New Paradigm for Charge Variant Characterization Using cIEF-MS and 'Discovery' Assisted Fractionation

Qi Wang, Ningning Wang, Chunni Lei, Guangwei Zhou, Yiqing Cui, Yongping Xu, Yongsheng Xiao

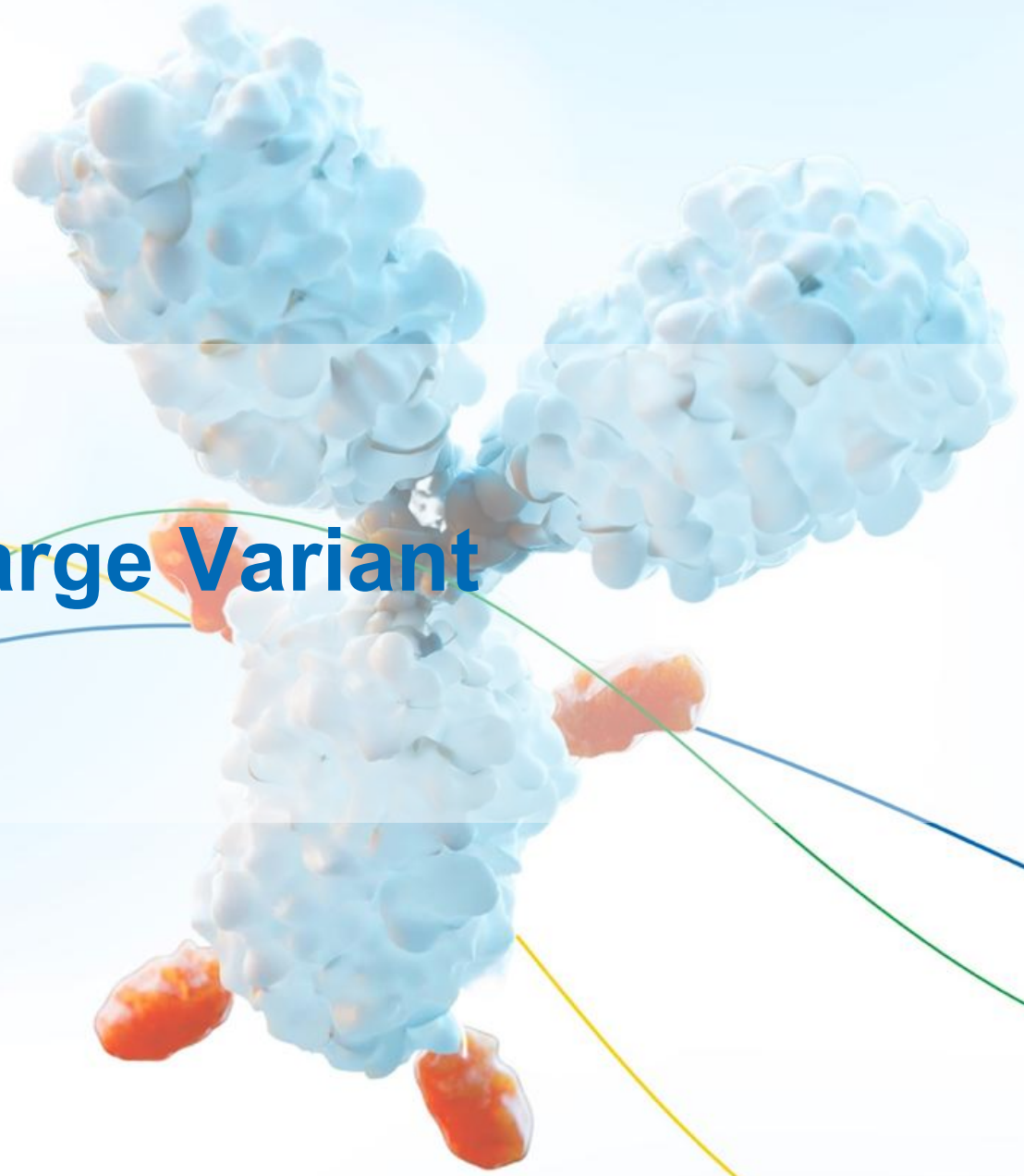
Department of BioDev Wuxi Process Development, WuXi Biologics

