Biologics Process Development Analytics

The Integration of Mass Spectrometry to the Process Development of Fusion Protein Therapeutics

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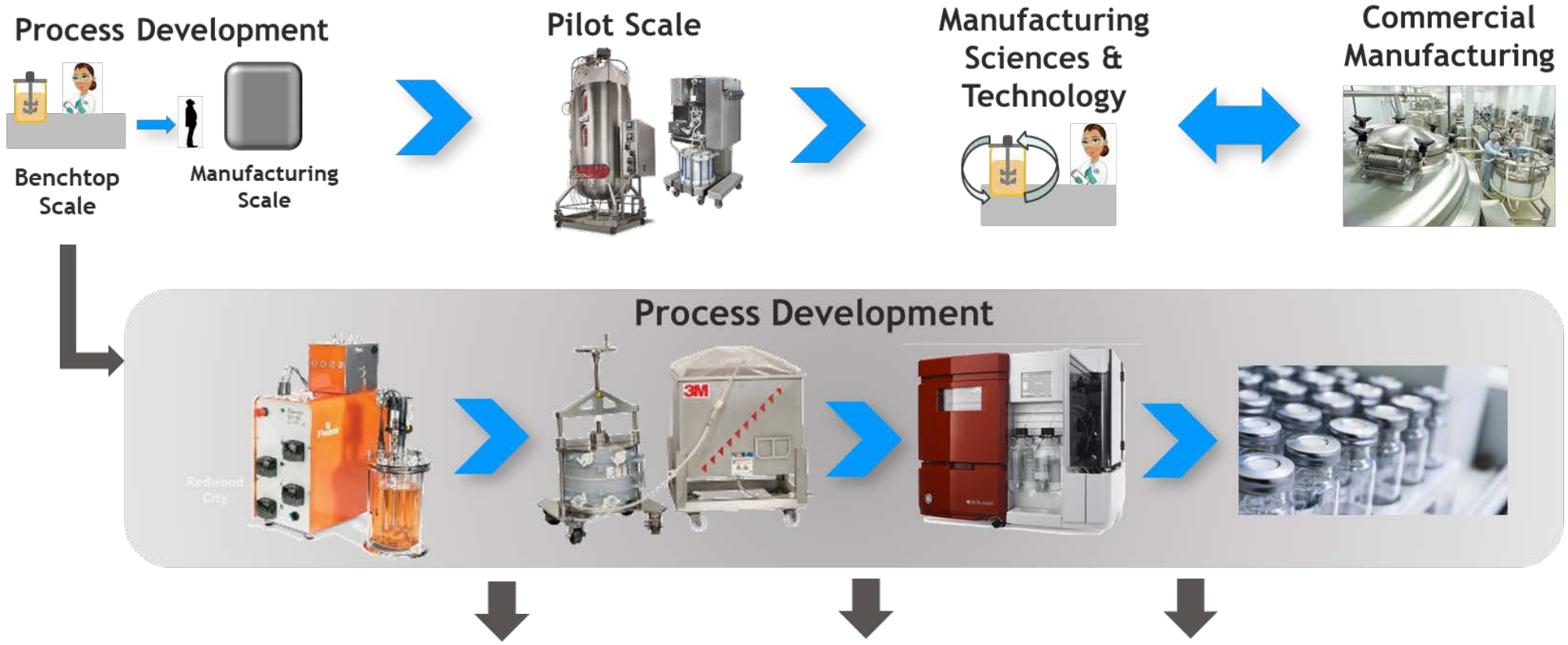
Outline

- **Introduction**
- **Case Studies**
  - Fab Site-Specific Glycosylation Monitoring
  - LMW Analysis for Fc-fusion Protein
  - Complex Charge Profile of Fc-fusion Protein
- **Summary**
- **Acknowledgement**

Bioprocess Development Analytical Support



Analytical Support

- High-throughput Platform Assays
- Sample Automation
- PAT for Real time Analytics
- **Mass Spectrometry for In-Process Support**

Fc-fusion Protein Therapeutics

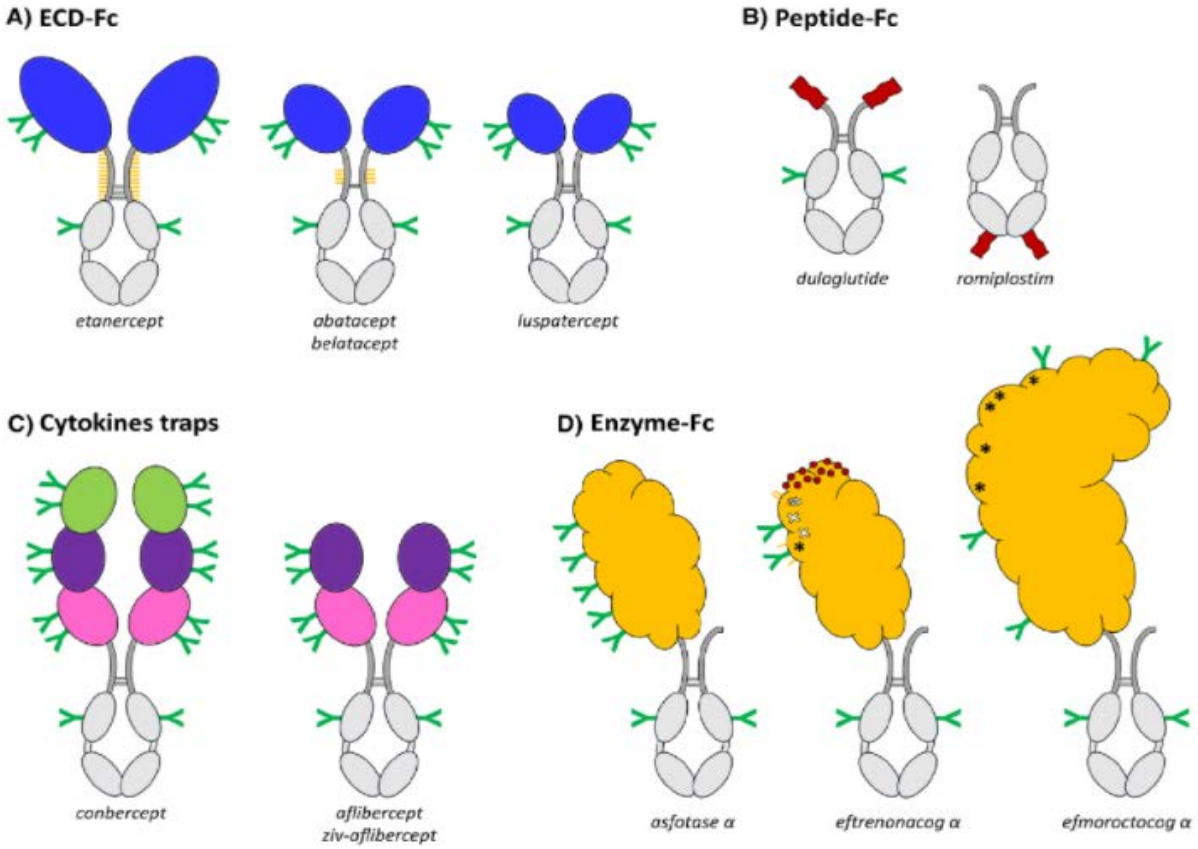
- Fc-fusion protein therapeutics are one of the most successful classes of IgG-based products;

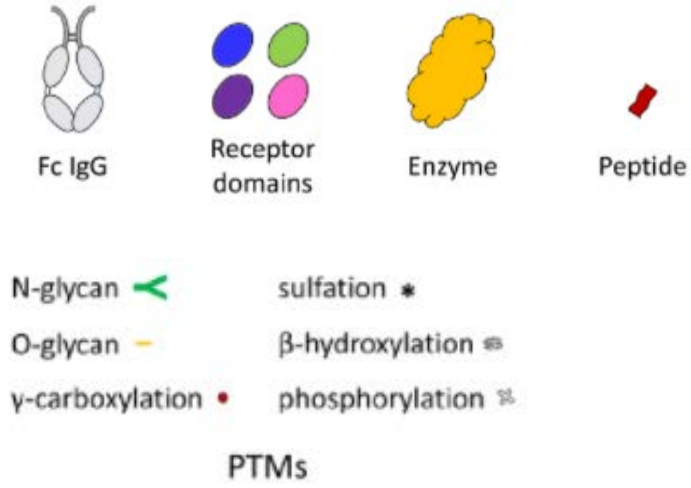
Product	Sales in 2019 (billion, USD)
Eylea	7.5
Enbrel	7.2
Trulicity	4.3
Orencia	3.2
Elocta	1.2

- Fc-fusion protein combine the pharmacological properties of biological ligands with the additional stability and inherent properties of IgG Fc domain;
- Fc-fusion protein can significantly improve the clinical potential of active protein drugs such as extend the plasma half life as well as engage immune-mediated effector functions;
- To date, ~**37** therapeutic fusion proteins are in clinical development and **13** products have been approved by the FDA, CFDA, and EMA;
  - Enbrel (TNFR-Fc fusion, FDA approval in 1998) and Orencia (CTLA4-Fc fusion, FDA approval in 2005) for treatment of rheumatoid arthritis;

Duivelshof BL, et al. J Sep Sci. 2021. Jan; 44(1):35-62.

