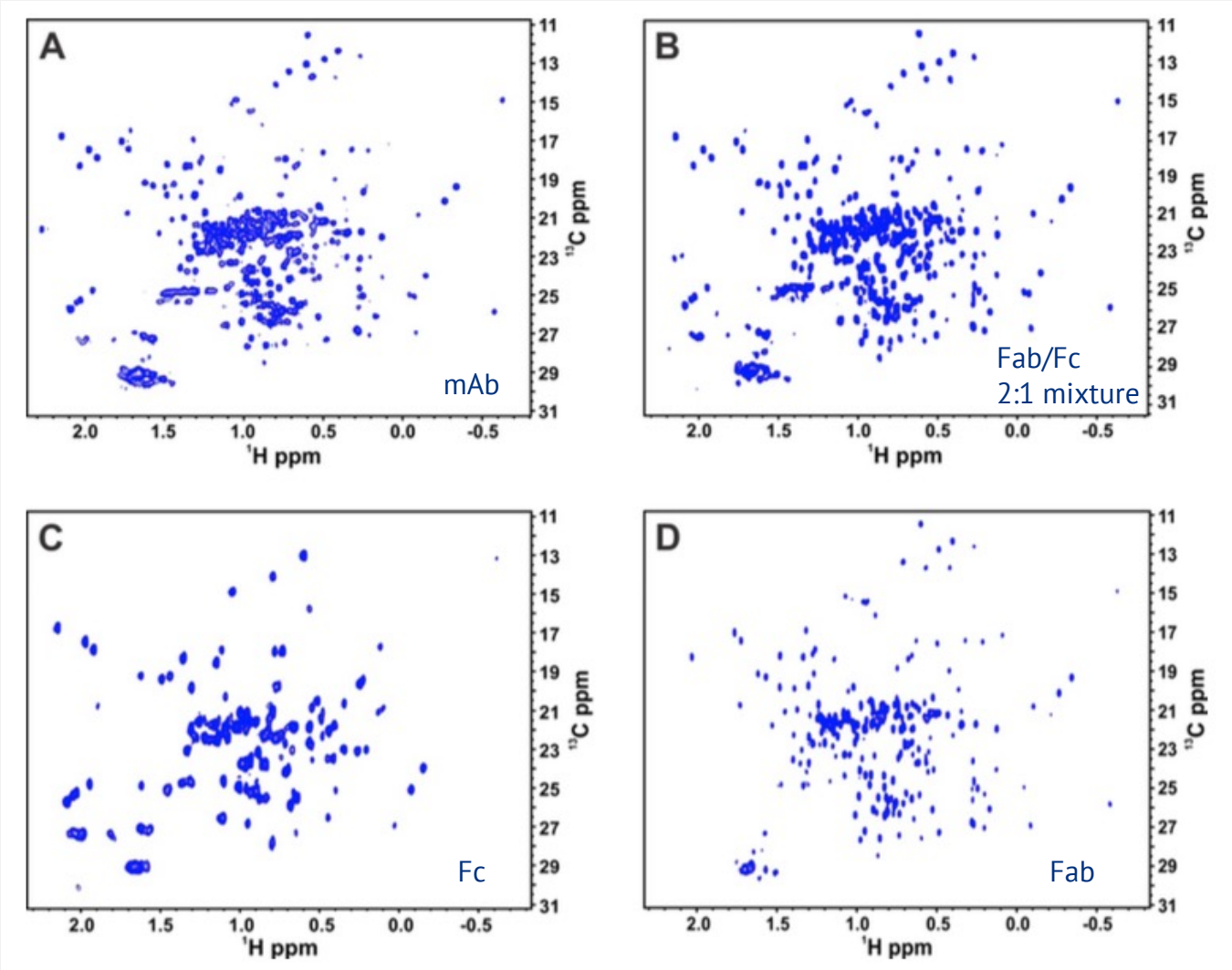


Of all the HOS techniques, 2D-NMR is the only analytical method that can simultaneously yield precise and accurate information about the primary, secondary, tertiary, and quaternary (i.e., HOS) structure of a biopharmaceutical in solution with atomic level resolution.

15N-SOFAST-HMQC spectrum of the biosimilar pegfilgrastim at 950 MHz for 30 h (acquired by SARomics Biostructures).