CASSS Mass Spec, 2025

Disclaimer

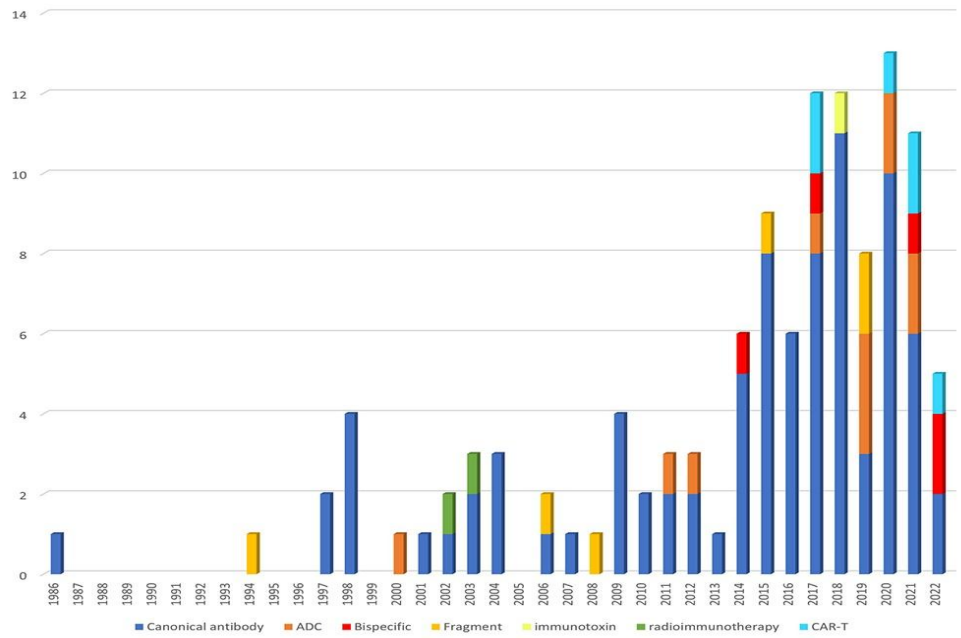
- All rights are reserved. The information in this PPT shall not be considered as information disclosure or investment advice.
- This document is strictly confidential to the recipient only, and may not be copied reproduced, redistributed, disseminated, or used or disclosed to any other person, or published , in whole or in part, for any other purpose without written permission from the Company and the author.
- This document has been prepared using information provided by the Company but without further investigation cannot be warranted as to its accuracy or completeness. Certain data in this document was obtained from external data sources, and the Company has not verified such data with independent sources. Accordingly, the Company makes no representations as to the accuracy or completeness of that data. Such data involves risks and uncertainties and is subject to change based on various factors. The use of registered trademarks, commercial trademarks and logos or photographic materials within this document are exclusively for illustrative purposes and are not meant to violate the rights of the creators and/or applicable intellectual property laws.

01 Introduction of Charge Variant Characterization



Introduction

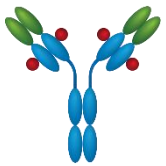
The drug market of protein therapeutics has shown a remarkable expansion in last two decades.



