Too Much Sugar! A Case Study for Development of a Complex Fusion Protein

2024 CASSS CE Pharm

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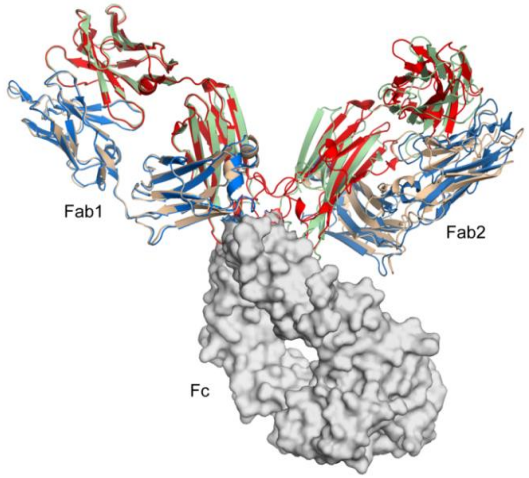
Agenda

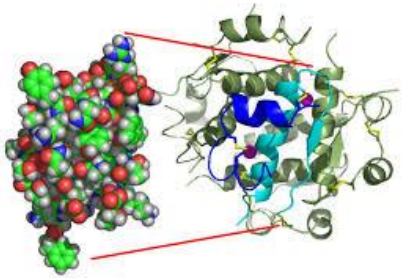
- Background
- Overview of Fusion Protein
- Case Study #1 Monitoring Fragmentation by CGE-SDS
- Case Study #2 Charge Variant Analysis via icIEF
- Conclusions/Summary

Beyond mAbs

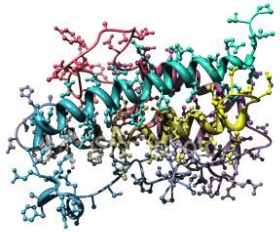


Monoclonal Antibody (mAb)
~150,000 Da

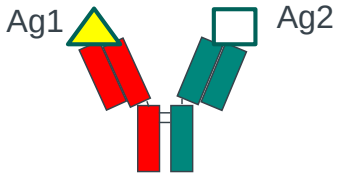
Growth Factors & Cytol
~ 15 -25,000 Daltons



Insulin- MW
~5,800 Da



Peptides- MW ~22,000 Da

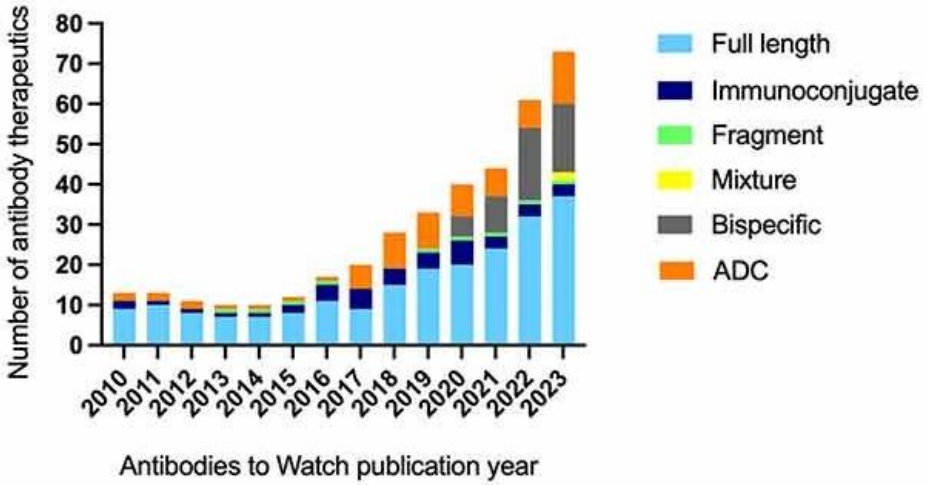


Bispecific
~150,000 Da

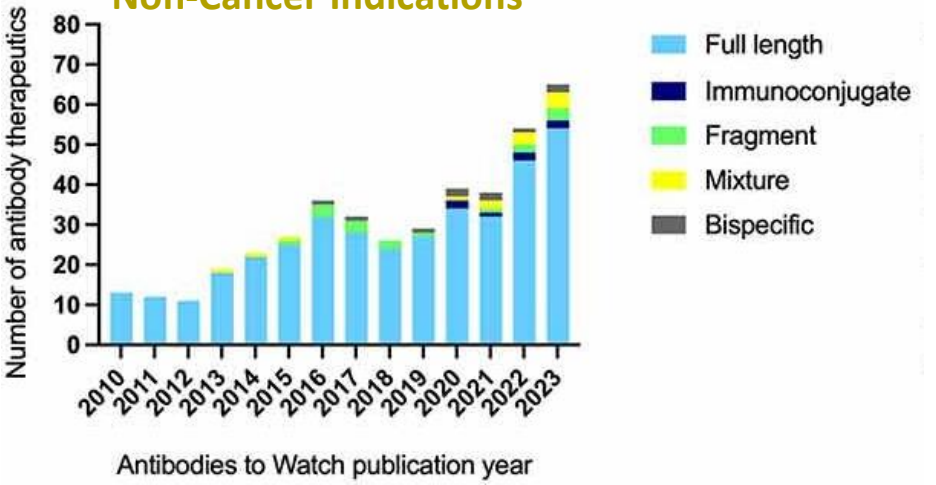
Nanobody

12-15,000 Da

Cancer Indications



Non-Cancer Indications



Kaplon, H., Crescioli, S., Chenoweth, A., Visweswaraiah, J., & Reichert, J. M. (2022). **Antibodies to watch in 2023**. *MAbs*, *15*(1).