Major Groups of Fc-fusion Proteins





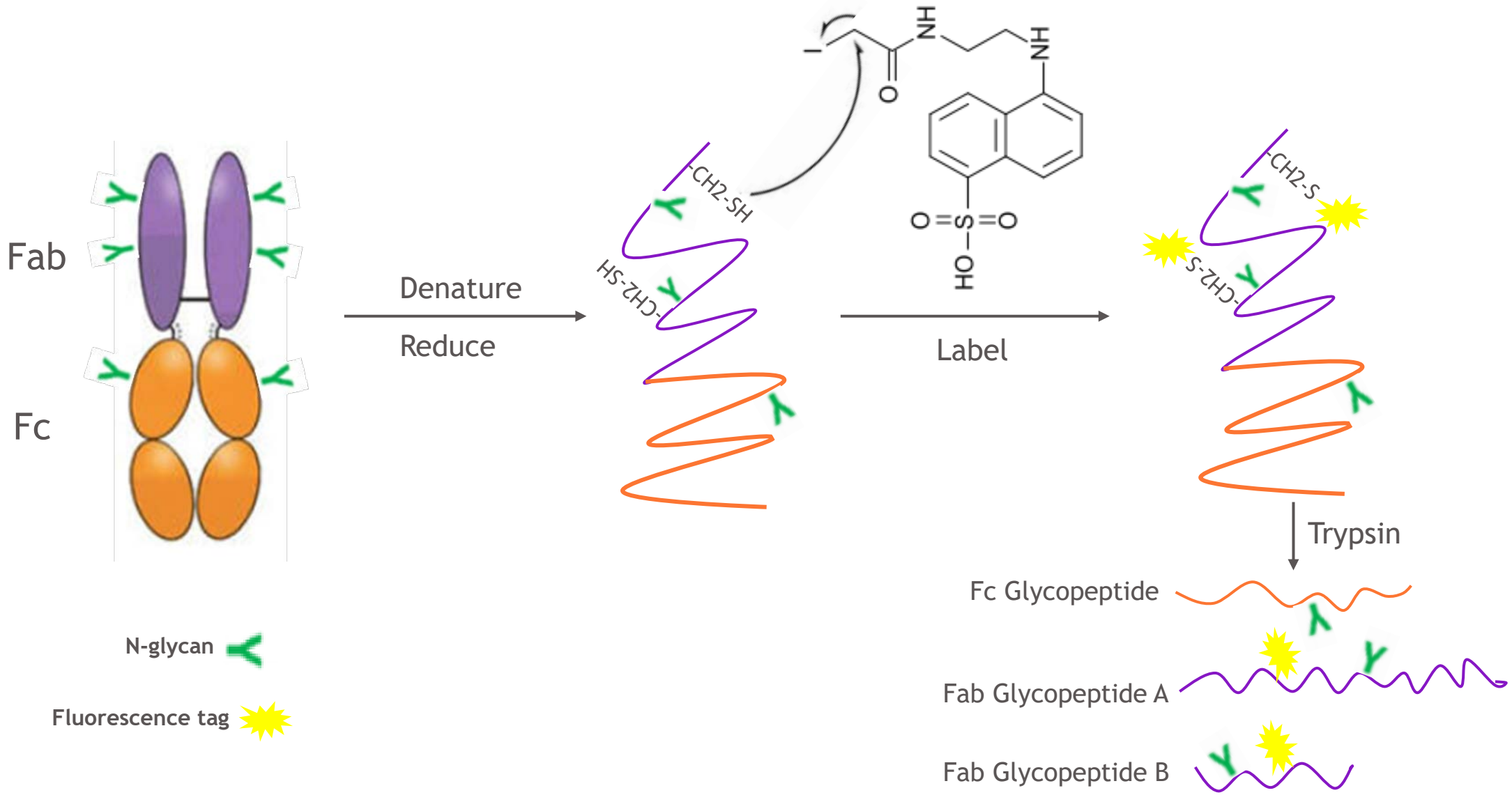
Therapeutic Fc-fusion proteins: Current analytical strategies
Duivelshof BL, et al. J Sep Sci. 2021. Jan; 44(1):35-62.

- Due to highly **heterogenous** structure (the presence of sialic acid, complex glycan structure, etc.), the analysis of Fc-fusion proteins is more challenging and complex than monoclonal antibodies;
- **Product-specific** methods over conventional generic or platform methods are often desirable to support process development;

Case Study I: Fab Site-Specific Glycosylation Monitoring

- A Fc-fusion protein with **four** *N*-glycosylation sites in Fab region and **two** *N*-glycosylation sites in the Fc region;
- A LC method was developed to monitor **Fab site-specific glycoforms** (G0F, G1F, G2F, G2FS1, and G2FS2) as the understanding about site-specific glycosylation as pCQA is continuously evolving during product development lifecycle;
- Mass spectrometry is coupled with LC for the peak identification of the complex chromatogram and the optimization of LC method parameters;
  - Mobile phase screening
  - Fluorescence tag screening
  - LC gradient optimization

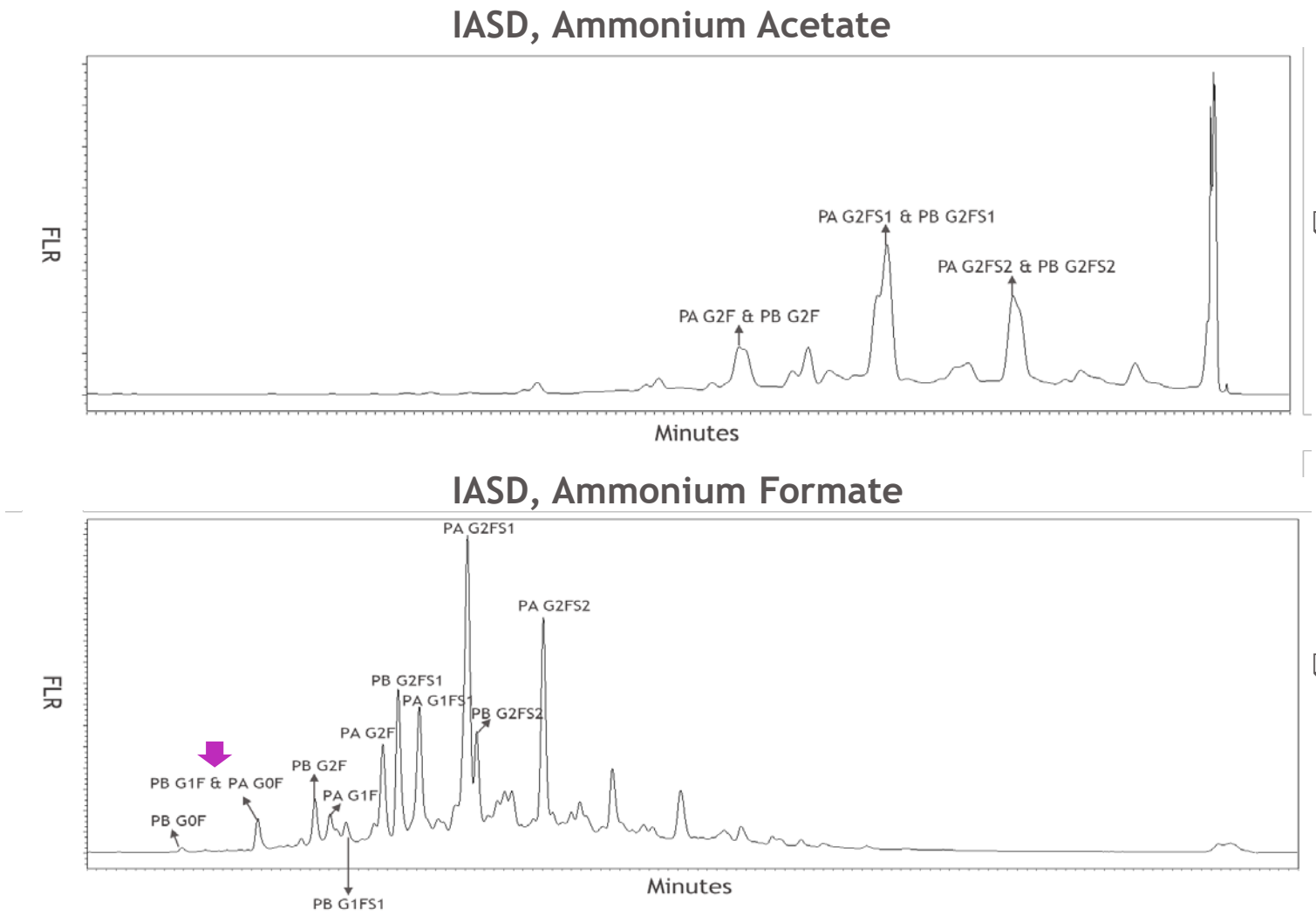
Glycopeptide Chemical Labeling



Workflow Overview

Label	• Denature • Reduce • **Label Cys with fluorescence tag (Alkylation)**
Desalt	• Sample desalt with SEC spin column
Proteolysis	• Trypsin digestion
LC Separation	• **HILIC for glycopeptide separation**
MS Identification & FLR Quantitation	• **MS for peak identification** • **FLR for Fab glycopeptide relative quantitation**

Ammonium Acetate *vs* Ammonium Formate as Mobile Phase

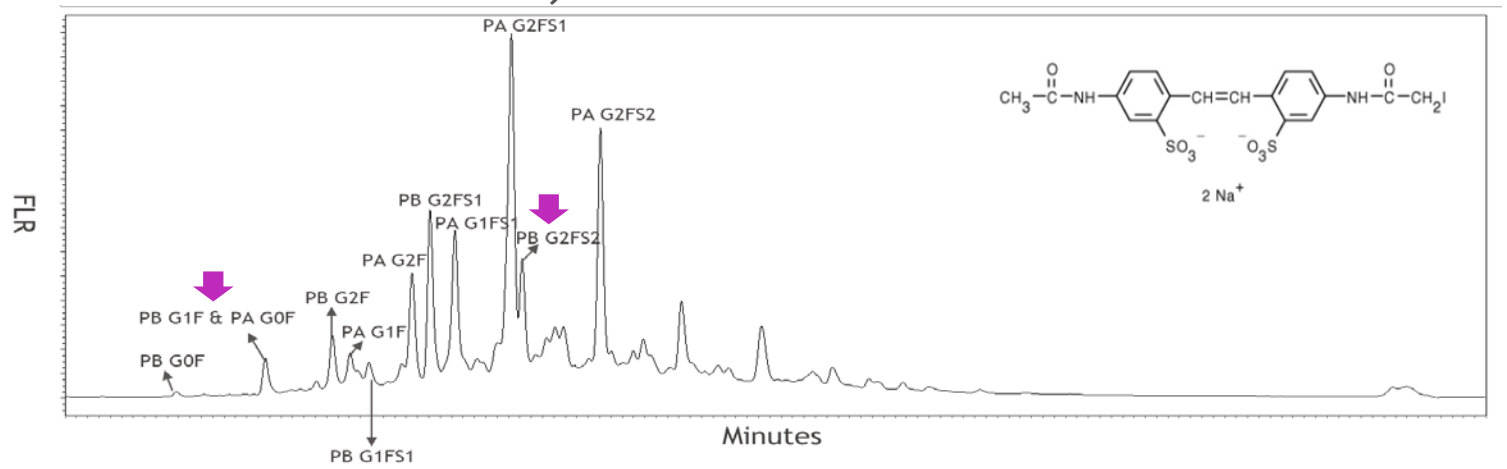


- ❑ Glycopeptide A and B coelution
- ❑ Only Glycopeptide A + G0F and Glycopeptide B + G1F coelutes