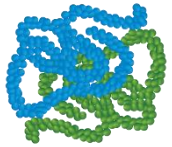
Monoclonal Antibody

Bispecific Antibody

Fc-fusion Protein

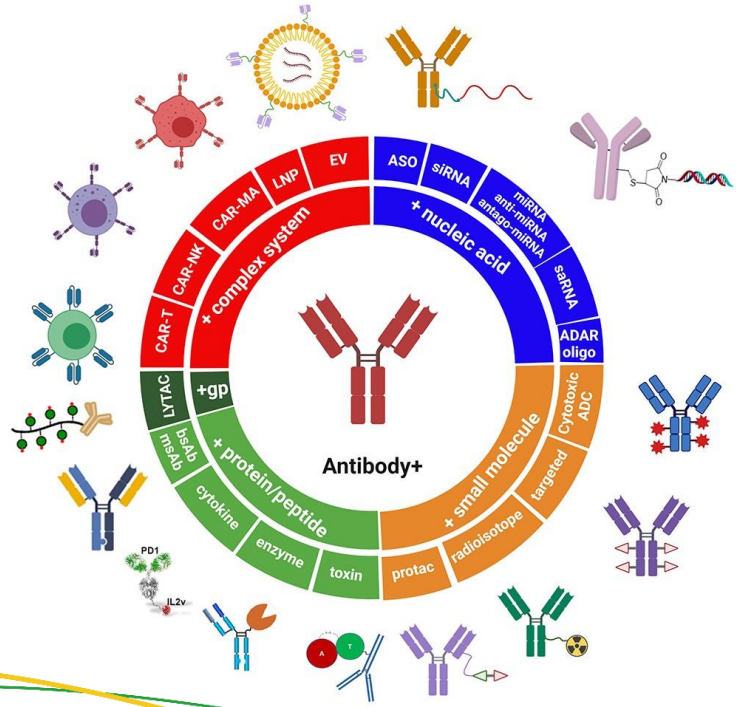


Antibody Drug Conjugate



Enzyme Replacement Therapy

Antibody Fragments



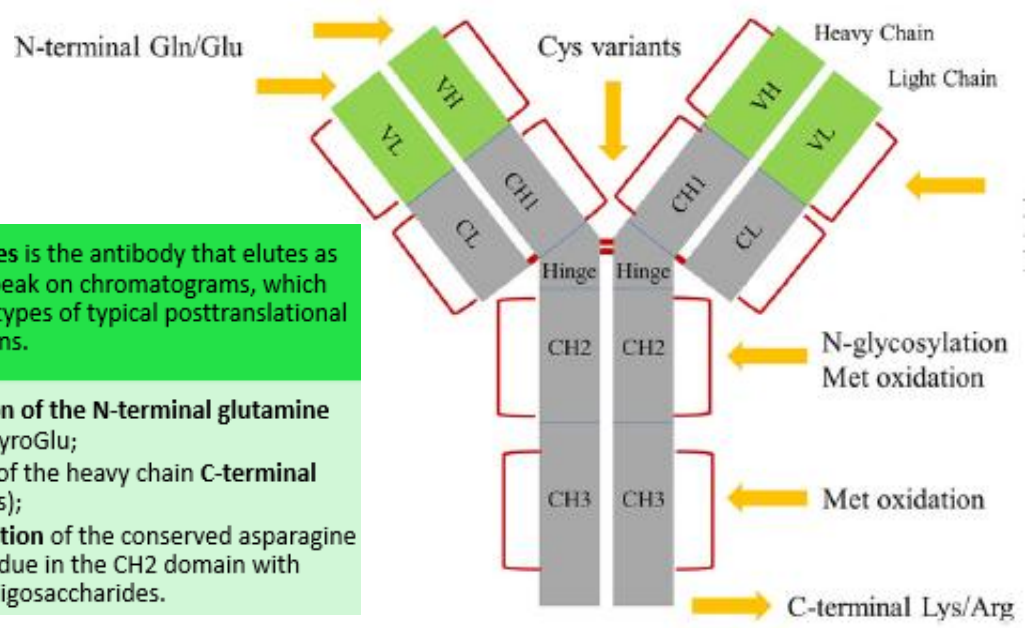
Antibody or antibody-containing therapeutics approved by the FDA as of 31 March 2022.

Antibody Ther, Volume 5, Issue 4, October 2022, Pages 280–287

Charge Variant Characterization: Acidic & Basic Species

- Charge variant is one of the most common heterogeneity types for biotherapeutics due to structural modifications and degradation during or after the production process.
- Charge variants including acidic and basic species are common CQAs for protein therapeutics.
- A variety of structural modifications are known to cause the formation of acidic or basic variants.

Acidic species
Sialic acid
Deamidation
Non-classical disulfide linkage
Trisulfide bonds
High mannose
Thiosulfide modification
Glycation
Modification by maleuric acid
Cysteinylation
Reduced disulfide bonds
Non-reduced species
Fragments



Main species is the antibody that elutes as the major peak on chromatograms, which with three types of typical posttranslational modifications.

- **Cyclization of the N-terminal glutamine** (Gln) to pyroGlu;
- Removal of the heavy chain **C-terminal lysine** (Lys);
- **Glycosylation** of the conserved asparagine (Asn) residue in the CH2 domain with neutral oligosaccharides.

Basic species
C-terminal Lys
N-terminal Glu
Isomerization of Asp
Succinimide
Met oxidation
Amidation
Incomplete disulfide bonds
Incomplete removal of leader sequence
Mutation from Ser to Arg
Aglycosylation
Fragments
Aggregates

Charge Variant Characterization by Online cIEF-MS

02

Characterization of Charge Variants at Different Drug Discovery and CMC Development Stages

Drug Discovery | Pre-IND and Phase I | Commercial Process Development | BLA Filing

CPD → PC → Pre - PPQ → PPQ → Commercial Launch / CPV

Understand the structure of each charge variant; limited functional testing

1. Identification of variants significantly changed during developability and stability, force degradation study
2. Troubleshooting / investigation during process and formulation development
3. Preliminary variants identification to define target range for CQA before major process change or process development for biosimilar product

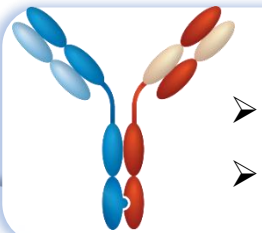
Anal. Chem. 2018, 90, 3, 2246−2254

Method Optimization:

- ❑ **Sample preparation**:
- ❑ **cIEF Separation:**
  - Capillary:Neutral-hydrophilic coatings and lengths.
  - Injection amount:
  - Focus voltage:
- ❑ **Interface:**
  - Tip position:
  - Sheath solution:

cIEF-MS to Support Early Stability and Developability Assessment

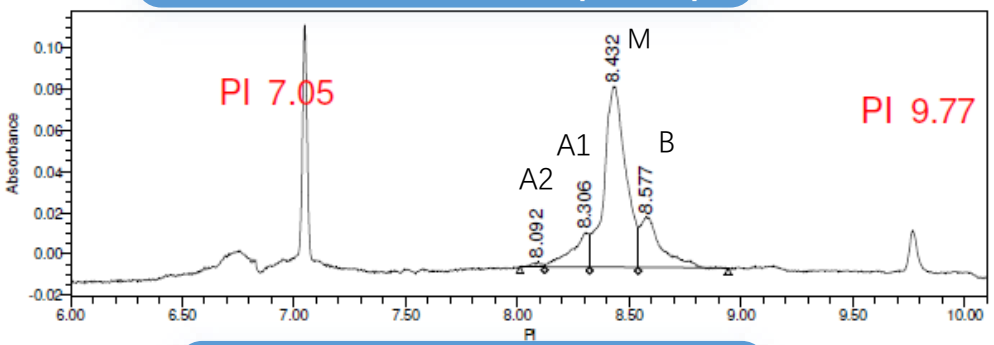
- Charge profile is the most susceptible attribute under thermo stress (40℃) condition.
- icIEF and cIEF-MS show consistent charge profile for unstressed and stressed samples