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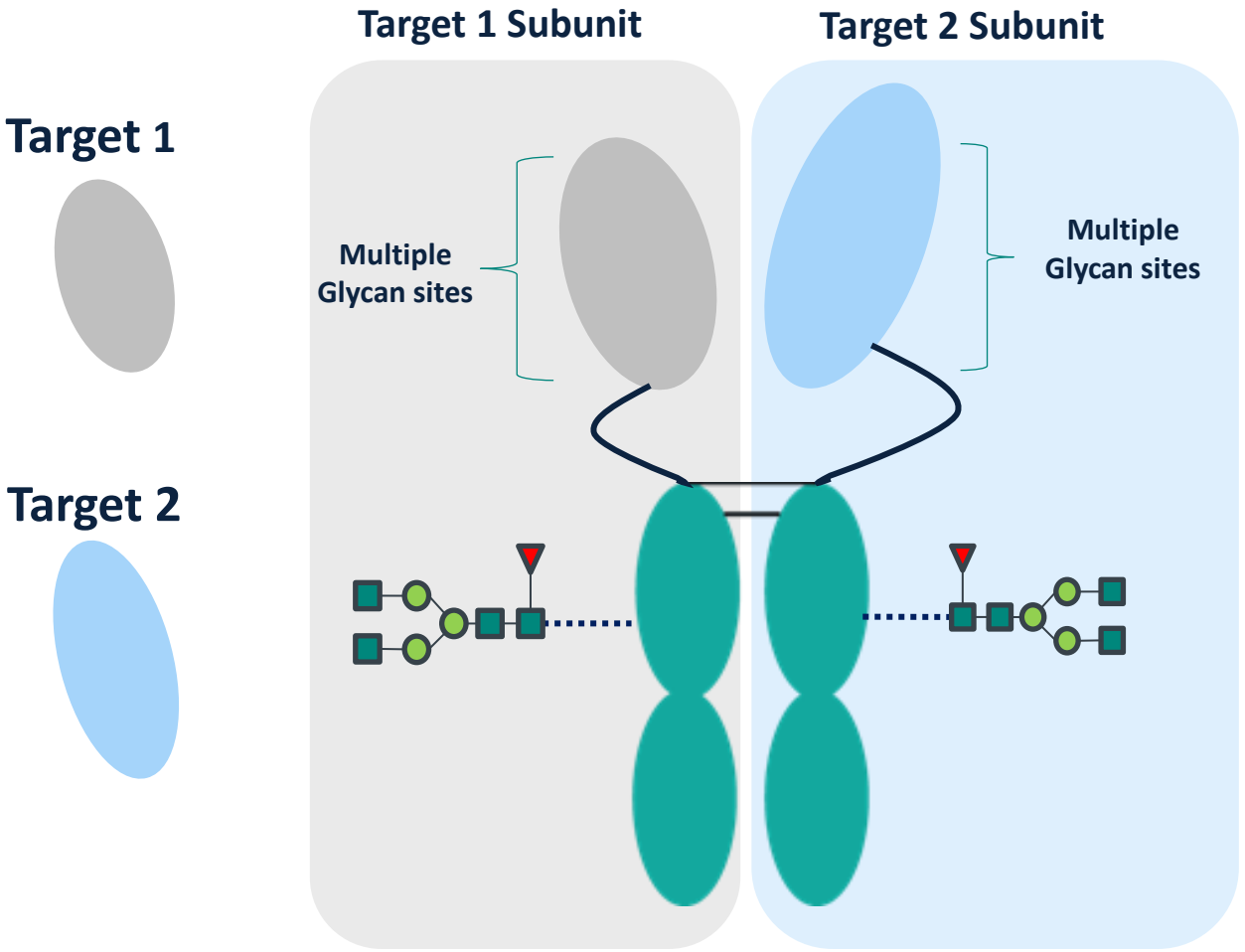
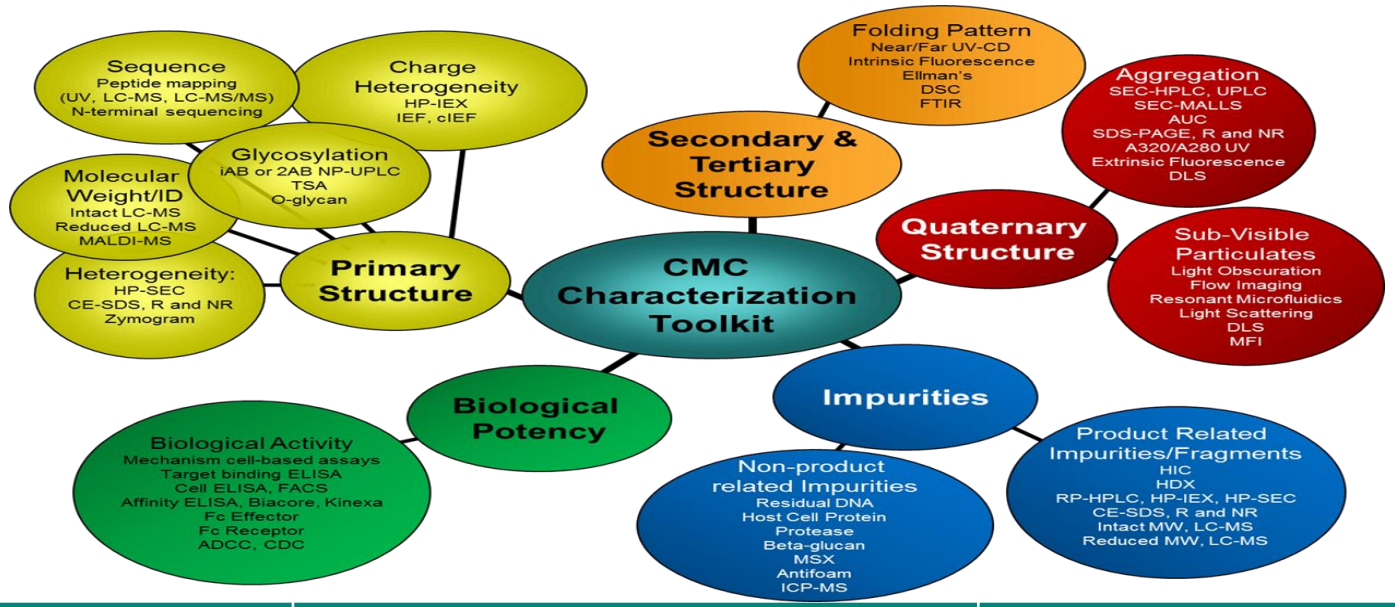
Structure of Fc Fusion Protein in Development



- Heterodimeric Fusion protein expressed as 2 single chains
  - Leads to complexity similar of a bi-specific mAb
- All glycan sites are N-linked glycans (no O-linked observed)
  - Some glycan sites in in Target 1 and Target 2 domains contain sialic acid