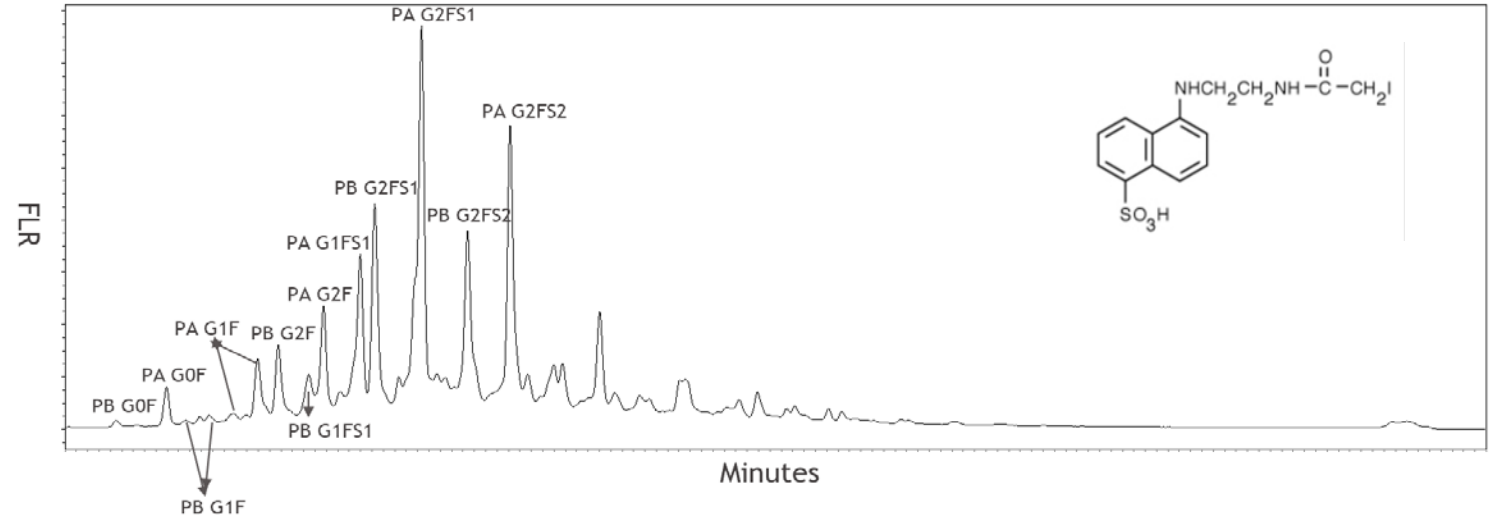
- Significant improvement in separation resolution with **ammonium formate** for IASD FLR tag;

IASD *vs* IAEDANS as Fluorescence Tag

IASD, Ammonium Formate



IAEDANS, Ammonium Formate

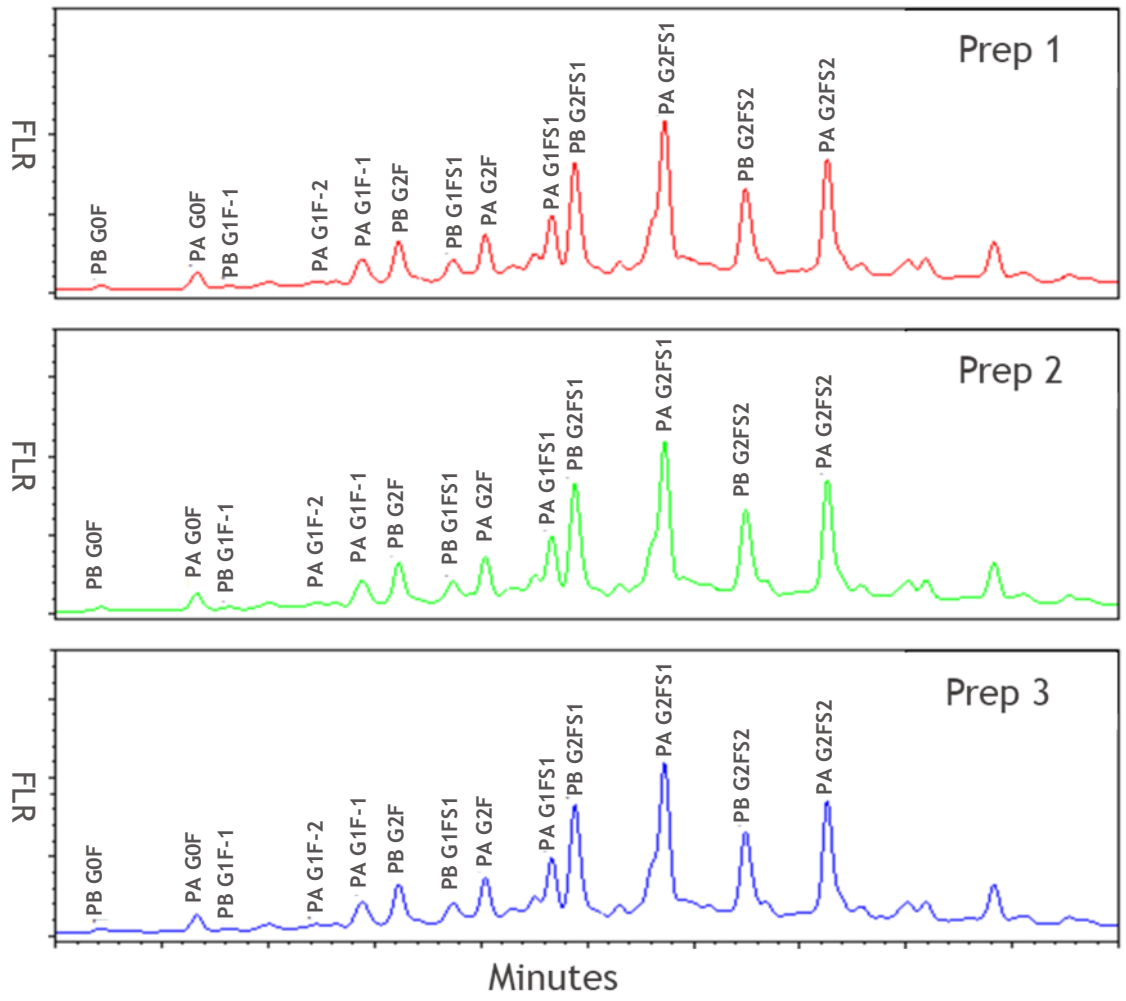


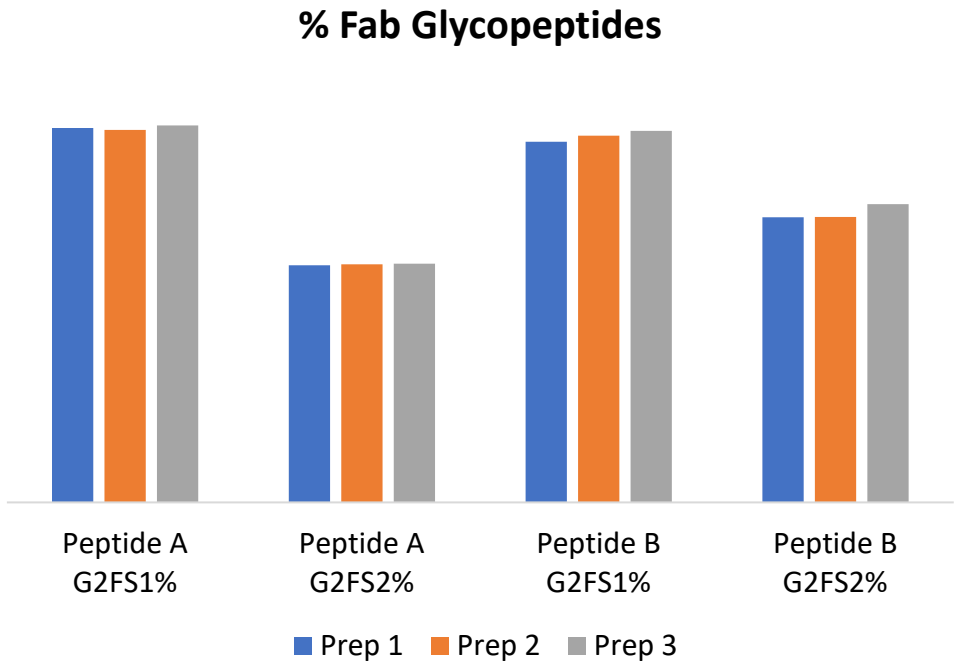
- ❑ Only Glycopeptide A + G0F and Glycopeptide B + G1F coelutes

- ❑ Glycopeptide A + G0F and Glycopeptide B + G1F were separated
- ❑ Better resolution between Glycopeptide A+ G2FS1 and Glycopeptide B+ G2FS2

- Different glycoforms of Fab glycopeptide A and B are fully resolved with **IAEDANS** FLR tag;

Quantitation of Fab Site-Specific Glycopeptides through Fluorescence Peak Intensity