NMR probes directly into the 3D structure of a protein

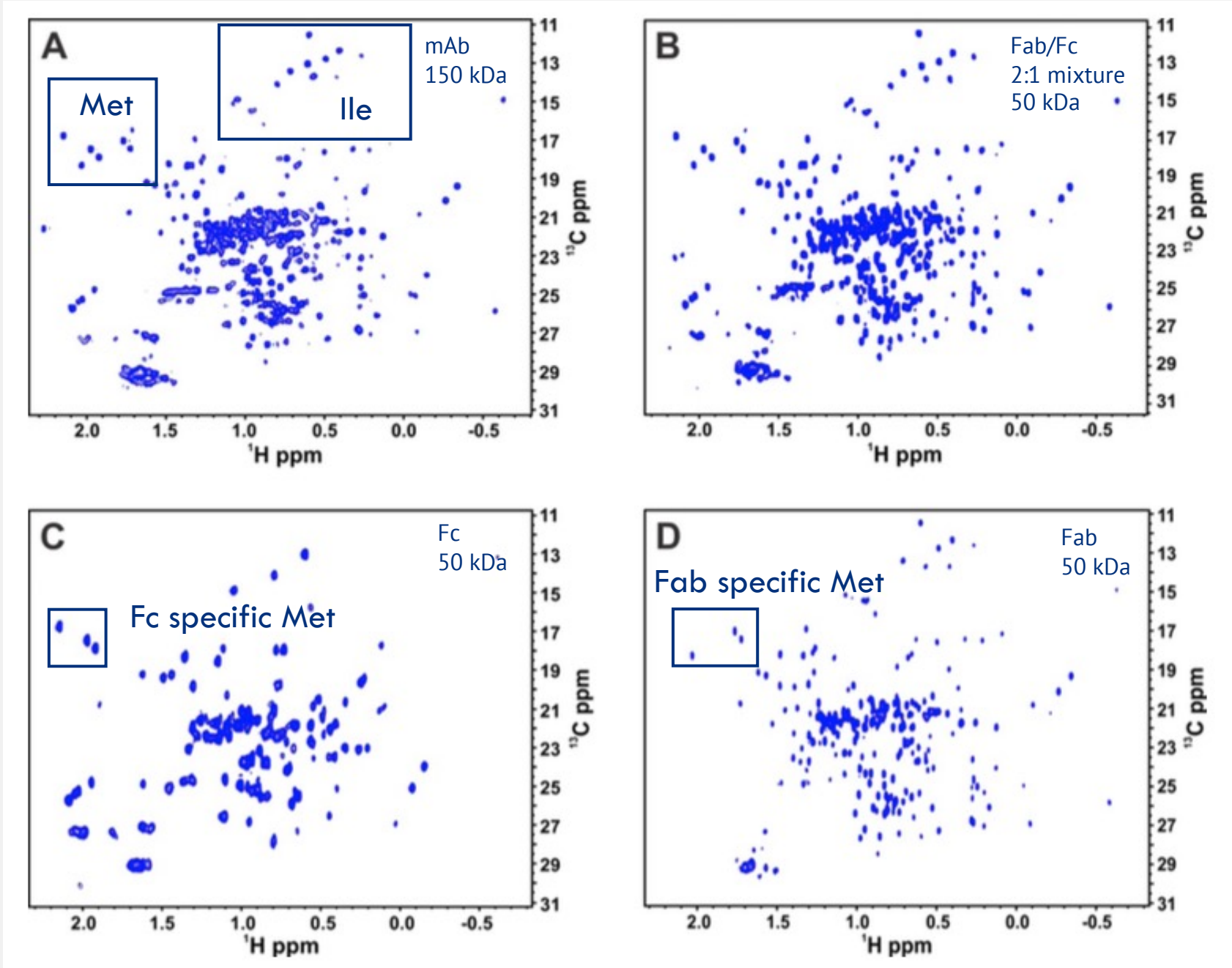
- The NMR spectrum is a fingerprint of the protein's 3D structure
- Natural abundance ^{13}C-isotope ratio ~= 1%
- Recent developments of NMR techniques allow natural abundance ^{1}H-^{13}C methyl NMR spectra to be acquired in minutes-hours[1]
- Applications of HOS NMR:
  - Batch-to-batch consistency
  - Biosimilar/originator comparability
  - Formulation optimization



NIST method:
[1]Arbogast LW, Brinson RG, Marino JP. Mapping monoclonal antibody structure by 2D 13C NMR at natural abundance. *Anal Chem.* 2015, 87, 3556-61.

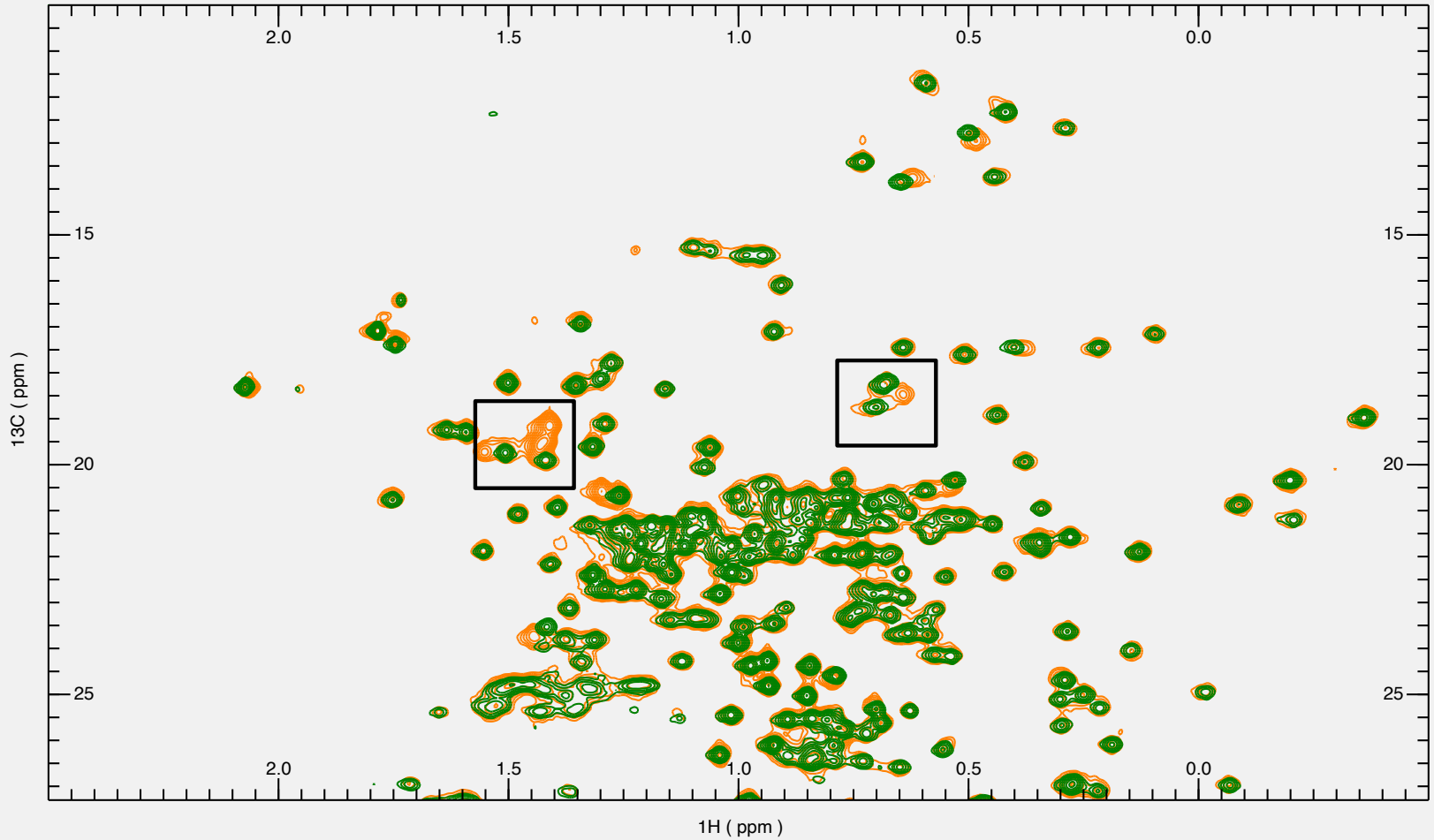
NMR spectroscopy

- NMR spectroscopy has atomic resolution
- Biological molecules in formulation can be directly studied
- Specific amino acid types can be identified and correlated to structure
- Biological molecules up to a size of 150 kDa can be studied
- Signal directly proportional to concentration