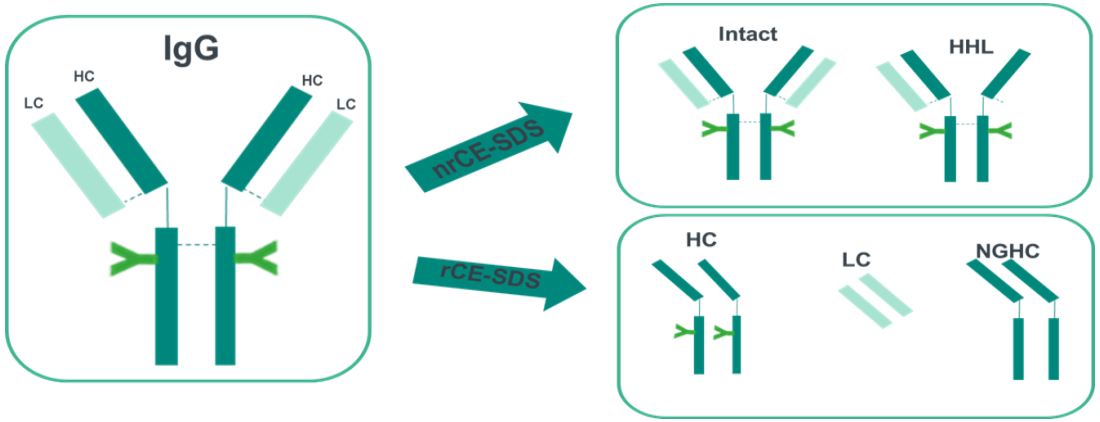
Typical Analytical Control Strategy

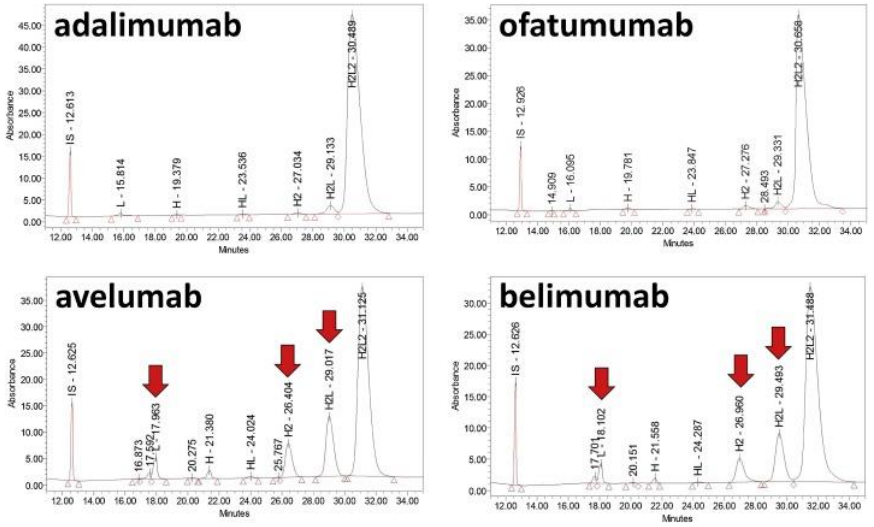


Attribute	Methodology	Rationale
Aggregation	Size Exclusion	Should be minimized as aggregation can be pose an Immunogenic risk
Fragmentation	Capillary Gel Electrophoresis Sodium Dodecyl Sulfate (CGE-SDS)	Fragmentation can have reduced efficacy, lead to potential immunogenic epitopes
Glycosylation	LC Based Methods with Fluorescent labeling	Depending on the MOA, may be considered CQA and impact efficacy and PK
Potency	ELISA or Cell Based Assay	Demonstrates the relative potency and ensures efficacy of product

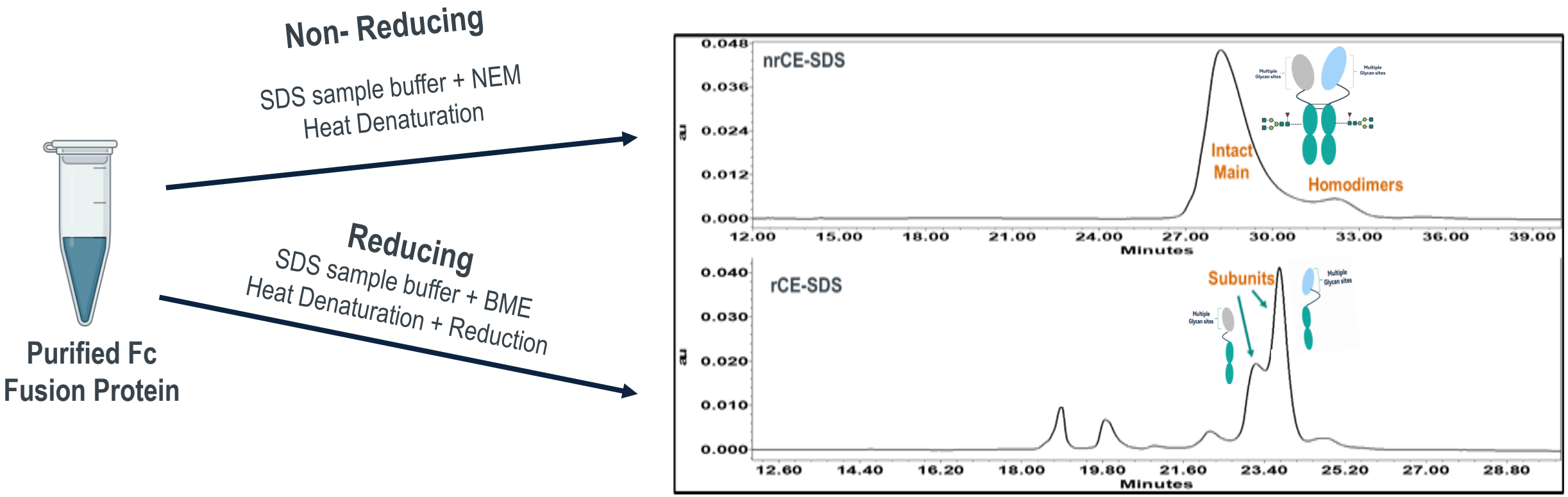
Fragmentation Analysis by CGE-SDS

- Fragmentation is a common Critical Quality Attribute (CQA) that should be controlled for biotherapeutics
- USP General Chapter 129 utilizes Sciex IgG Purity Assay Kit
- Most companies leverage ‘Platform’ method for early phase development





Wagner, E., Colas, O., Chenu, S., Alexandre Goyon, Amarande Murisier, Cianférani, S., Francois, Y., Fekete, S., Davy Guillarme, D’atri, V., & Beck, A. (2020). Determination of size variants by CE-SDS for approved therapeutic antibodies: Key implications of subclasses and light chain specificities. *Journal of Pharmaceutical and Biomedical Analysis*, *184*, 113166–113166.



Challenges

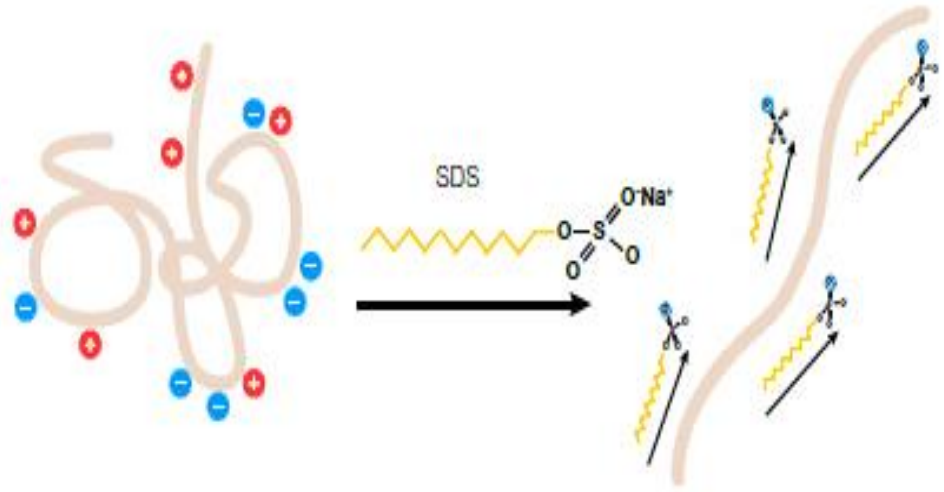
1. Significant amount of low molecular species in reducing CGE-SDS
2. Higher levels of homodimers observed compared to orthogonal assays (e.g. size exclusion)

Case Study #1 – Monitoring Fragmentation by CGE-SDS

Challenge #1 - Understanding the LMW Profile in rCGE-SDS

Optimization of Sample Preparation

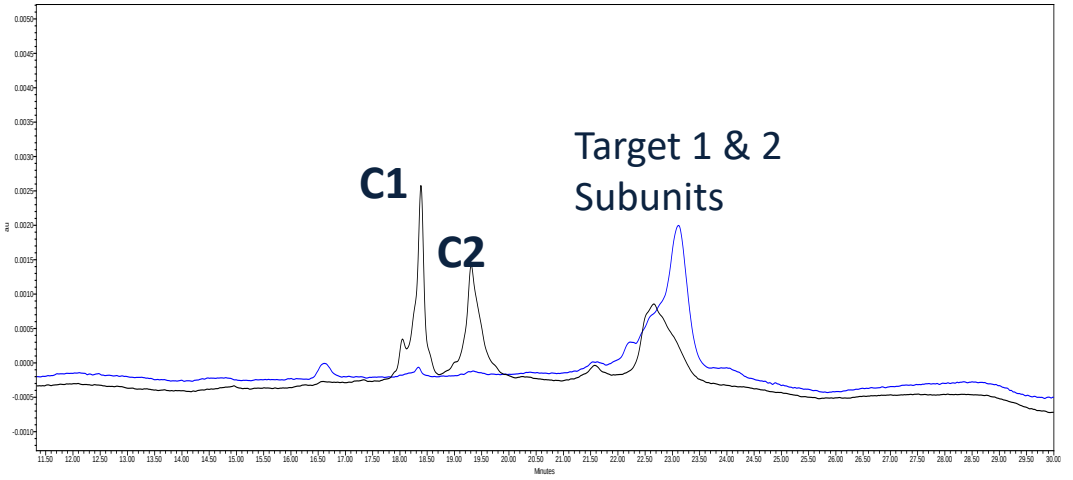
- ✖ [SDS] or other denaturants – confirmed via DSC
- ✖ Alkylating agent
- ✖ Heating temperature/time