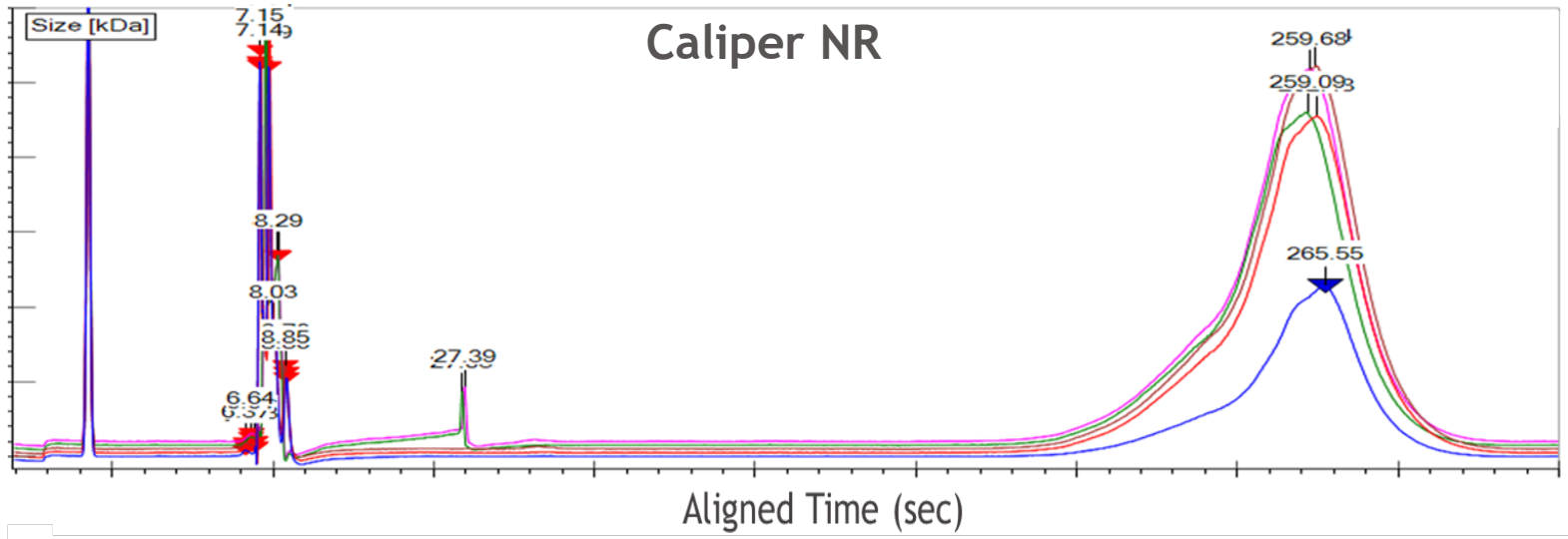
- **Reproducible** quantitation for glycopeptides;

Case Study I Conclusions

- A HILIC LC-FLR-MS method was established to separate glycopeptides and quantify Fab site-specific glycosylation for a Fc-fusion protein;
- Mobile phase and fluorescent tags played significant role in glycopeptide separation with HILIC column;

Case Study II: LMW Analysis for Fc-fusion Protein

- A Fc-fusion protein with >10 *N*-glycosylation sites;
- Caliper under non-reducing (NR) condition is the platform method for in-process sample support to determine the purity of monoclonal antibody with high throughput capability;
- Poor resolution of platform Caliper NR method for separating low molecular weight (LMW) fragments due to the *N*-glycan heterogeneity;

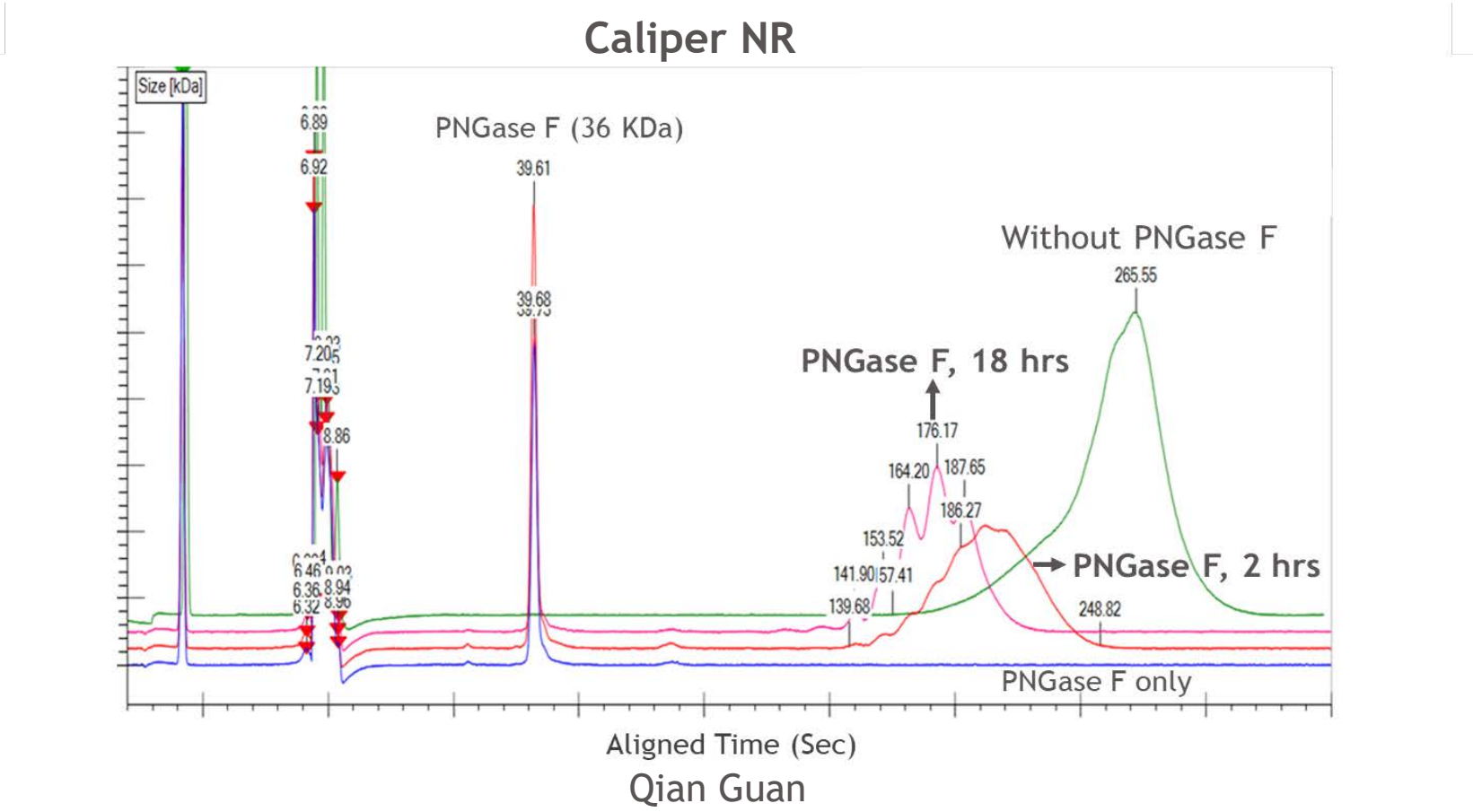


- ❑ Super broad peak;
- ❑ No separation for LMWs;

Qian Guan

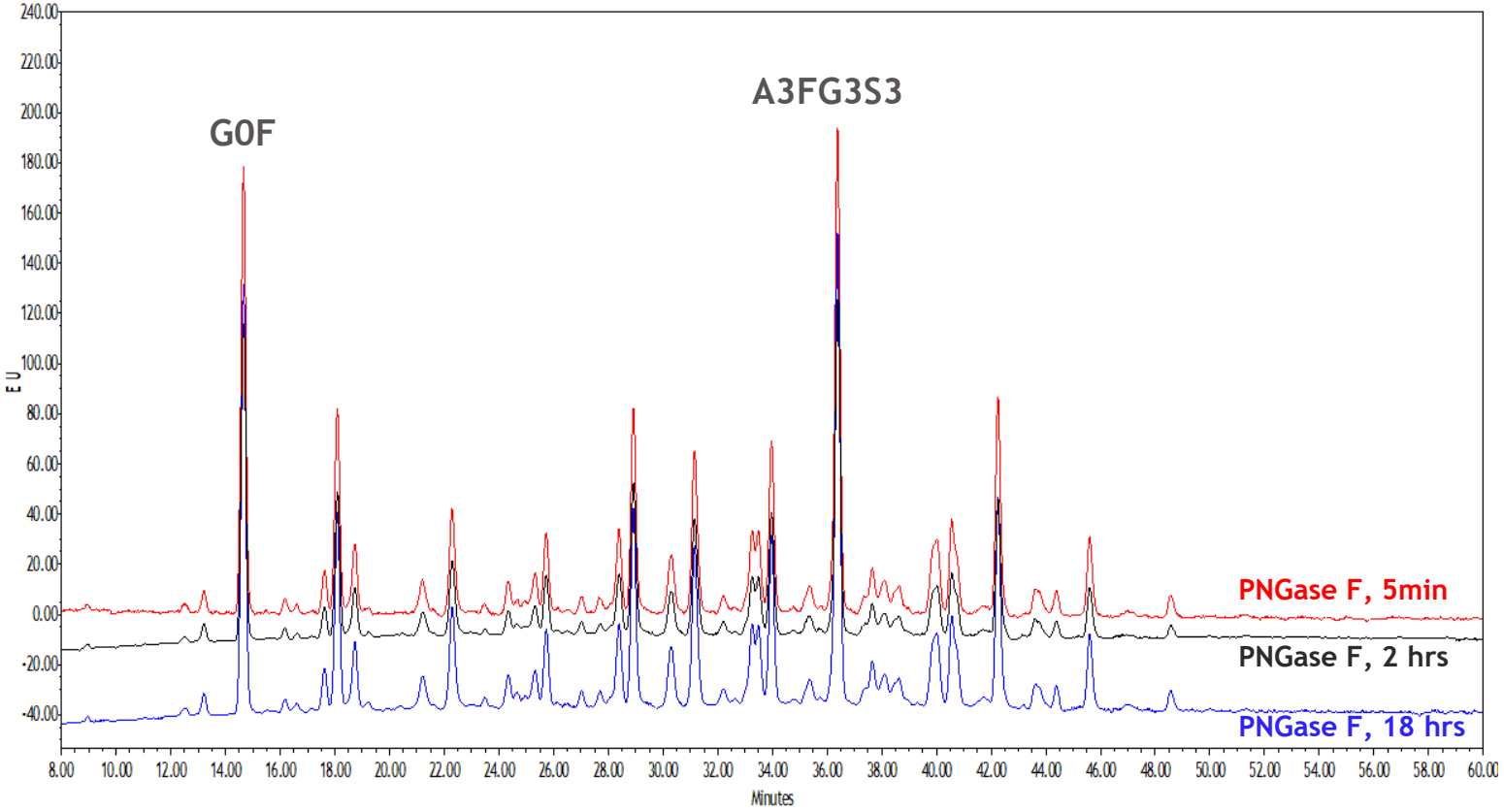
De-glycosylation by PNGase F with Native Conditions