Analysis of NMR spectra

- Visual inspection
  - If you know NMR ☺
- Peak analysis
  - Chemical shifts
  - Integrations
  - Peak widths
- Correlation plots
- Chemometrics
  - Principal component analysis (PCA)
  - Clustering



^{1}H-^{13}C 2D NMR spectra of NIST Fab produced in 20%-^{13}C-labeled *P. pastoris* (orange) or murine (green) expression media. Boxes indicate where differences for methyl groups can be detected in the spectra.

Interlaboratory study for HOS assessment of mAbs

- 26 NMR laboratories worldwide
- Coordinated by NIST
- Experimental system
  - NIST Fab
- 39 different NMR spectrometers
  - 500-900 MHz
- SARomics Biostructures acquired data sets at 800 and 900 MHz
  - Swedish NMR Center Gothenburg
- Highly reproducible across different laboratories and spectrometers

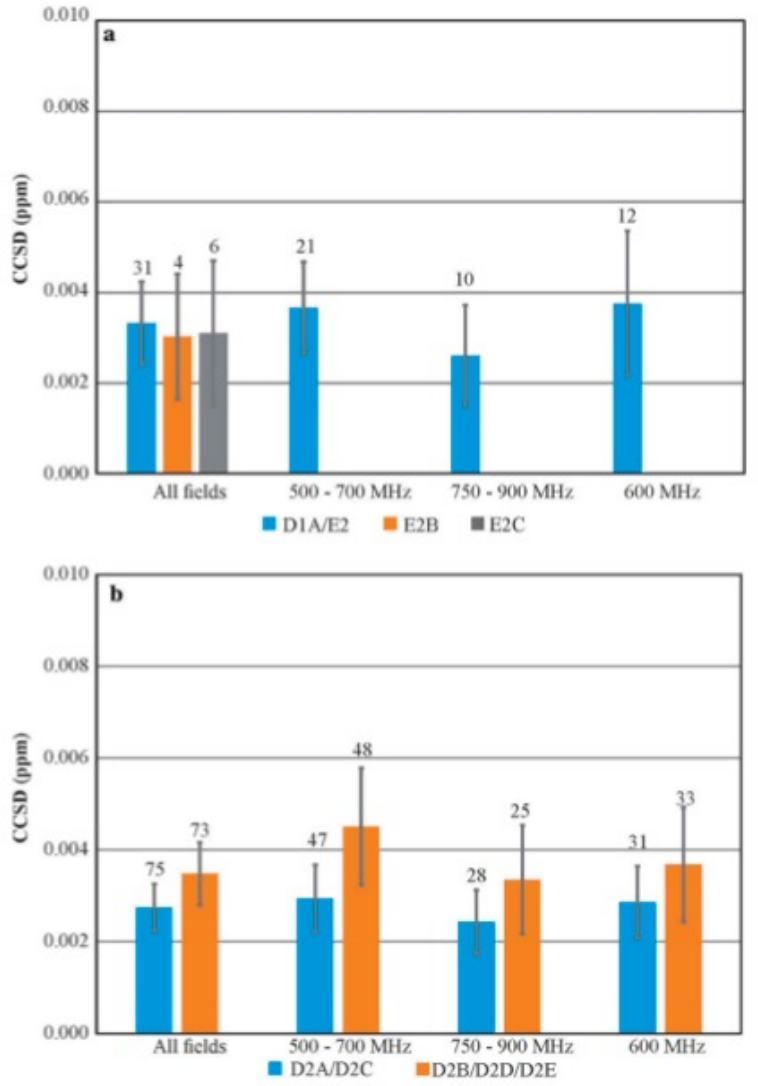


Figure 3. Average Combined Chemical Shift Deviation (CCSD) precision plots of required experiments at 37 °C. (**a**) CCSD of $^{1}H,^{15}N$ D1A/E2, E2B, and E2C spectra; (**b**) CCSD of D2A/D2C and D2B/D2D/D2E $^{1}H,^{13}C$ spectra. All $^{1}H,^{15}N$ spectra and $^{1}H,^{13}C$ D2A/D2C spectra were acquired with uniform sampling, while $^{1}H,^{13}C$ D2B/D2D/D2E spectra were 50% non-uniformly sampled. The number above each bar represents the total number of spectra included in the analysis for each respective experimental type. For $^{1}H,^{15}N$ CCSD plot, E2 experiments were included since the only parameter change from D1A was a smaller ^{15}N spectral width. Errors bars represent 95% confidence intervals of SEM. For detailed breakdown of experimental codes, see **Table S10.**

Robert G. Brinson, John P. Marino, Frank Delaglio, Luke W. Arbogast et al (2019) Enabling adoption of 2D-NMR for the higher order structure assessment of monoclonal antibody therapeutics, mAbs, 11:1, 94-105, DOI: 10.1080/19420862.2018.1544454

Case study
SARomics Biostructures - Genovis
Infliximab originator and biosimilar

Preparation of Fab and Fc fragments from infliximab originator and biosimilar

FabALACTICA (IgdE), cloned from *Streptococcus agalactiae* (Genovis), was used to generate Fab and Fc fragments of infliximab, both from an originator and biosimilar. The mAbs were digested using Immobilized FabALACTICA on spin columns at room temperature under non-reducing conditions. The fragments generated were then purified by Fc or CH1 affinity chromatography (*Fig. 1*).