- ✖ Unable to leverage historical experience
- ✖ Direct characterization not feasible due to SDS containing gel buffer
- ✖ Development of orthogonal methods to achieve similar resolution (reducing conditions)

- **Leverage standards, chemical treatment and enzymatic digestion to better understand profile**

Peak Identification – Leveraging Processing Conditions



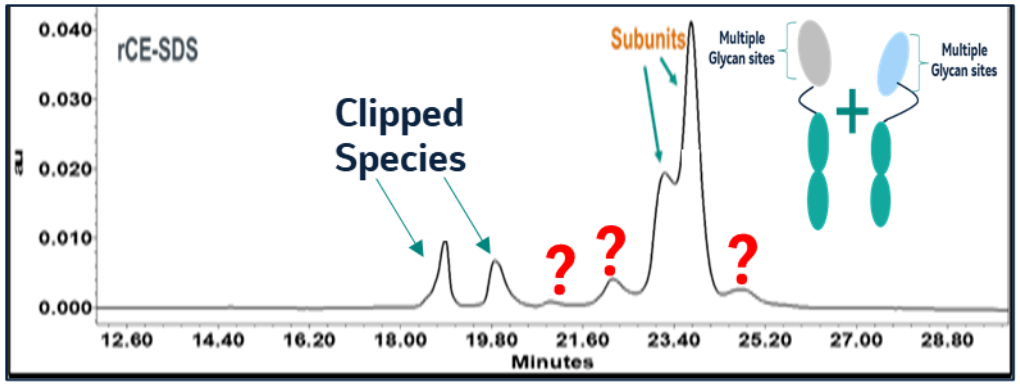
- Typical fermentation process =14d
- Increased upstream process > 20d
  - Known clipping site increases observed (confirmed via RPLC-MS)

<u>Observations with extended fermentation</u>

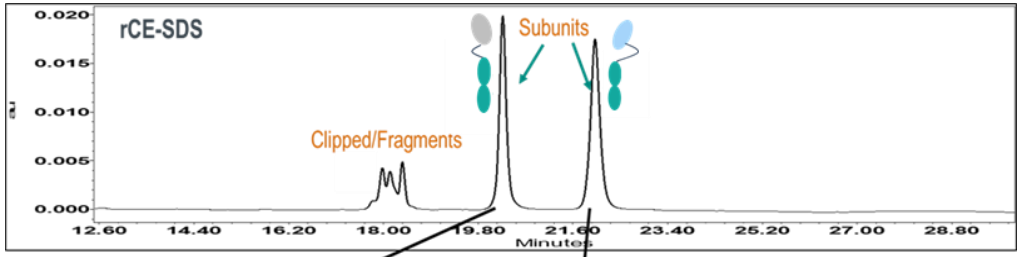
1. Reduction in peak area for Target 1 and 2 subunits
2. Increase in clipped species (C1 and C2)

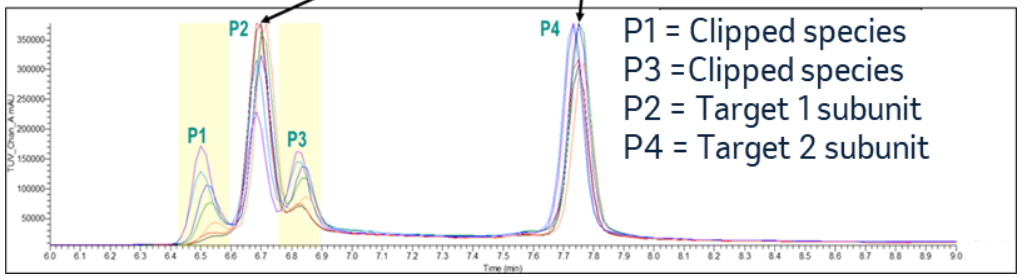
❑ Known clipped species identified in e-gram, what about all the other peaks in the profile?

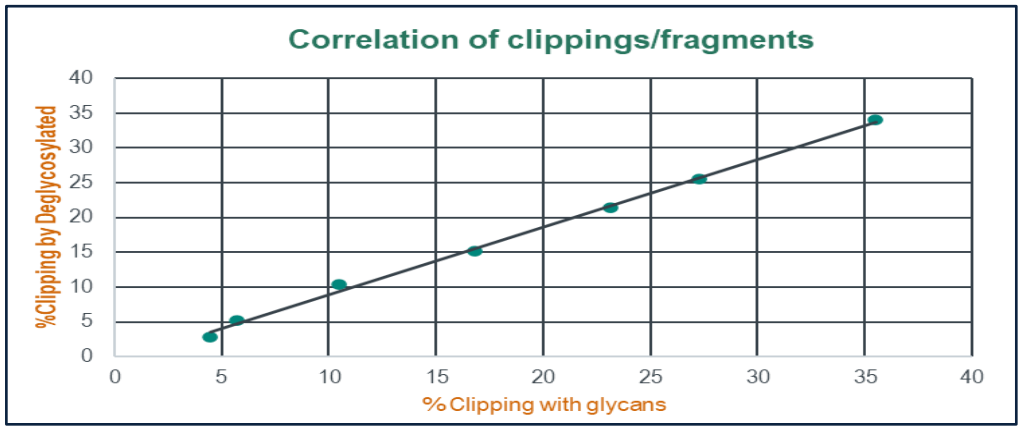
Peak Identification -Enzymatic Digestion to Simplify Heterogeneity



PNGase F







- PNGase F digestion simplifies the peak profile and allows for accurate detection of fragments
- Unknown peaks are removed following PNGase F digestion
  - Several clones with different levels of clipped species analyzed by rCGE-SDS and Reversed Phase LC-MS
- Glycan removal allowed for simplified analysis of fragments and aligns with ATP

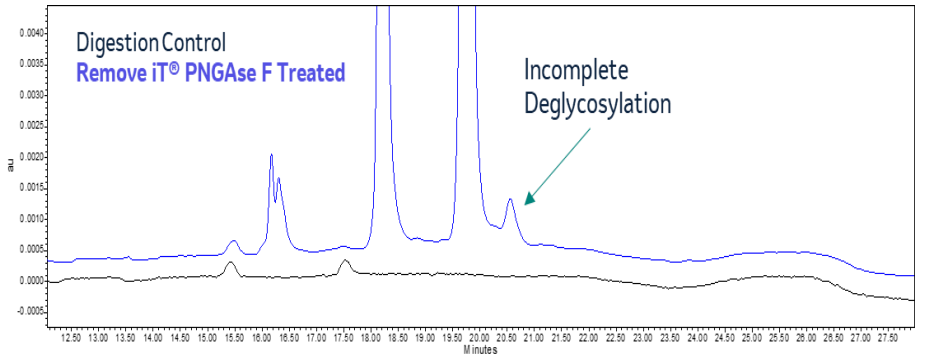
CGE-SDS data generated by Jaymin Patel and RP-LC-MS data by Anita P Liu.