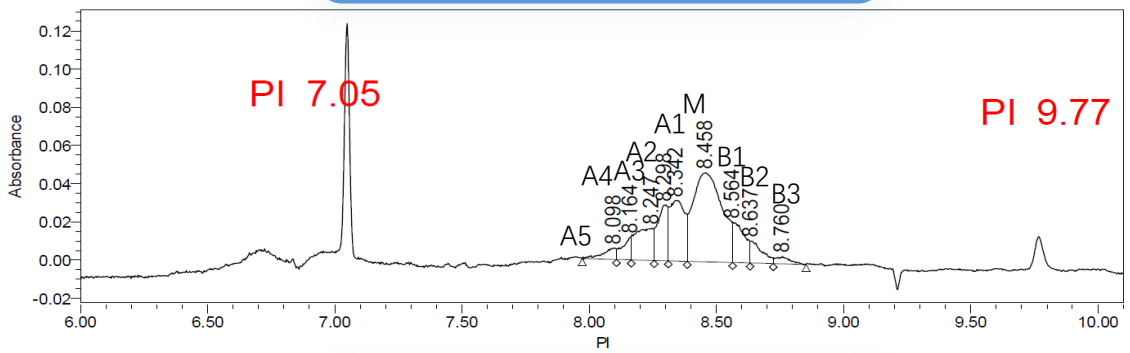
Molecular Information

- Bispecific antibody
- 2 N-glycosylation sites

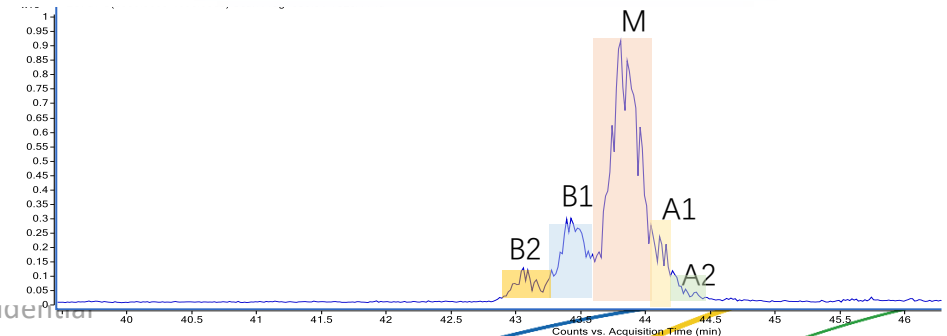
icIEF result of unstressed sample sample