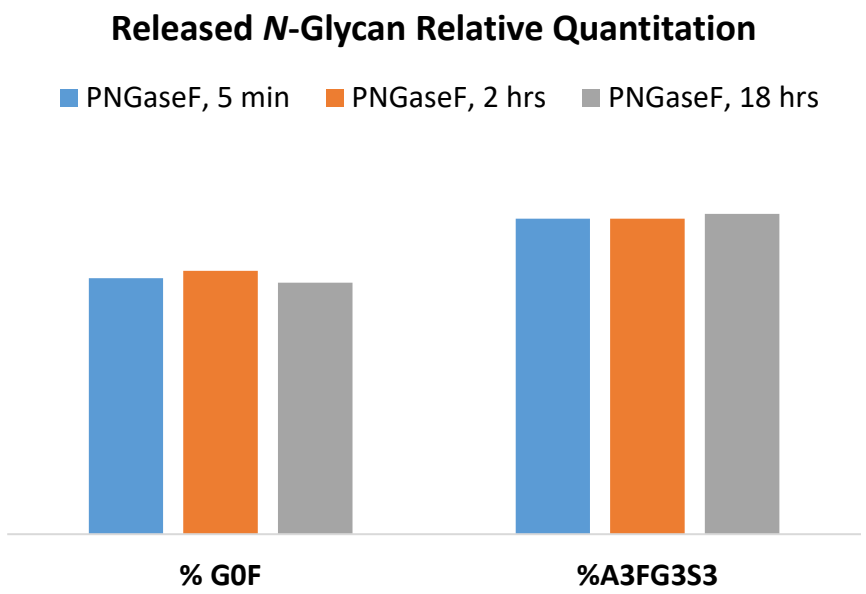
- Peaks started to split but still broad, after 18-hour PNGase F digestion under native conditions (37°C);
  - Is the digestion complete?



Qian Guan

Consistent *N*-glycan Profile with PNGase F Digestion under Native Conditions





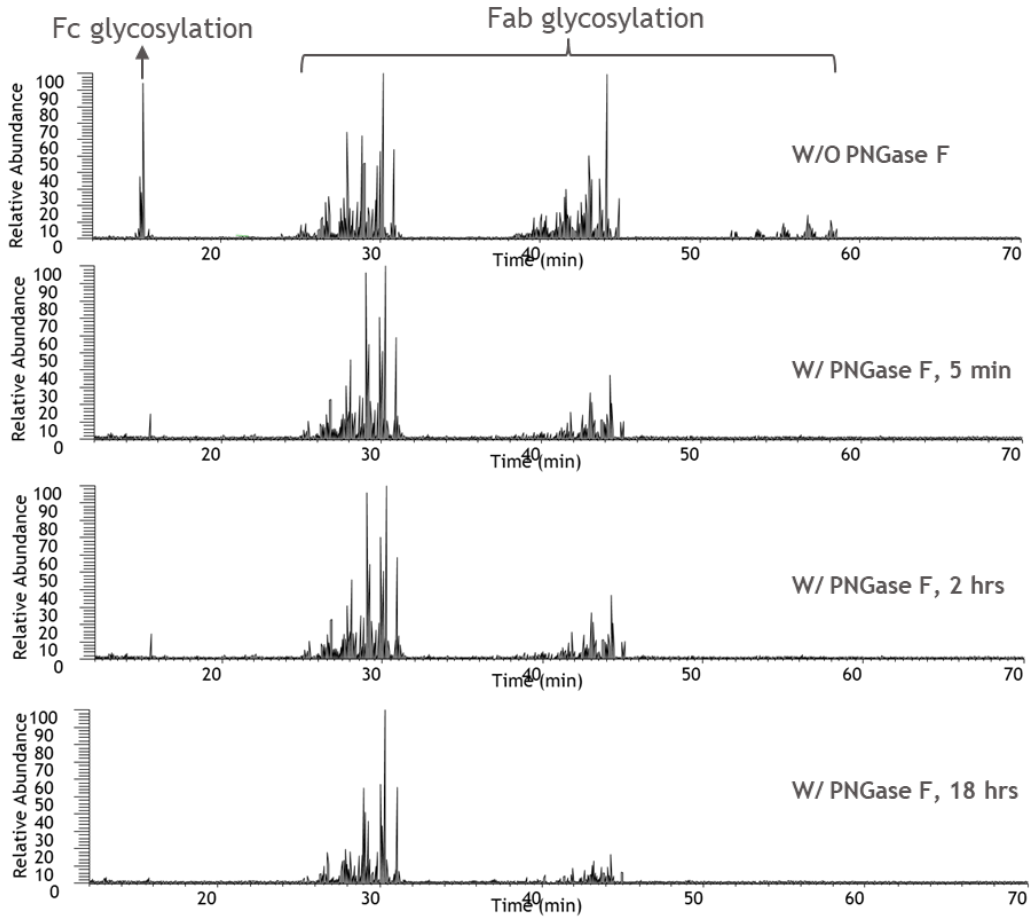
Xia Xu

- Whether *N*-glycans from all the glycosylation sites were completely removed remains **inclusive**;

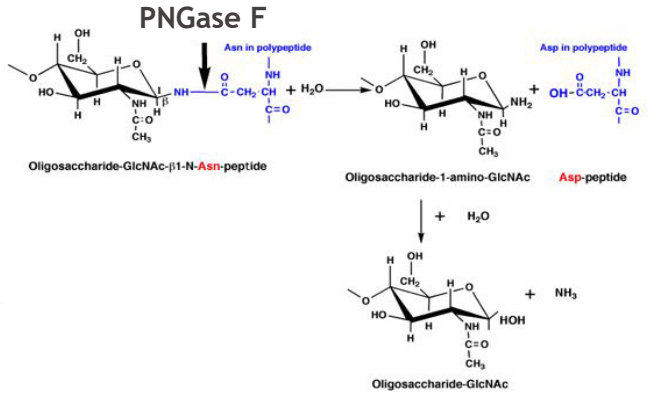
Glycopeptide Site-Occupancy Analysis by LC-MS

EIC of m/z=204.0867

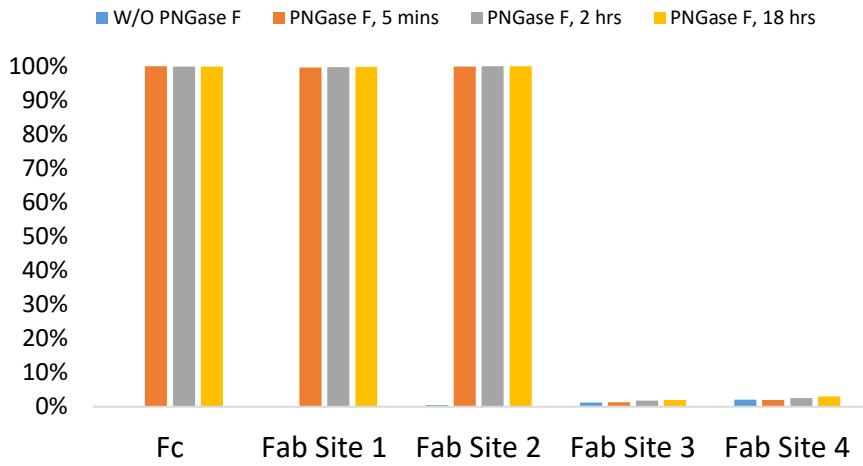
Signature ions of glycopeptide fragmentation



- PNGase F cleaves between Asn and the innermost GlcNAc, transforming Asn to Asp;