To simulate aging, the originator was also treated with H_2O_2 to induce oxidation prior to digestion. All samples were buffer exchanged to PBS and concentrated with a high yield for 2D-NMR studies.

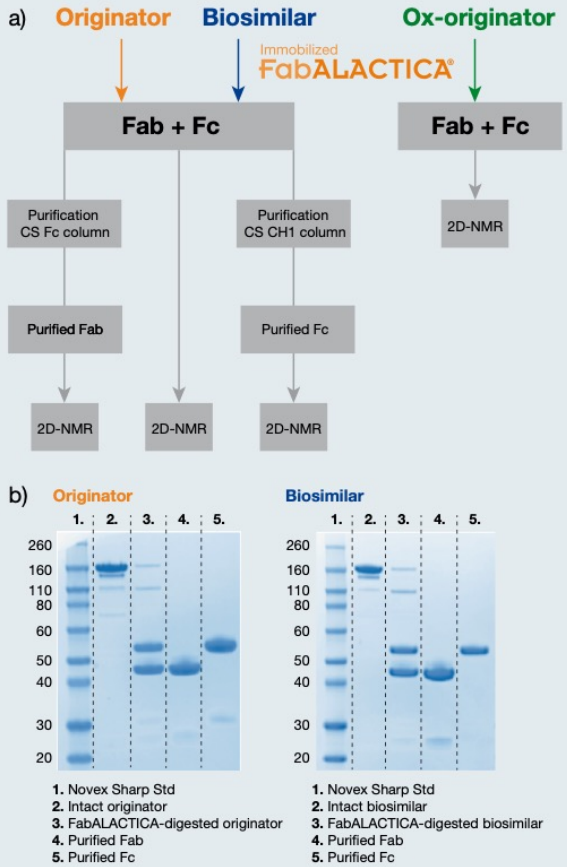
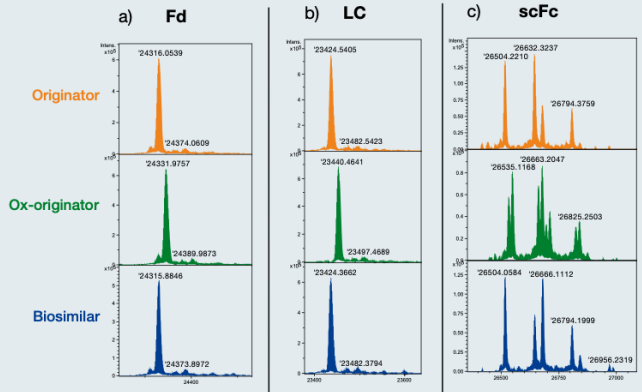


Figure 1. a) Schematic overview of the sample prep process including digestion with Immobilized FabALACTICA and separation by affinity chromatography where the flow through was collected and analyzed. Ox-originator was generated by treating the originator with 0.3% H2O2 at 37°C, 2.5 h. **b)** Non-reducing SDS-PAGE showing the fractions of the Fab and Fc fragments generated by digestion with Immobilized FabALACTICA.

LC-MS analysis of the samples after FabALACTICA digestion

To verify the quality and masses of the fragments generated, the digested samples of infliximab as originator and biosimilar were analyzed by LC-MS. The detected masses confirmed that the specific digestion site was in the heavy chain at the sequence DKT_{226} / $H_{227}TC$ above the hinge, as expected. The mass data of the oxidized originator sample confirmed that oxidation occurred in both the Fc and Fab fragments. (*Fig. 2*, Table 1).



Fragment (modifications)	Fd 1-226	Fd 1-226 Mox	LC	LC Mox	scFc 227-449 G0F	scFc 227-449 2Mox G0F
T (Mr)	24315.9572	24331.9221	23424.3946	23440.3895	26504.0967	26535.0786*
Originator E (Mr)	24316.0539	-	23424.5405	-	26504.2210	-
Mass Δppm	4.0	-	6.2	-	4.7	-
Ox. Originator E (Mr)	-	24331.9757	-	23440.4641	-	26535.1168
Mass Δppm	-	2.2	-	3.2	-	1.4
Biosimilar E (Mr)	24315.8846	-	23424.3662	-	26504.0584	-
Mass Δppm	-3.0	-	-1.2	-	-1.4	-

* dehydro

Table 1. Experimental (E) and theoretical (T) masses (Da) of the infliximab drug products. Originator, oxidized originator and biosimilar. The G0F glycoform of the scFc subunit was chosen for mass accuracy determination.

Figure 2. LC-MS analysis of infliximab digested with FabALACTICA. Deconvoluted spectra of the three subunits **a)** Fd **b)** LC and **c)** scFc obtained from the originator, oxidized originator and biosimilar after digestion and separation on a C4 column (Waters). MS analysis was performed on an Impact II Q-TOF (Bruker).