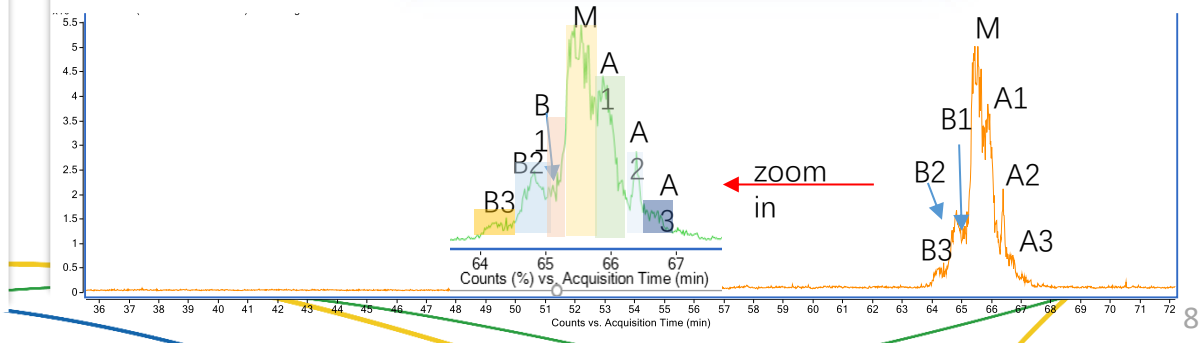
icIEF result of stressed sample sample



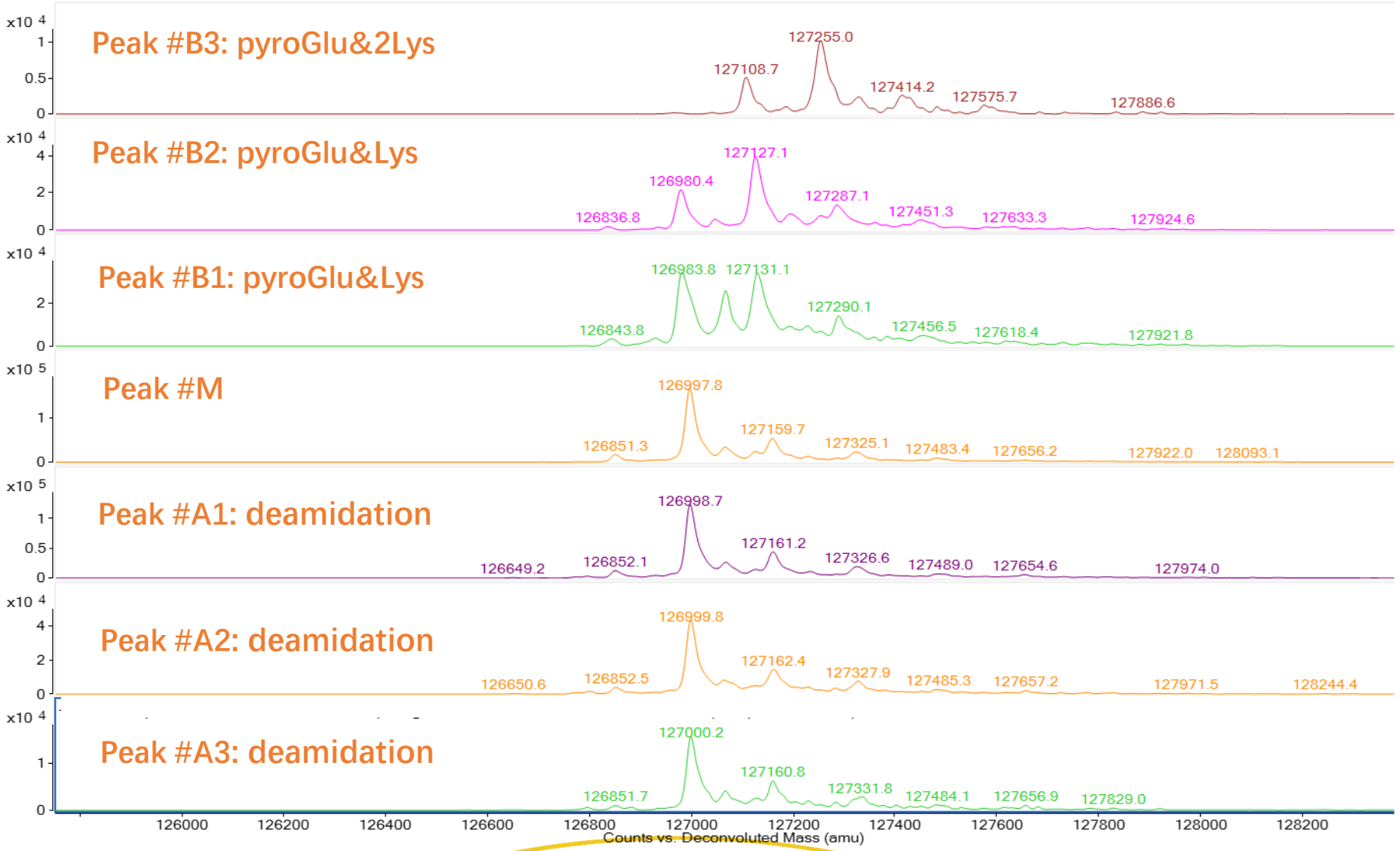
cIEF-Mass result of unstressed sample



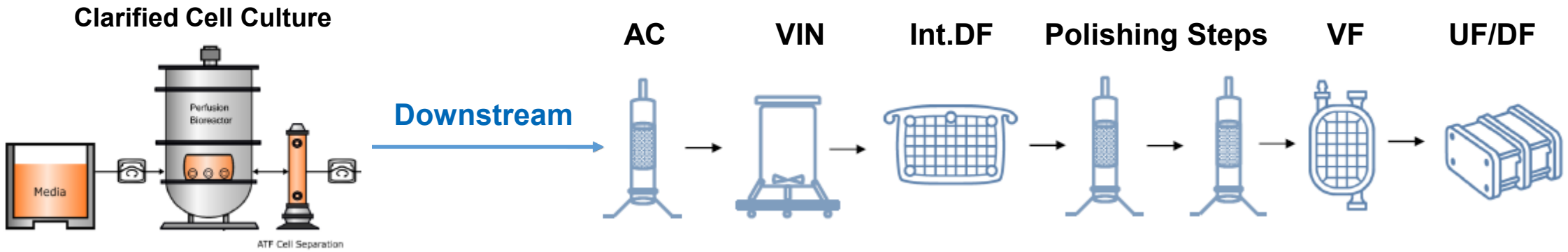
cIEF-Mass result of stressed sample



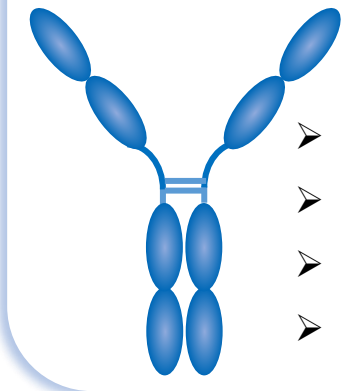
icIEF VS cIEF-MS: Unstressed & Stressed Sample



cIEF-MS to Support Process Development and Manufacturing Investigation/Troubleshooting