Optimization of Deglycosylation for QC Implementation

Option 1 Remove iT® PNGase F

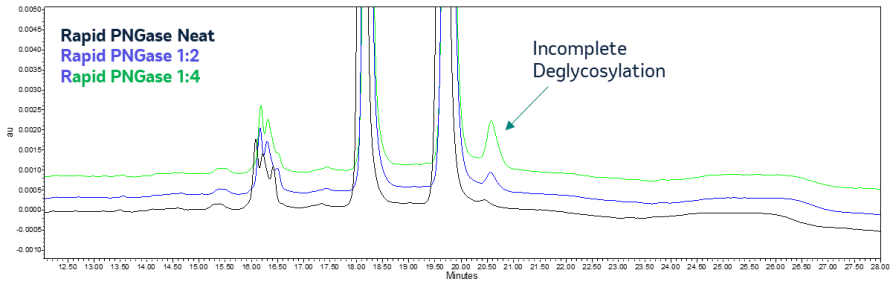
Remove-iT®PNGase F is an engineered N-glycanase containing Chitin Binding Domain (CBD)

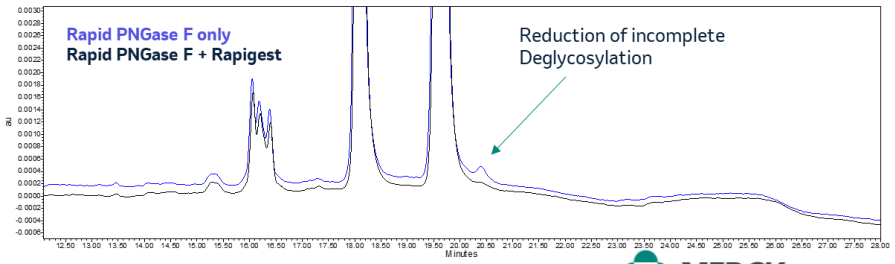
Protein Denaturation → Deglycosylation (Incubation @ 37°C for 1h) → Enzyme Removal → CGE-SDS Analysis



Option 2 Rapid PNGase F + Rapigest SF

Protein Denaturation (Incubate @ 80°C for 2 min) → Deglycosylation (Incubation @ 50°C for 10 min) → CGE-SDS Analysis

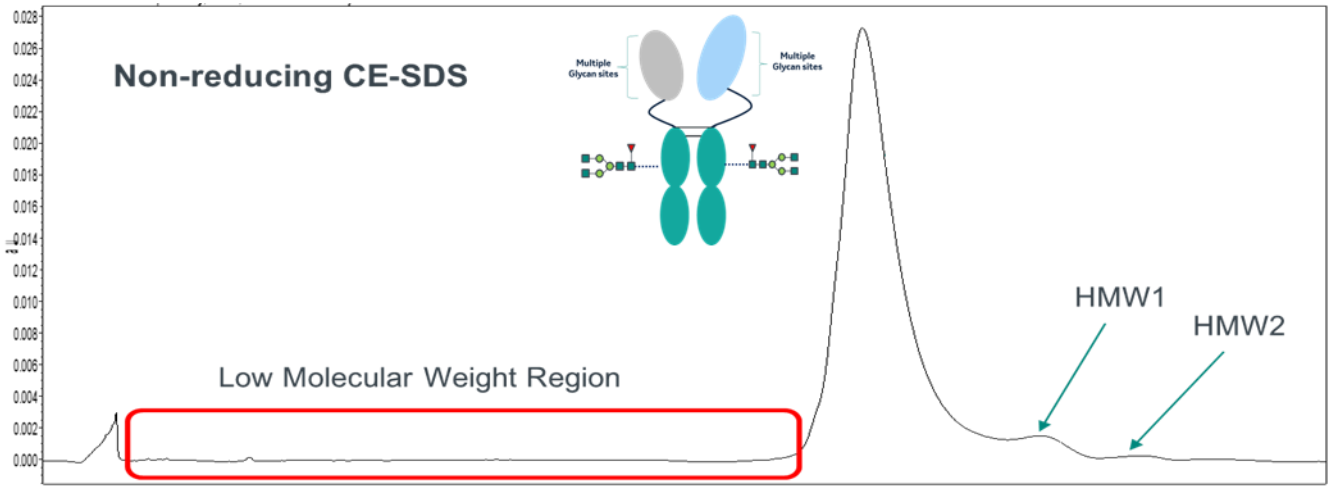




- ❑ Rapid PNGase F + Rapigest SF removes all ‘sticky’ glycans prior to CGE-SDS Analysis

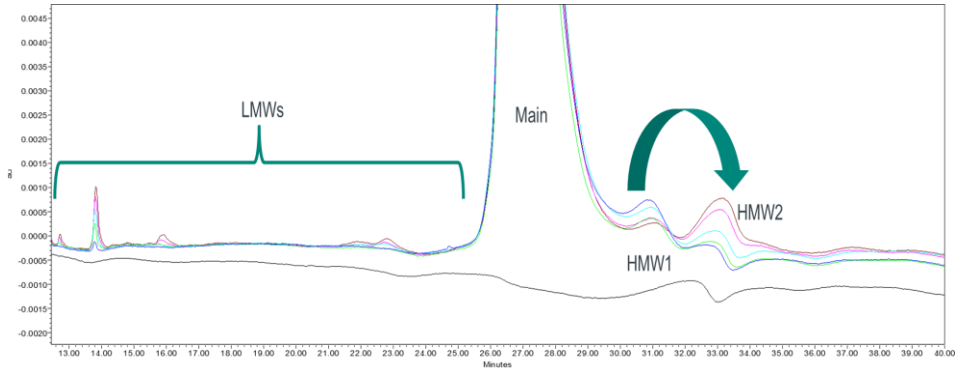
Data generated by Priya Venkatakrishnan

Challenge #2 – HMW Artifact in nrCGE-SDS



Level	Main Peak		HMW 1	
	% Purity	Recovery	% Purity	Recovery
DS-50%	98.2	103	1.1	38
DS-100%	96.7	100	3.0	100
DS-150%	95.6	97	4.2	139

Thermal Stressed Samples



Observations During Qualification

- CGE-SDS HMW > Size exclusion
- Non-Linear response during method qualification
- Conversion of HMW 1 →HMW 2

Data generated by Jaymin Patel

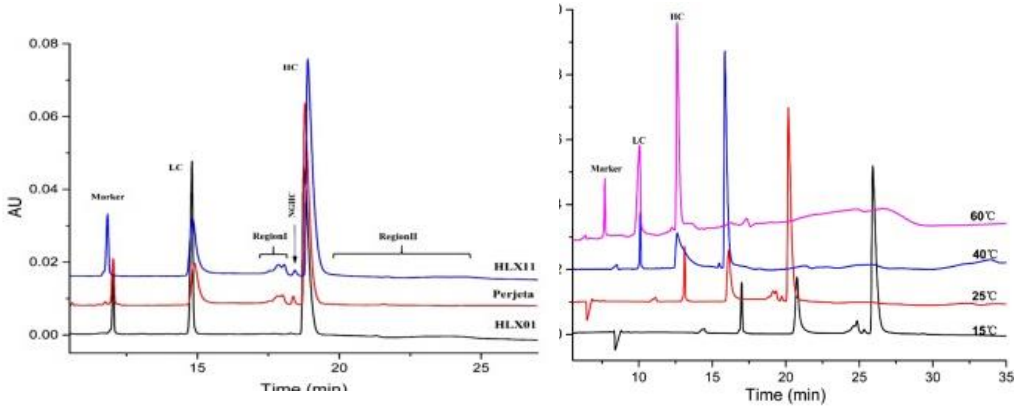
Elimination of HMW Artifacts in nrCGE-SDS