2D NMR spectra are very information rich but can be hard to interpret

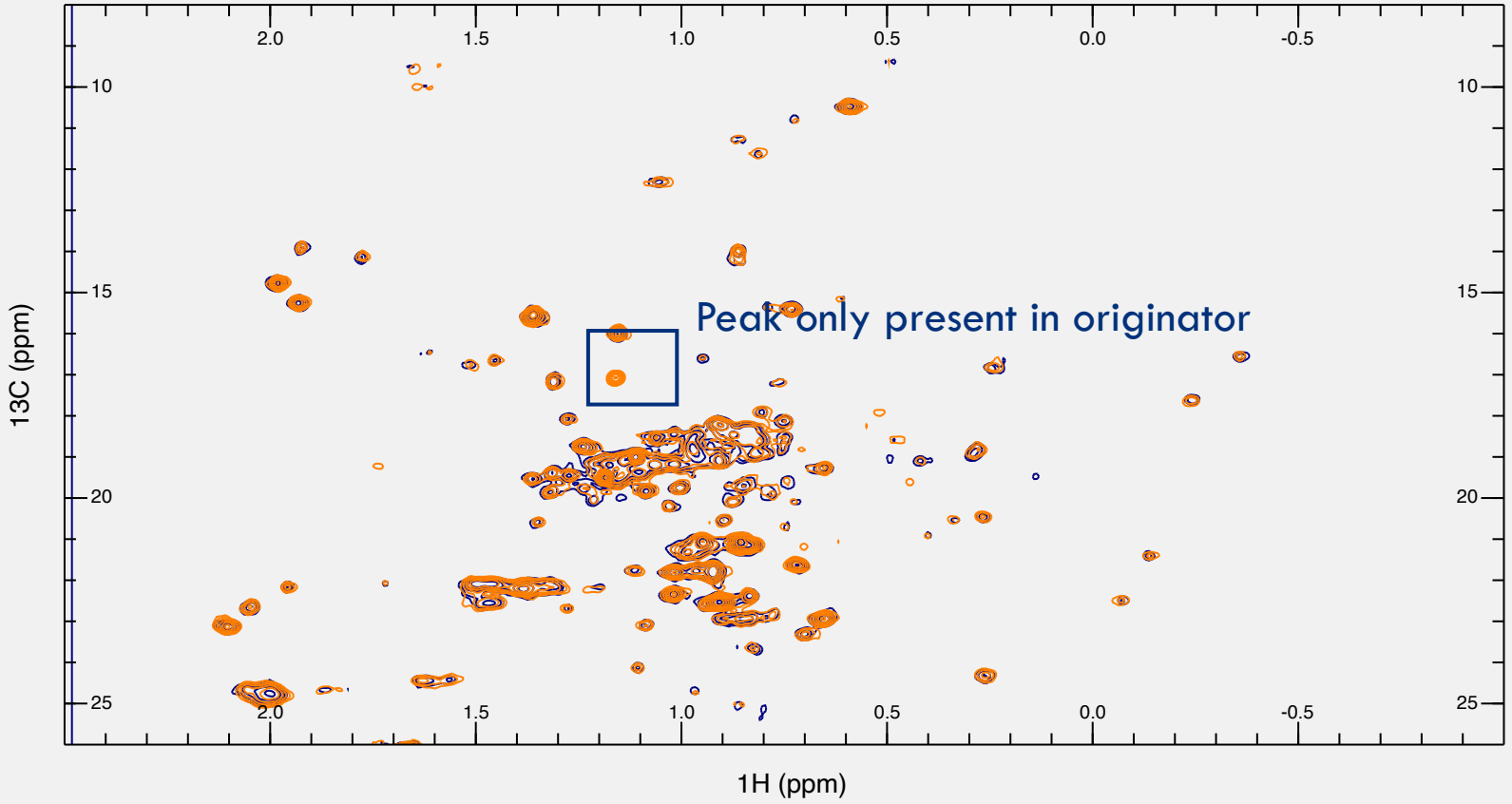
Expectations on spectra based on sample:

- 2:1 intensity ratio for Fab:Fc domains since a mAb contains 2 Fab and 1 Fc domains
- Identical fingerprint spectra for originator and biosimilar

Results:

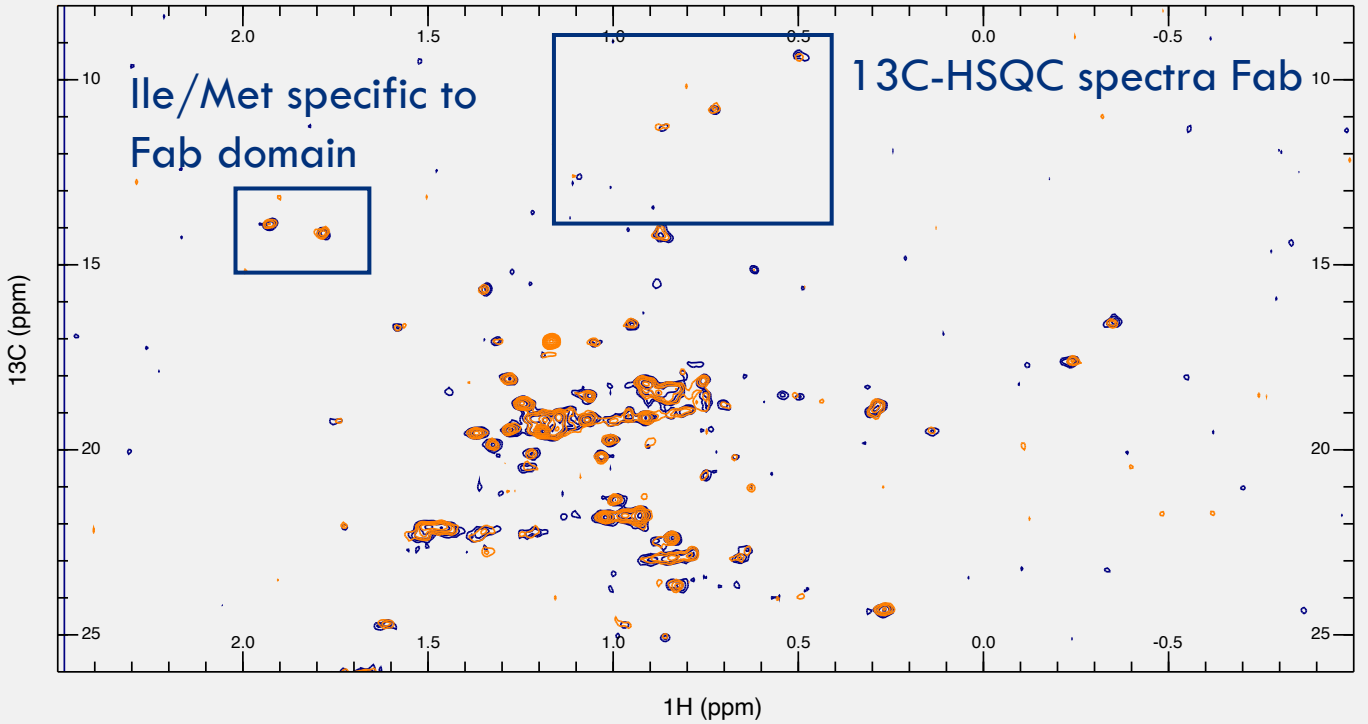
- Less peaks than expected for a 150 kDa protein
- Large variation in peak intensities indicates presence of dynamical processes
- At least one peak only detected in originator
- Same HOS for both samples => Same 3D structure