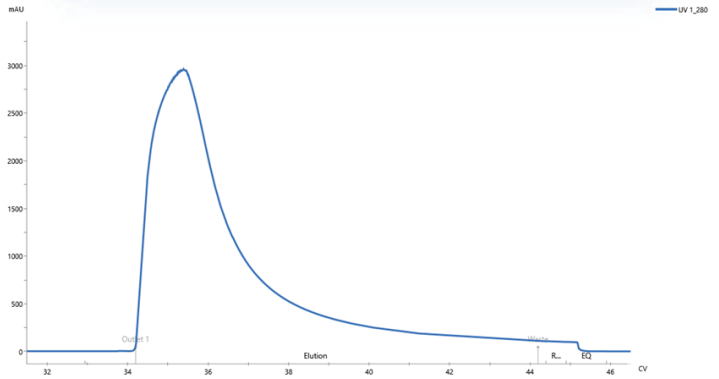
Molecular Information



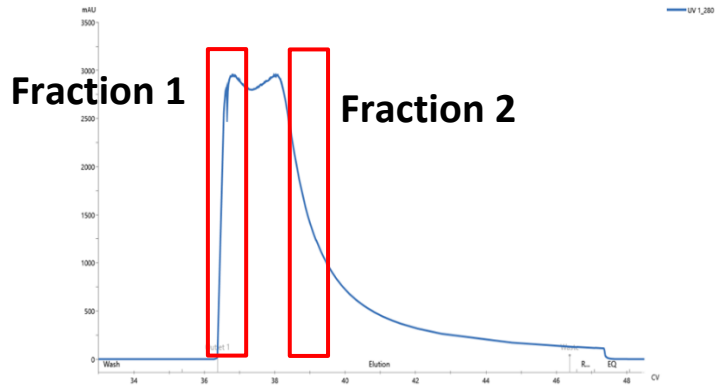
- Fc fusion Protein
- 2 N-glycosylation sites
- 4 O-glycosylation sites
- 20 disulfide bonds