Journal of Pharmaceutical and Biomedical Analysis
Volume 190, 25 October 2020, 113527

Short communication

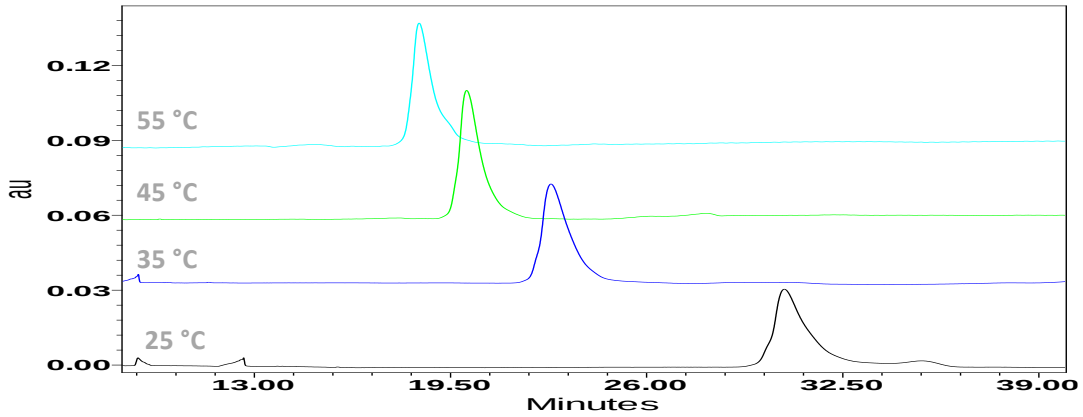
Characterization and elimination of artificial non-covalent light Chain dimers in reduced CE-SDS analysis of pertuzumab

Lei Zhang [1], Mengdan Fei [1], Yanqing Tian, Sipeng Li, Xiaoqi Zhu, Linlin Wang, Yanpeng Xu, Michael Hongwei Xie

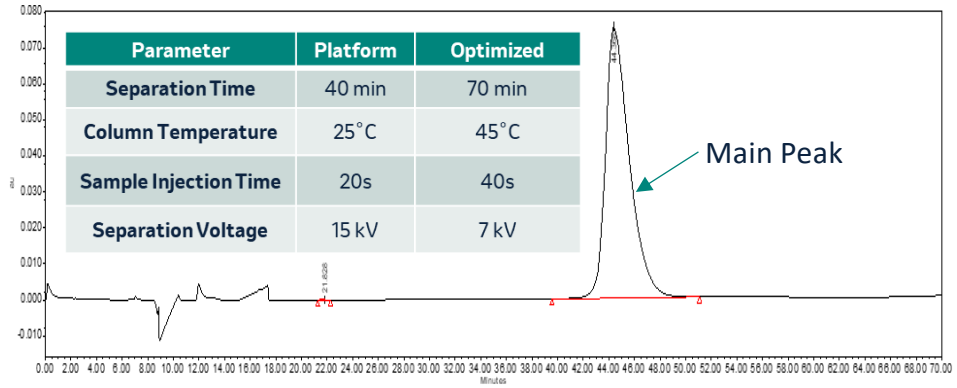


Zhang, L., Fei, M., Tian, Y., Li, S., Zhu, X., Wang, L., Xu, Y., & Michael Hongwei Xie. (2020). Characterization and elimination of artificial non-covalent light Chain dimers in reduced CE-SDS analysis of pertuzumab. *Journal of Pharmaceutical and Biomedical Analysis*, *190*, 113527–113527.

Fusion Protein Development



Parameter	Platform	Optimized
Separation Time	40 min	70 min
Column Temperature	25°C	45°C
Sample Injection Time	20s	40s
Separation Voltage	15 kV	7 kV



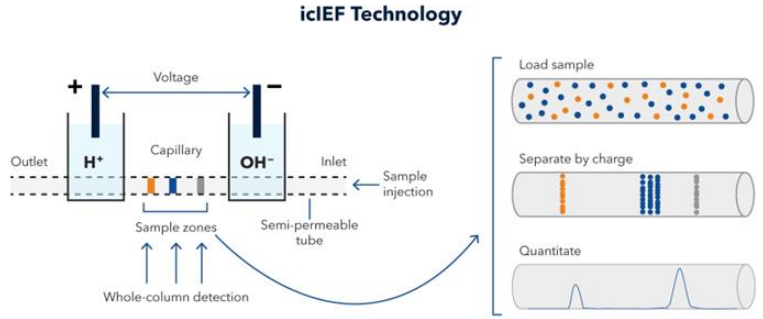
Level	% Purity
150	99.8
125	99.8
100	99.8
75	99.8
50	99.7

- Increasing capillary temperature (during separation) removed sample induced artifact
  - Decrease separation voltage to maintain similar resolution
  - Optimized method had improved linearity, accuracy and precision

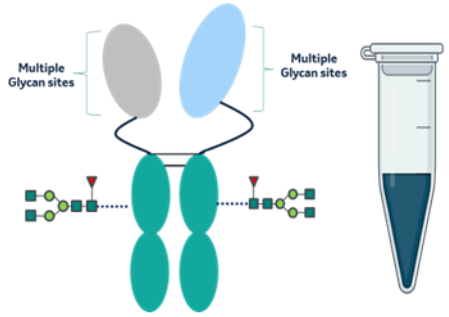
Data generated by Monica Haley

Case Study 2- Charge Variant Analysis via icIEF

- Charge variants are a common CQA monitored for biotherapeutics as they provide information on PTM's and changes on stability
- Most common use for early phase is icIEF or CZE due to their 'platformability'

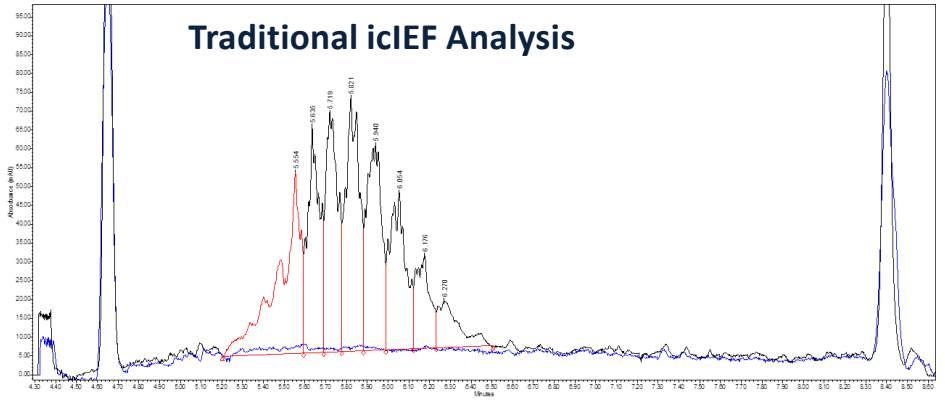


Case Study 2 - Complex icIEF Profile

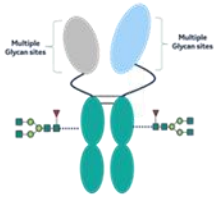


Some glycan sites contain sialic acid

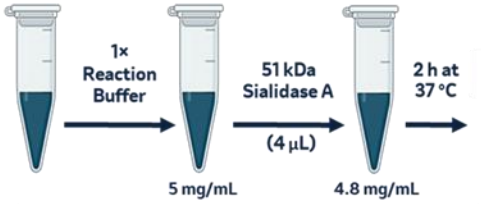
Reagent
Pharmalyte 3-10
Pharmalyte 5-8
10 mM L-Arginine
8 M Urea
1% Methyl Cellulose
Milli-Q Water