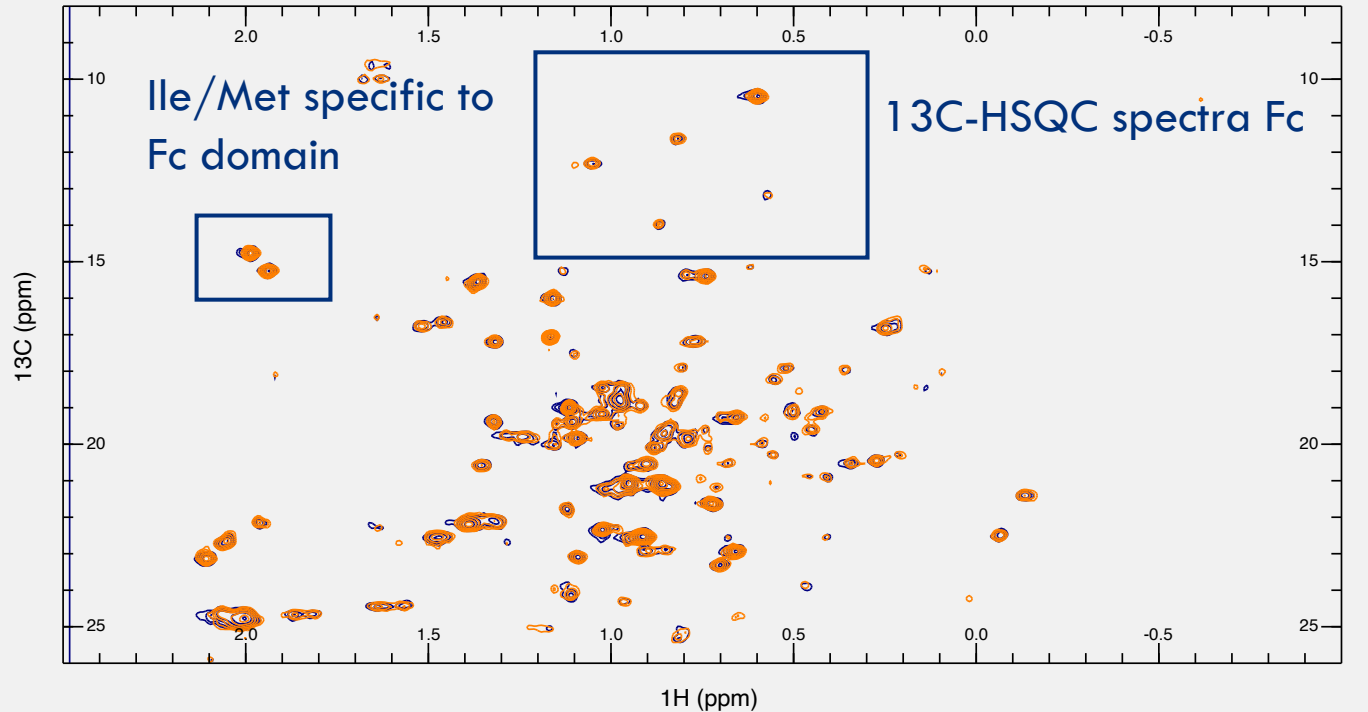
13C-HSQC spectra of cleaved Fab + Fc



Deconvolution of Fab + Fc spectra

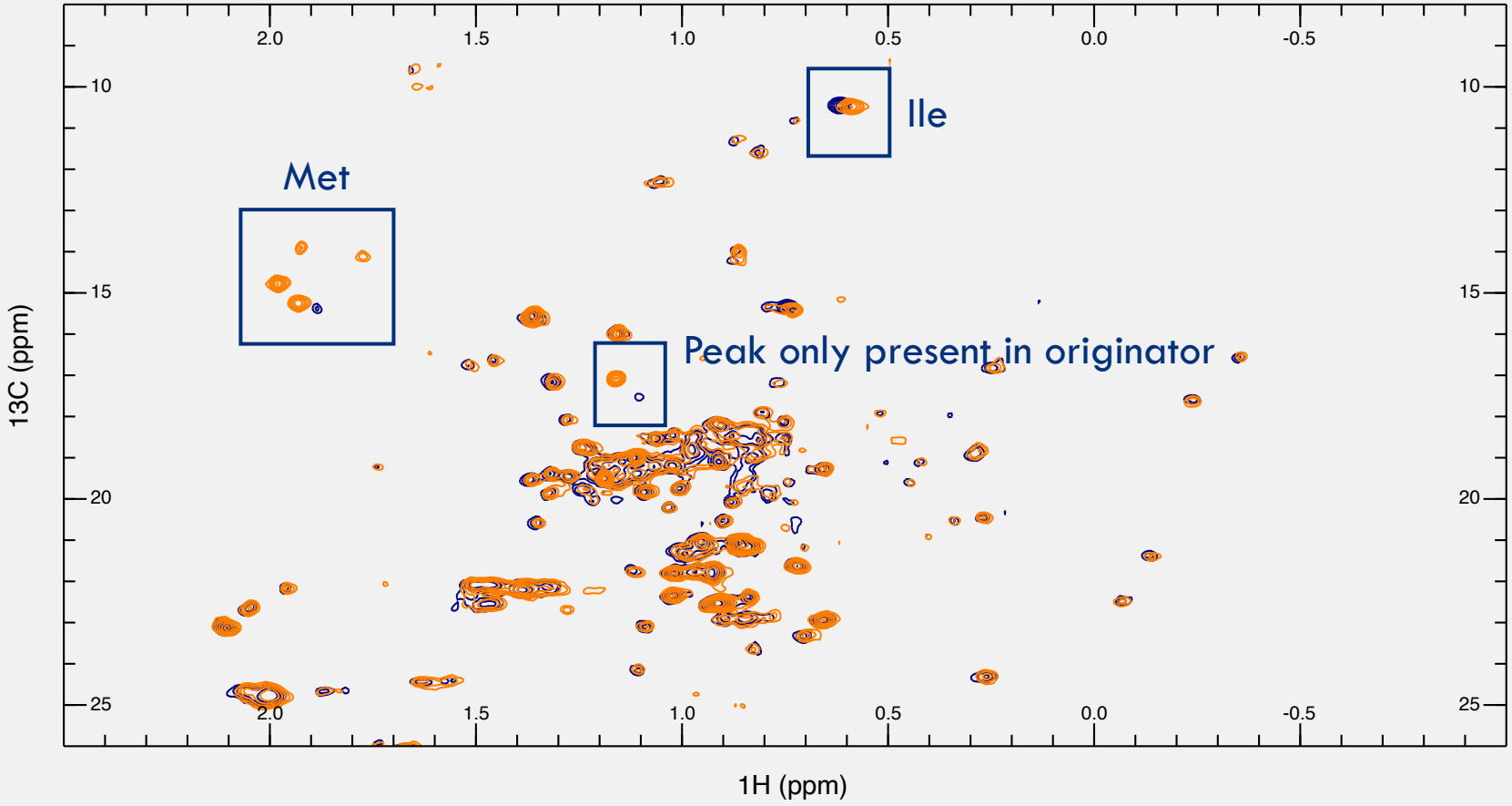
- Isolated peaks can be identified to Fab or Fc domain
- Met and Ile residues can be mapped on protein structure
  - Two Met in Fc domain
  - Four Met in Fab domain
- Weaker peak intensities for Fab domain versus Fc domain
  - Two out of four Met missing
  - Dynamic process specific to Fab domain?
- All available Met and Ile in Fc residues can be identified in spectra