Doublet Peak Issue during Downstream Process

Low Loading with single peak



High Loading with doublet peak

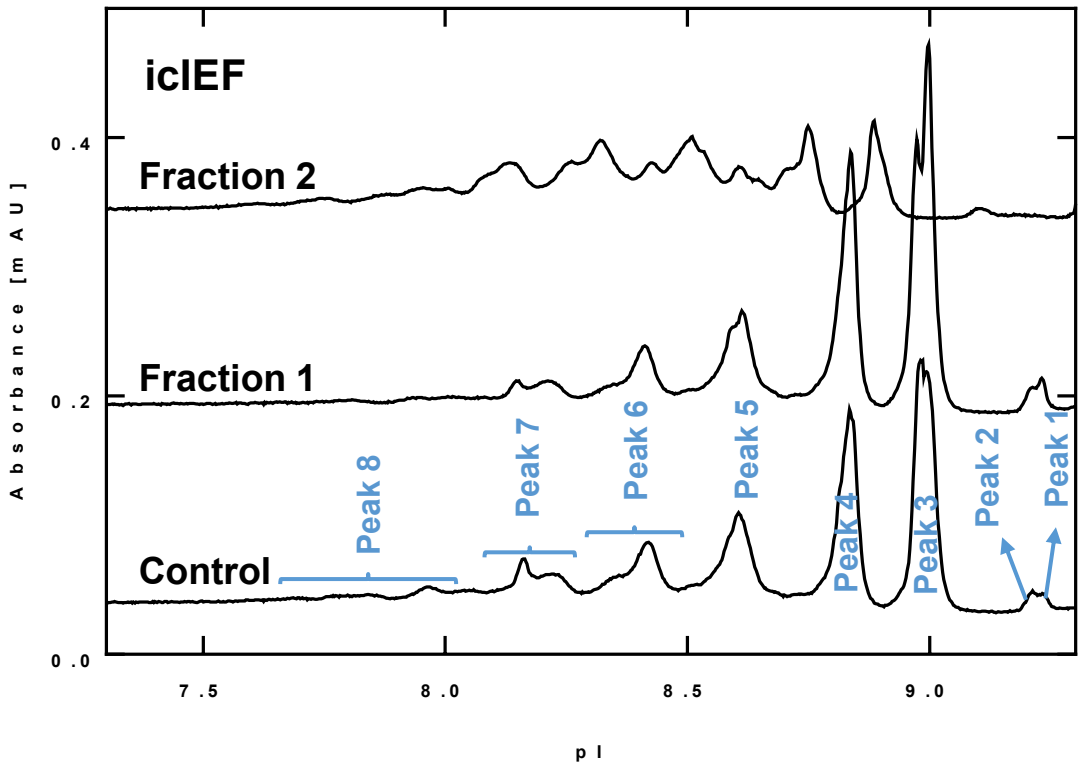


Process Investigation: cIEF-MS to Decipher the ID of Doublet Peaks

WuXi Biologics
Global Solution Provider

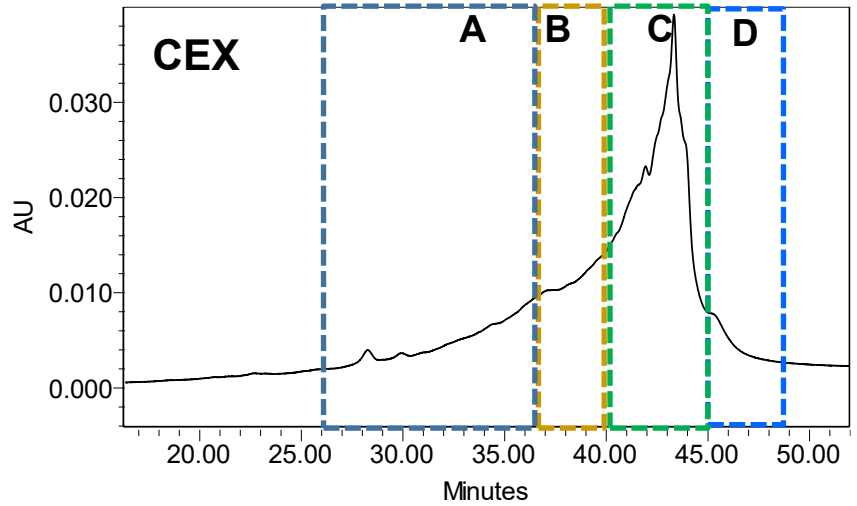
Investigation:

- ✓ Fraction 1 shows higher peak 3-4
- ✓ Fraction 2 shows higher peak 5-8



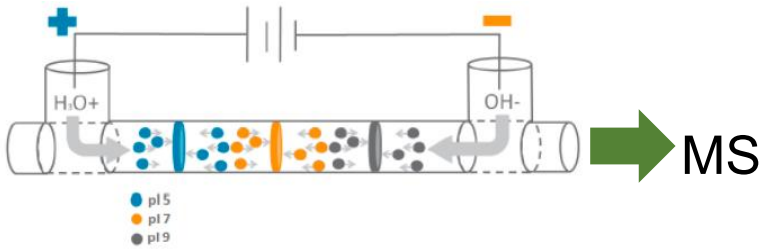
Challenge

- ➢ CEX fractionation profile is not aligned with icIEF profile



Orthogonal cIEF-MS method become the only choice to identify each charge variants in icIEF profile

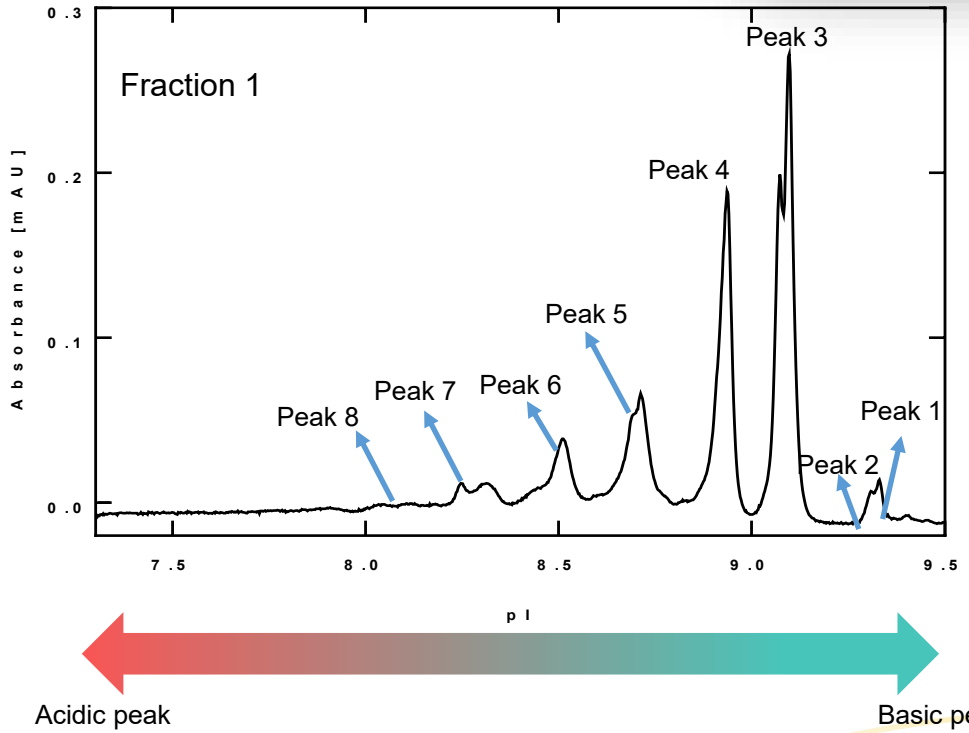
Process Investigation: cIEF-MS to Decipher the ID of Doublet Peaks