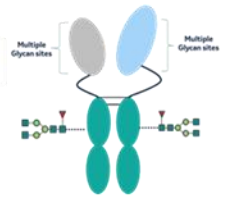


- Complex heterogeneous profile observed due to presence of sialic acid



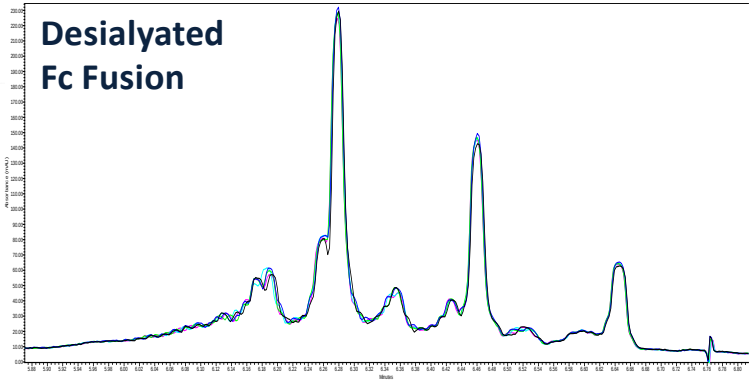
Sialic acid on glycans





Sialic acid removed from glycans

Reagent
Pharmalyte 3-10
Pharmalyte 5-8
10 mM L-Arginine
8 M Urea
1% Methyl Cellulose
Milli-Q Water

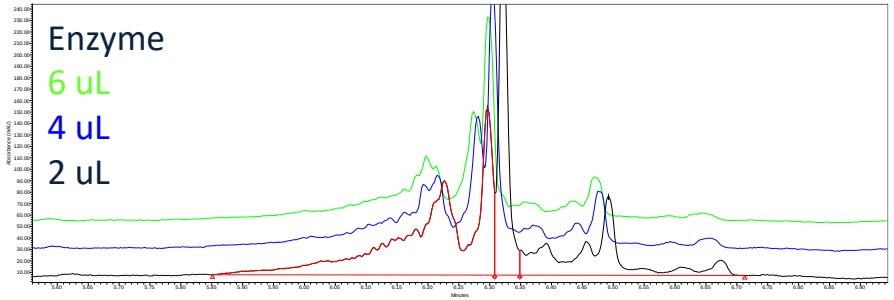


- Removing sialic acid simplifies icIEF trace, leading to a more traditional profile

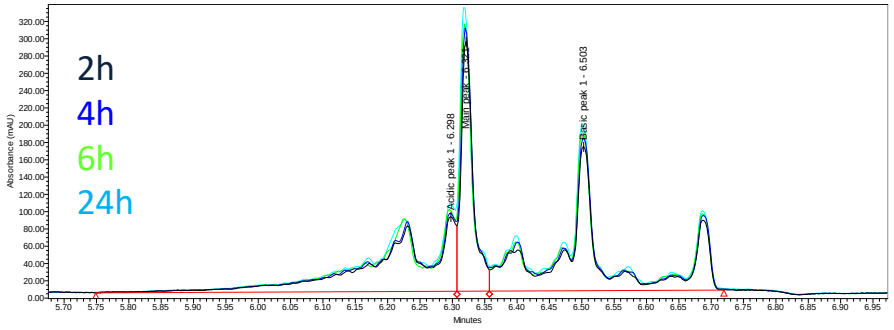
Data generated by Michael Grasso

Can the icIEF Peak be further characterized by enzymatic digestion?