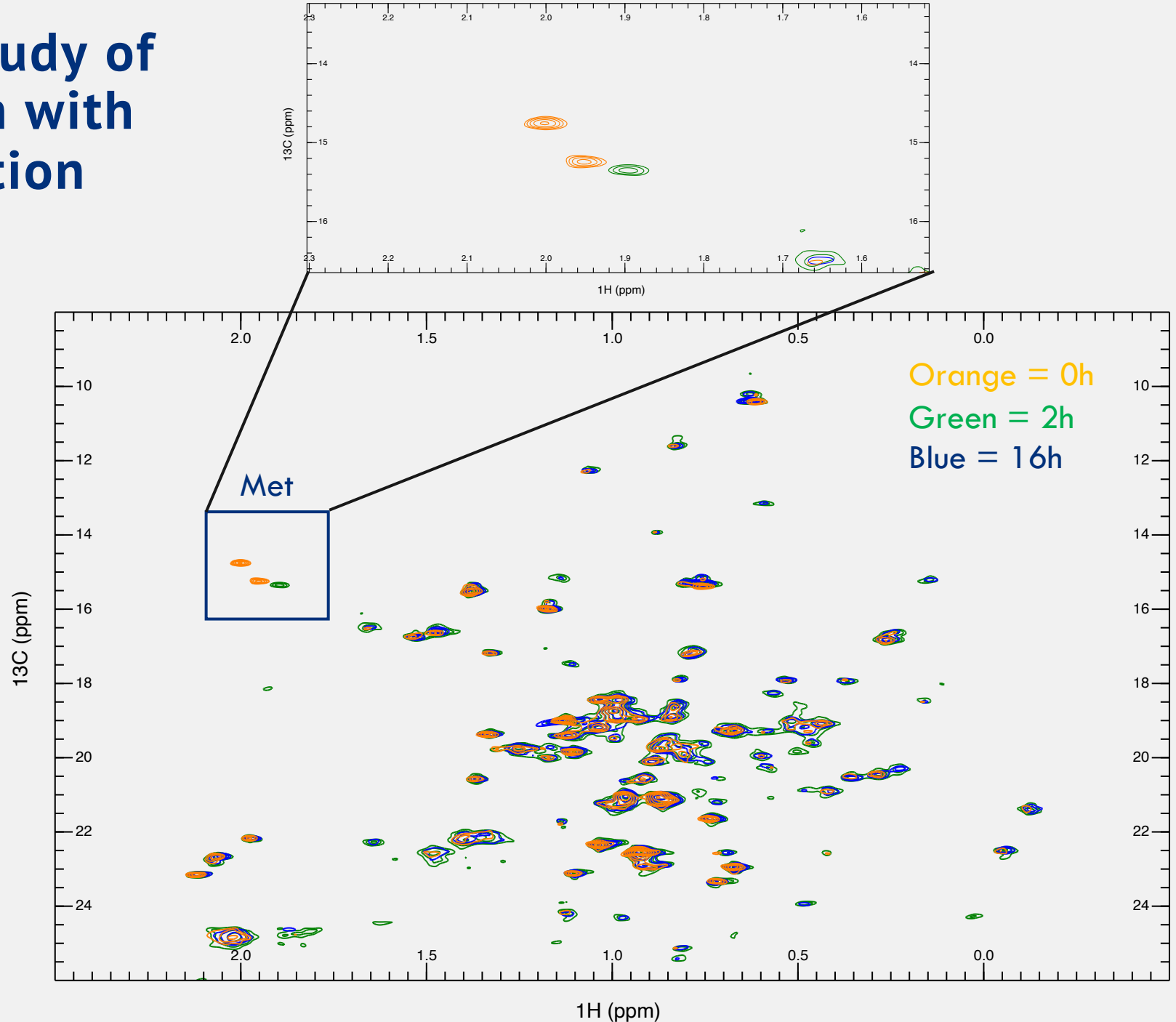
Forced oxidation of originator Fab + Fc

- Forced oxidation of mAb using H_2O_2
- Digestion of mAb after oxidation
- Met residues almost fully oxidized
- No major peak differences => Same 3D structure before and after oxidation
- Peak only present in originator disappears upon oxidation
- Ile adjacent to Met in Fc domain identified with no large peak shift