% Deamidation of Glycopeptides after PNGase F

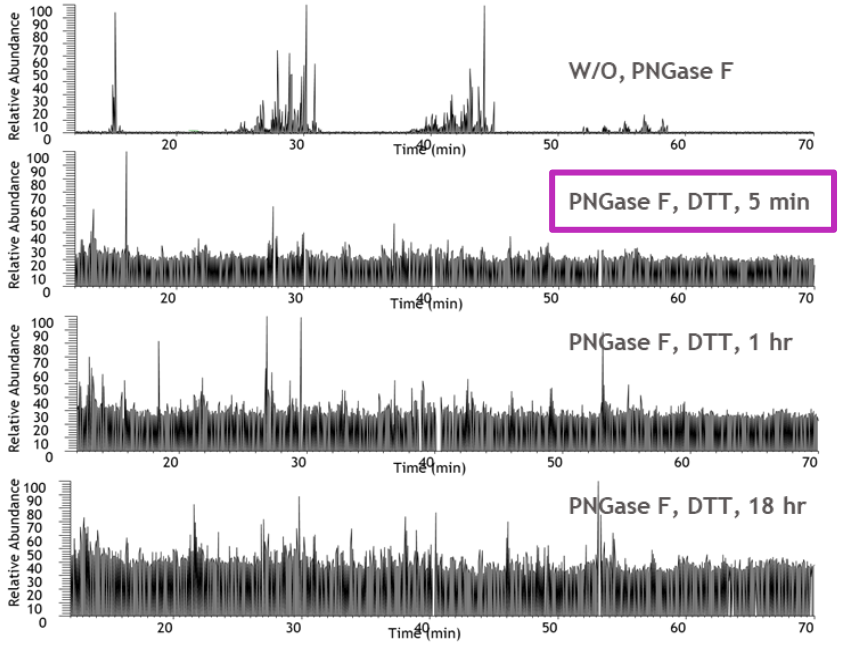


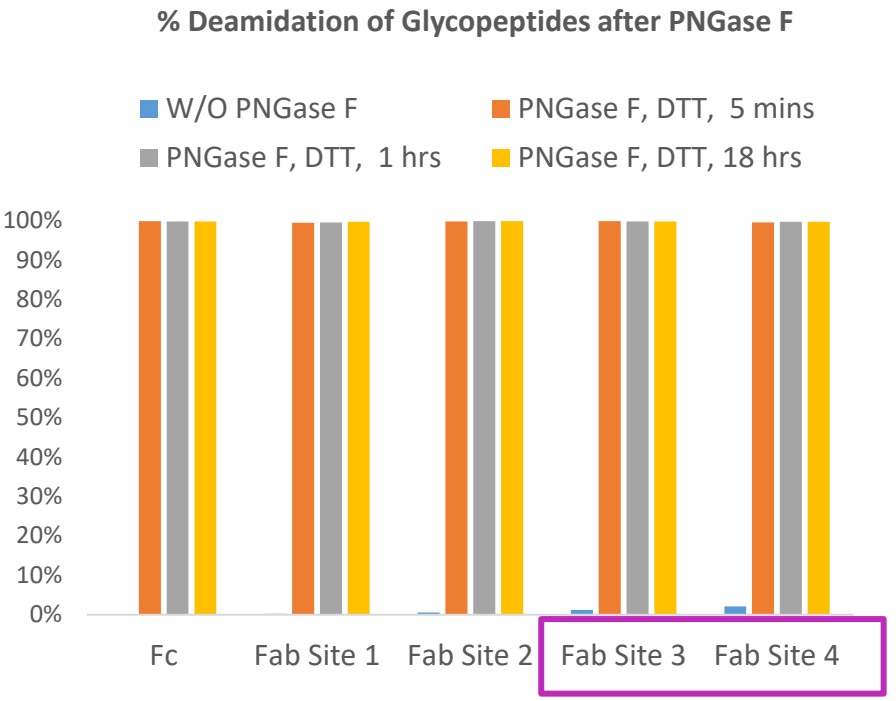
- **Fab site-dependent** preference by PNGase F under native conditions;

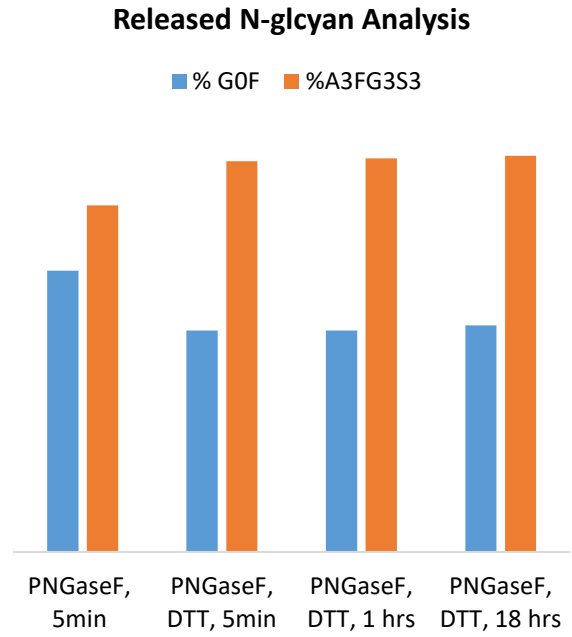
PNGase F Digestion under Reducing Conditions Completely Removes *N*-glycans

- Fab glycosylation site 3 and site 4 are surrounded by **disulfide bonds**, which could potentially block the enzyme access to the glycosylation sites;
- Therefore, PNGase F digestion under reducing conditions was evaluated;

EIC of m/z=204.0867



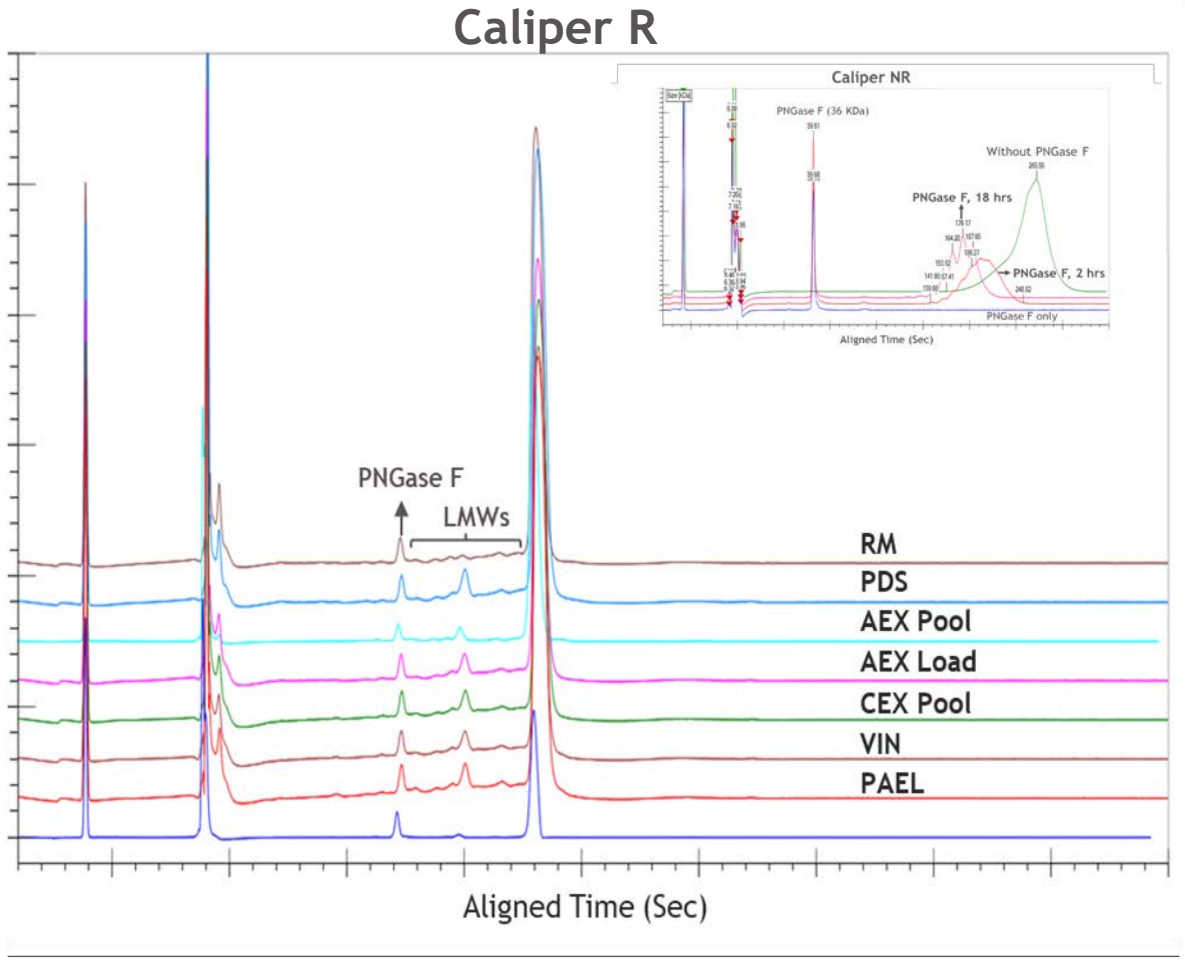


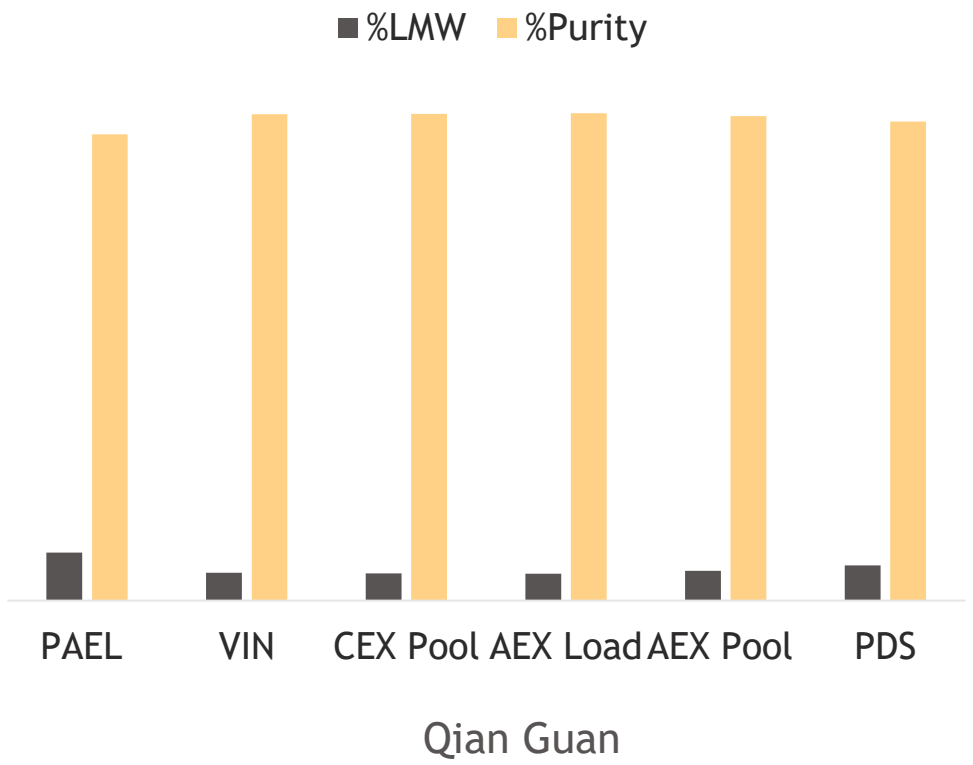


- **PNGase F digestion for 5min with DTT** is sufficient to removes *N*-glycans from all the modification sites;

Optimized Caliper (Reduced) Method for In-Process Sample Support

- PNGase F digestion protocol under reducing condition was applied to Caliper sample preparation;
- VIN step removed LMWs; As a result, % purity increased;