Amount of Sialidase Enzyme

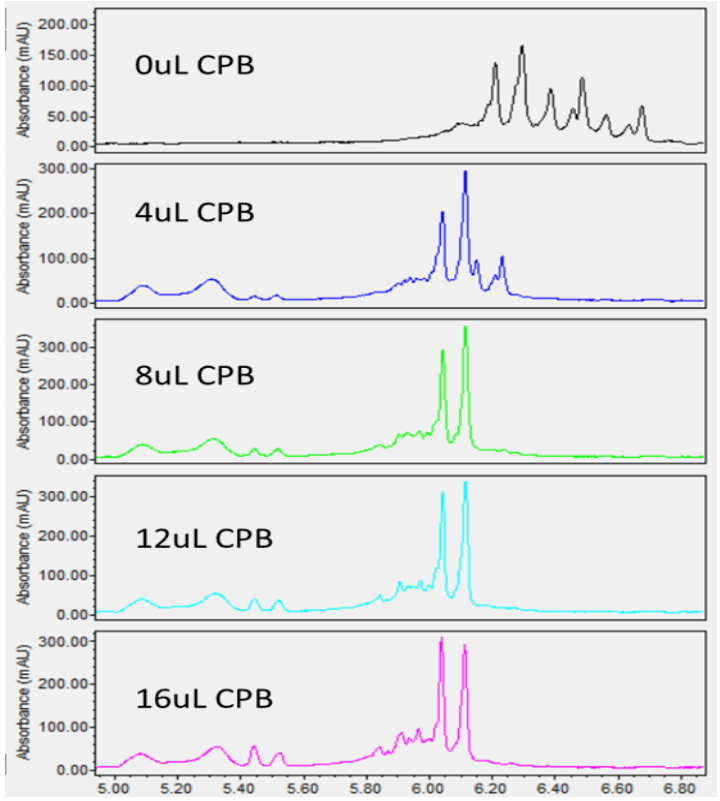


Desialylation Digestion Time



- No changes in profile from increase enzyme and digestions time

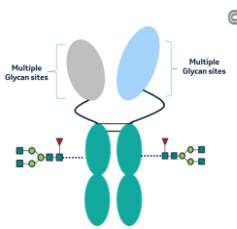
Carboxy Peptidase B

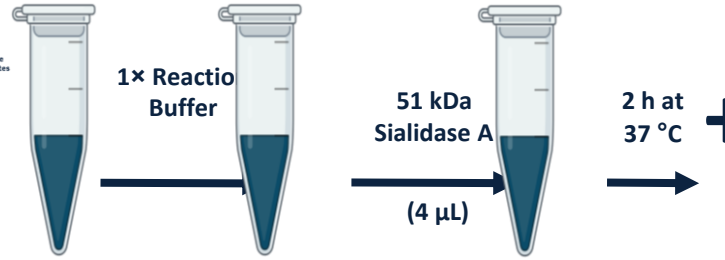


- Digestion with CPB simplifies profile indicating presence of C-Term Lysine

Data generated by Michael Grasso

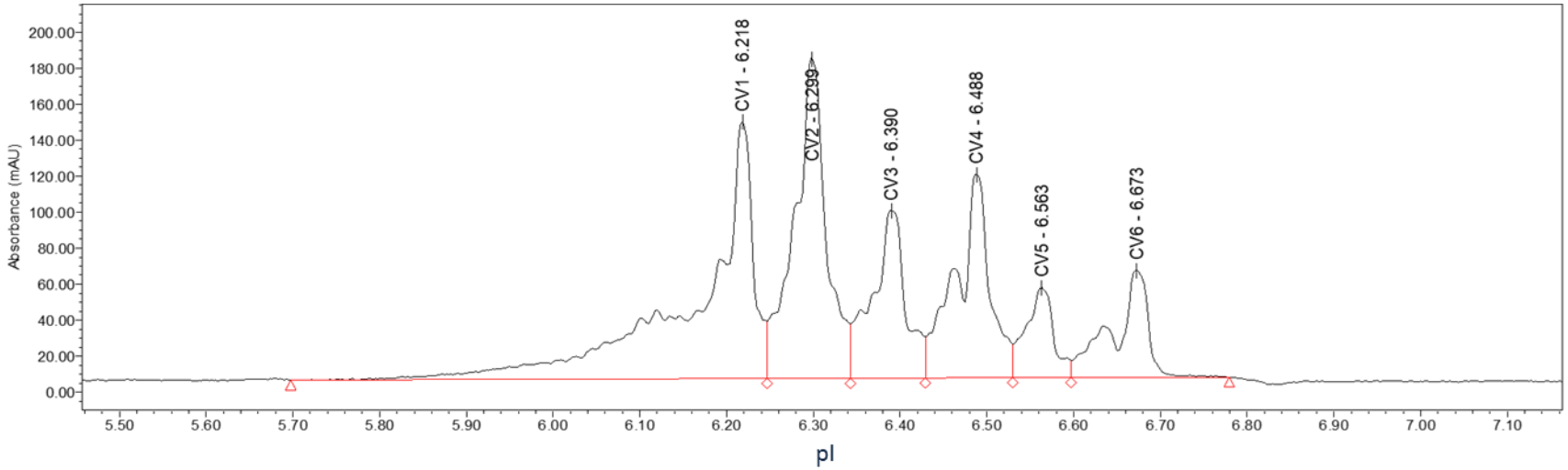
icIEF method for highly glycosylated and sialylated Fusion Protein





De-Sialylation by Sialidase Enzyme

Reagent
Pharmalyte 3-10
Pharmalyte 5-8
10 mM L-Arginine
8 M Urea
1% Methyl Cellulose
Milli-Q Water



- ❑ Simplified profile achieved by sialidase treatment
- ❑ Updated peak report to report charge variant groups vs. traditional (acidic, main, basic)

Conclusion and Future Directions

- Understanding of complex rCGE-SDS profile - incorporation of enzymatic step and importance of understanding sample prep
- Artifact in nrCGE-SDS mitigated through optimization of separation conditions
- icIEF profile identified and implemented enzymatic digestion and updated peak reporting
- Platforms analytics will be challenged with addition of fusion proteins and other novel modalities
- New characterization approaches/tools will be required to support analytical control strategy

Acknowledgements

❑ AEC-Mass Spectrometry
- Anita P. Liu
- Hongxia Wang

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- Michael Grasso
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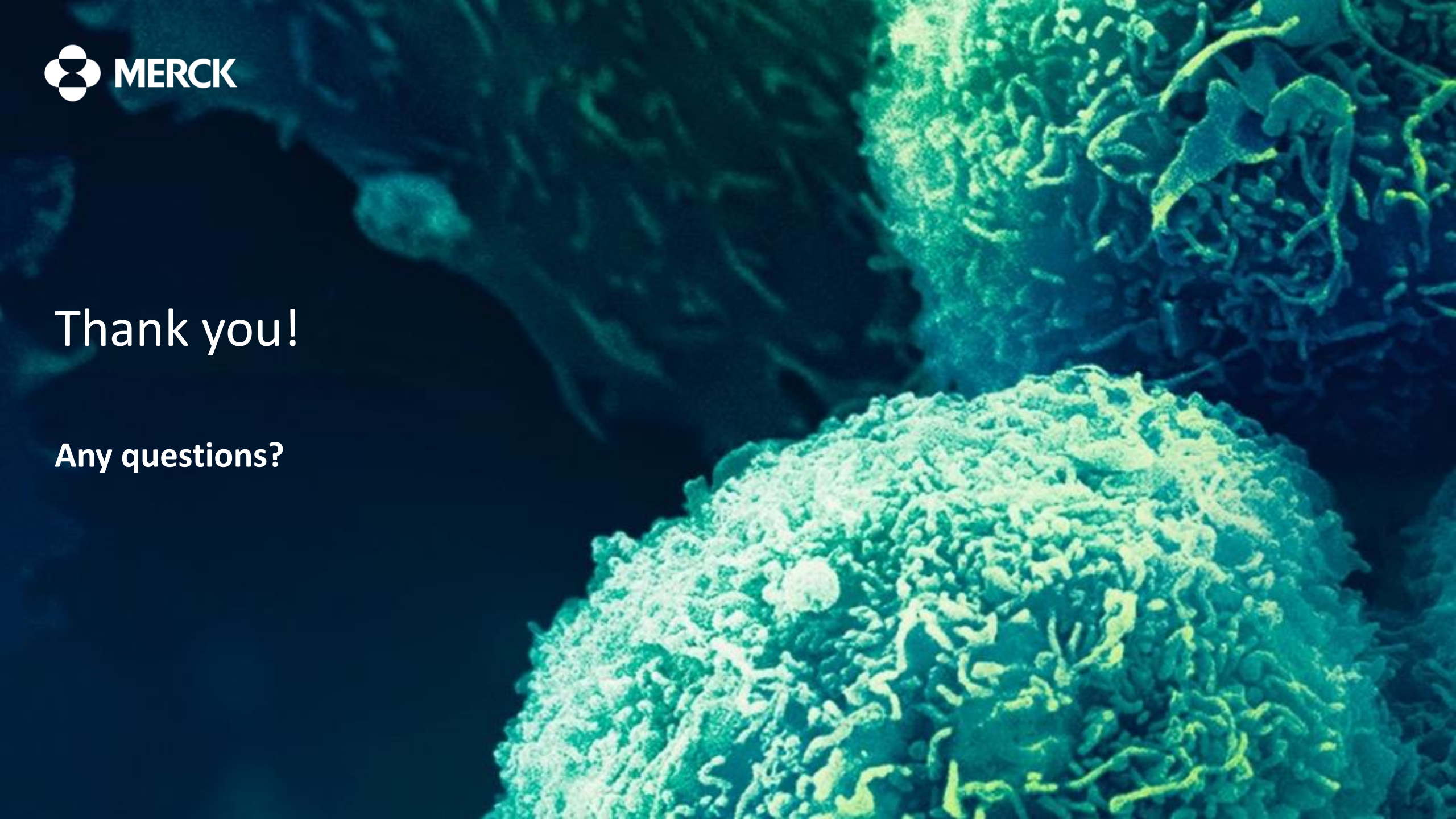
- Alex Pavon
- Xinliu Gao
- Jason Cheung
- Eric Routhier

❑ Preclinical Development
- Veronica Juan

❑ Biologics Process R&D
- Michael Iammarino
- Jessica Pan
- Tiffany Tang
- Julie Robinson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Thank you!
Any questions?