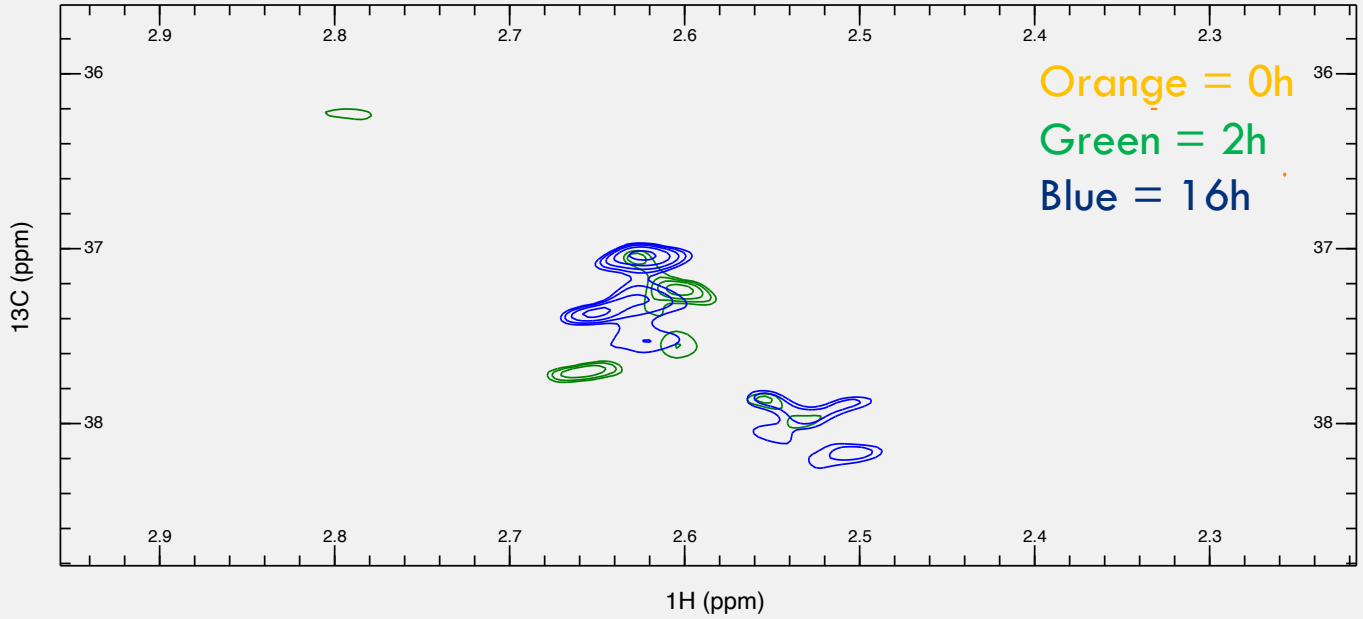
Time-resolved study of forced oxidation with atomic resolution

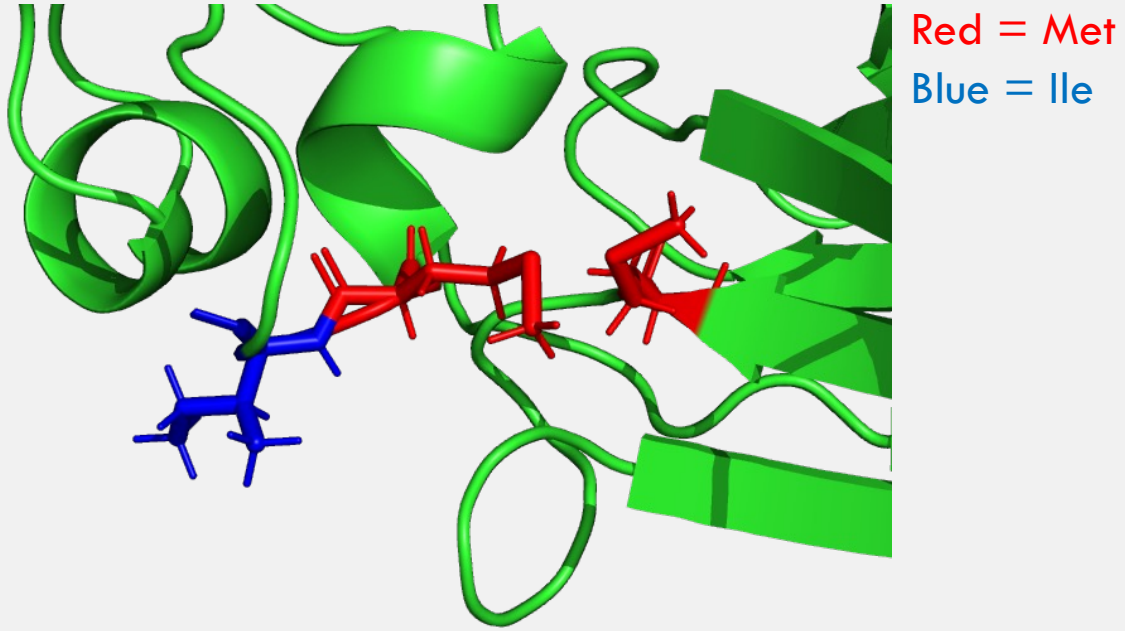
- Spectra acquired on Fc domain
- SOFAST-HMQC with 50% NUS ratio
- Acquisition time per spectra = 1-2h
- Implementation by Swedish NMR Center
- Oxidation profile different for the two methionines
- One methionine fully oxidized after 2h
- One methionine in intermediate shift after 2h
- Full oxidation after 16h



Methionine oxidation products studied by NMR

- Oxidized methionine reactant products detected in NMR spectra
- The two methionines in the Fc domain are closely located in space
- Different oxidation profiles for the two methionines
- Heterogenous response after 16h indicating multiple populations of oxidized methionines





Summary

- NMR spectroscopy is another tool in the toolbox for the characterization of biopharmaceuticals
  - One of few techniques with atomic resolution in solution state
  - Same biopharmaceutical in formulation which is injected into patients can be studied by NMR spectroscopy
  - Highly flexible and information rich
- Higher order structure (HOS) characterization
  - Easy validation of HOS for comparing originator and biosimilar
  - Can be easily extended into chemometrics
  - Dynamic processes can easily be detected from simple 2D spectra
  - Adds detailed information to other biophysical techniques
  - Easily correlates changes with protein structure
  - Time resolved processes of intact protein can be easily and relatively rapidly acquired

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