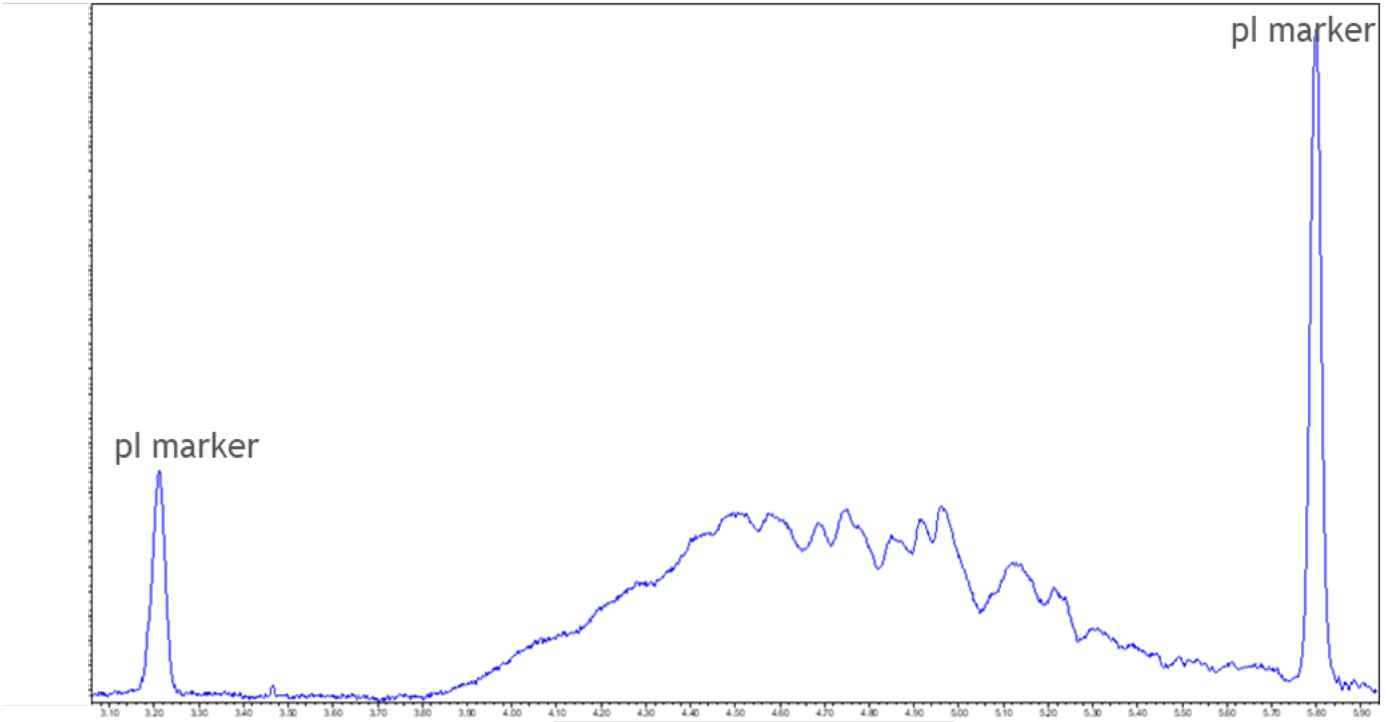
Qian Guan

Case Study II Conclusions

- LMWs were not resolved from intact protein with Caliper for a Fc-fusion protein when *N*-glycans are present;
- PNGase F digestion under reducing condition was implemented during sample preparation to enable the purity analysis with Caliper for in-process samples;
- Site-specific glycopeptide analysis with LC-MS is a useful tool for establishing molecule specific method to analyze the sample purity;

Case Study III: Complex Charge Profile of Fc-fusion Protein

- A Fc-fusion protein with >10 *N*-glycosylation sites;
- Complex charge profile due to sialic acid modification;

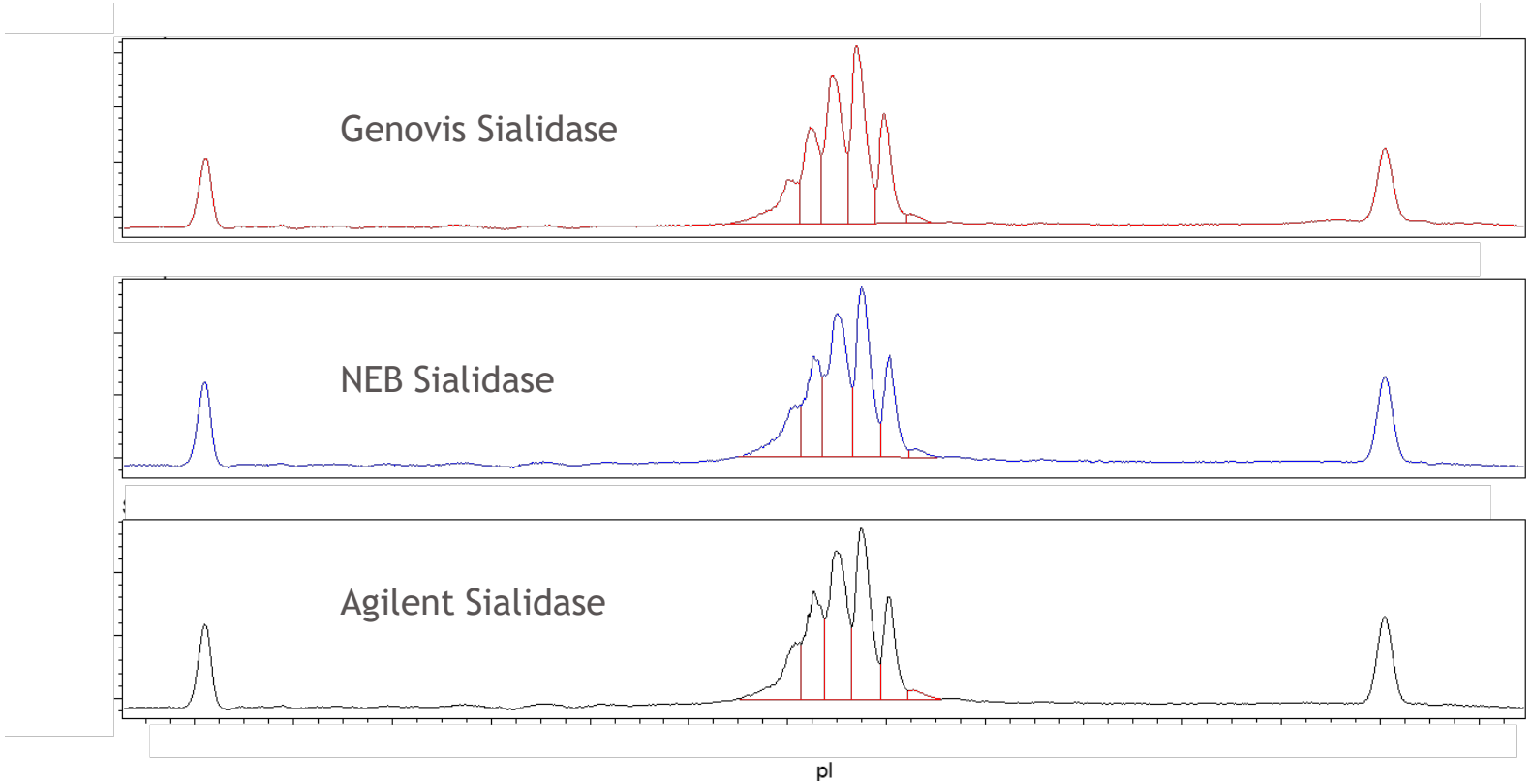


iCIEF Profile

- ❑ Difficult for peak integration;
- ❑ Challenging for results reporting;
- ❑ Limited information for process;

Simplified Charge Profile by Sialidase Treatment

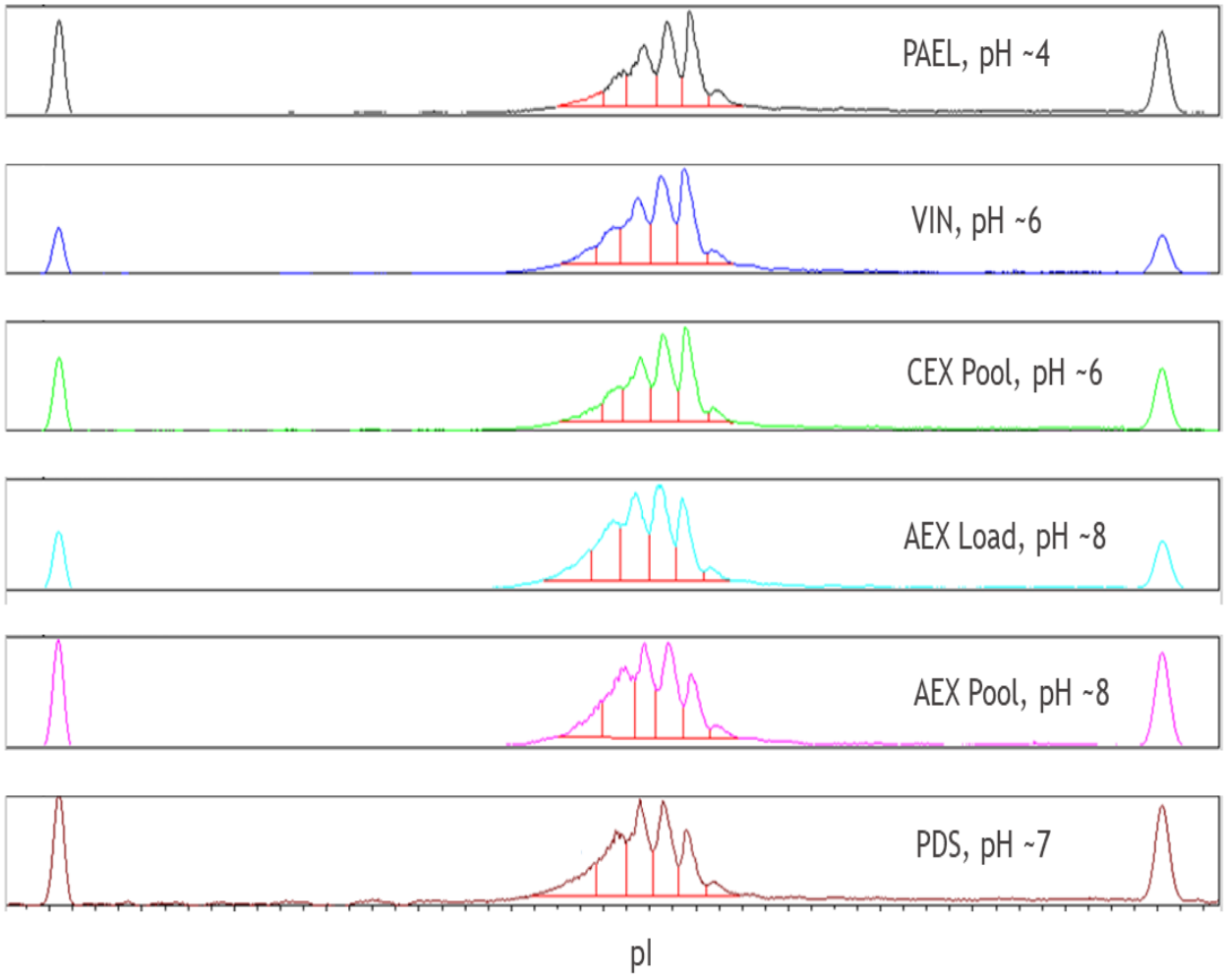
- In-process sample testing strategy
  - Reduce the charge heterogeneity by sialidase treatment;
  - Desialyated iCIEF is for monitoring other attributes that could impact the charge profile;
  - Sialic acid can be monitored by SA quantitation method, *N*-glycan method, and/or glycopeptides method;

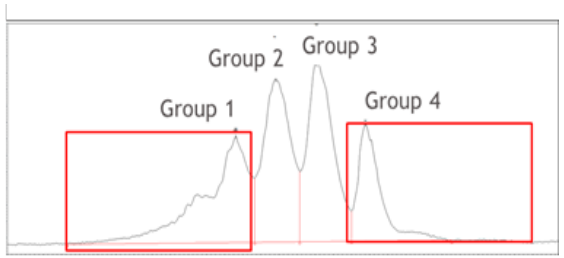


- ❑ 30 mins Sialidase digestion;
- ❑ Comparable results for three vendors' sialidase;

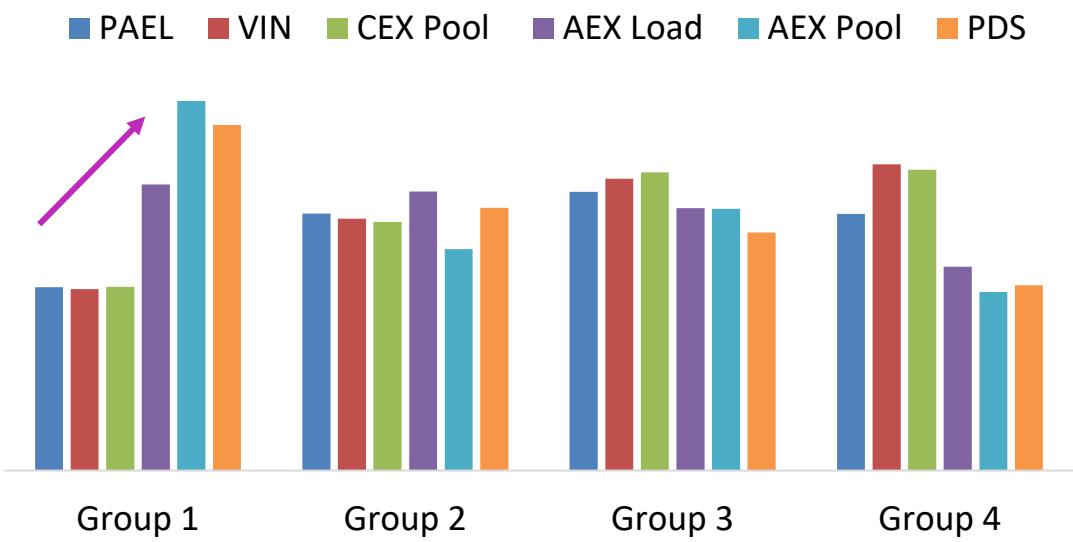
Helen Zhao, Li Zhang, Joann Max

iCIEF Profile for Downstream Steps





iCIEF Grouped Peak Intensity (%)

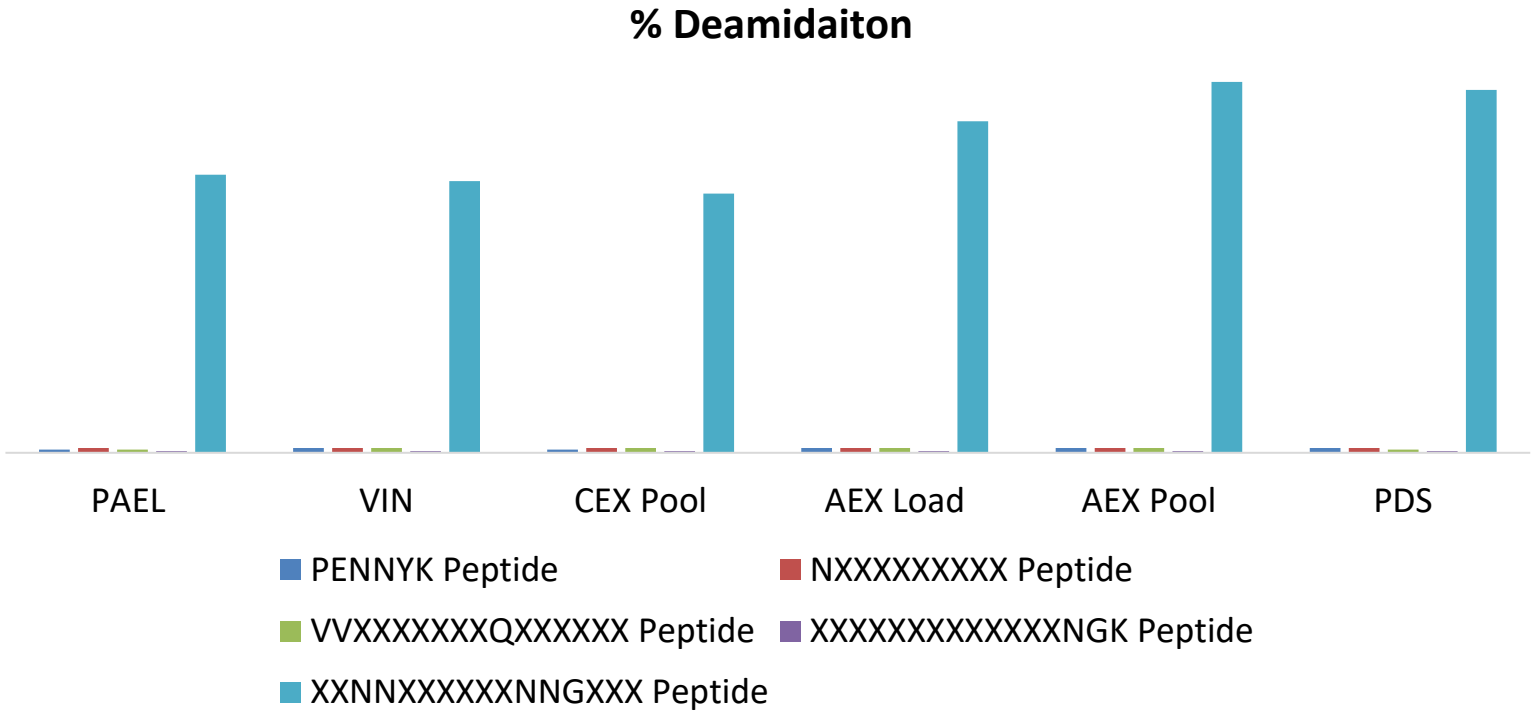


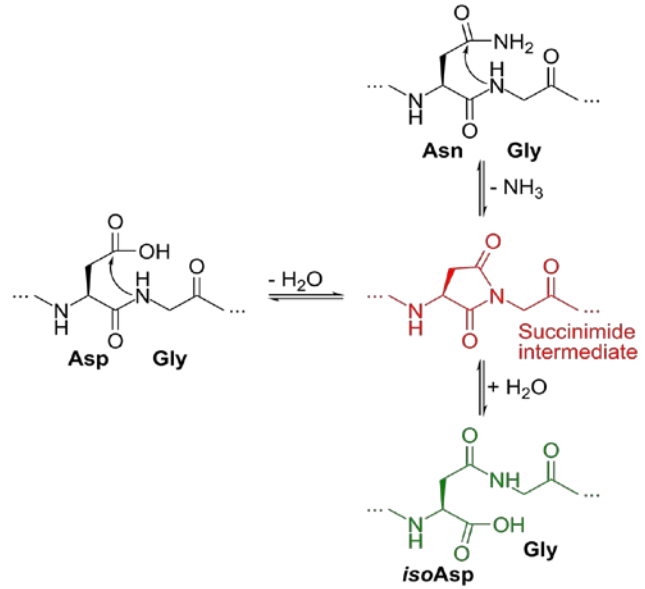
- % Group 1 **increases** starting from AEX load

Li Zhang, Helen Zhao

Deamidation by LC-MS Peptide Mapping

- CEX pool and AEX load sample pH is different: pH ~6 *vs* pH ~8;
- Deamidation is checked by peptide mapping because it's known as acidic variant and can be trigged under basic pH;
- XXNNXXXXXXNNGXXX peptide deamidation increase during downstream process;





https://en.wikipedia.org/wiki/Deamidation

Case Study III Conclusions

- Complex iCIEF profile due to terminus sialic acid;
- Simplified iCIEF profile after sialidase treatment is useful to monitor other attributes (e.g. deamidation) that could impact the charge profile;
- Peptide mapping suggested the deamidation of peptide XXNNXXXXXXNNGXXX could potentially contribute to the increase in iCIEF acidic region during downstream steps;

Summary

- The analysis of Fc-fusion proteins is challenging;
- Molecule specific methods are often required to support the process development of complex protein therapeutics;
- Mass spectrometry is a critical analytical technique to assist the development of non-platform methods for process development support;

Acknowledgement

Partnership among Process Development Analytics, Upstream, and Downstream

- **Qian Guan**
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- Mengxiao Lu

Thank You for Your Attention!


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