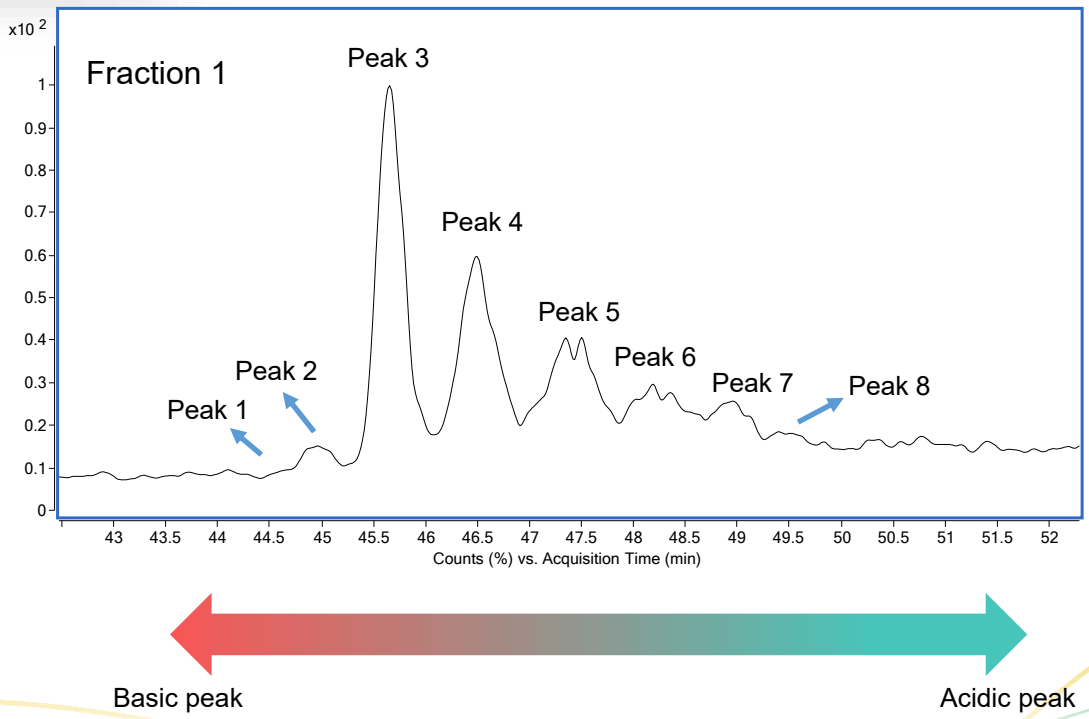
- icIEF and cIEF-MS show very consistent charge profile

Consistent charge profile

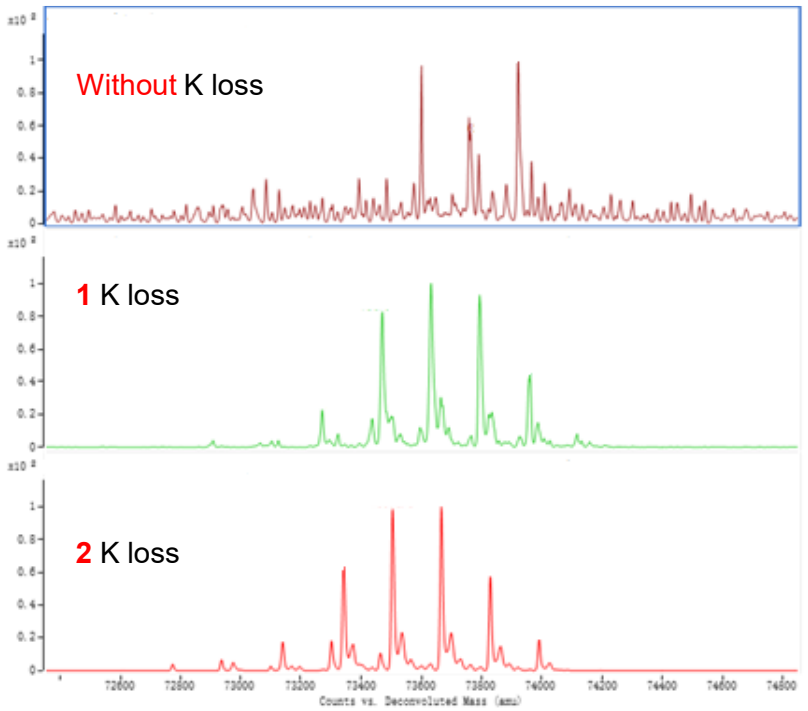
icIEF (UV) result

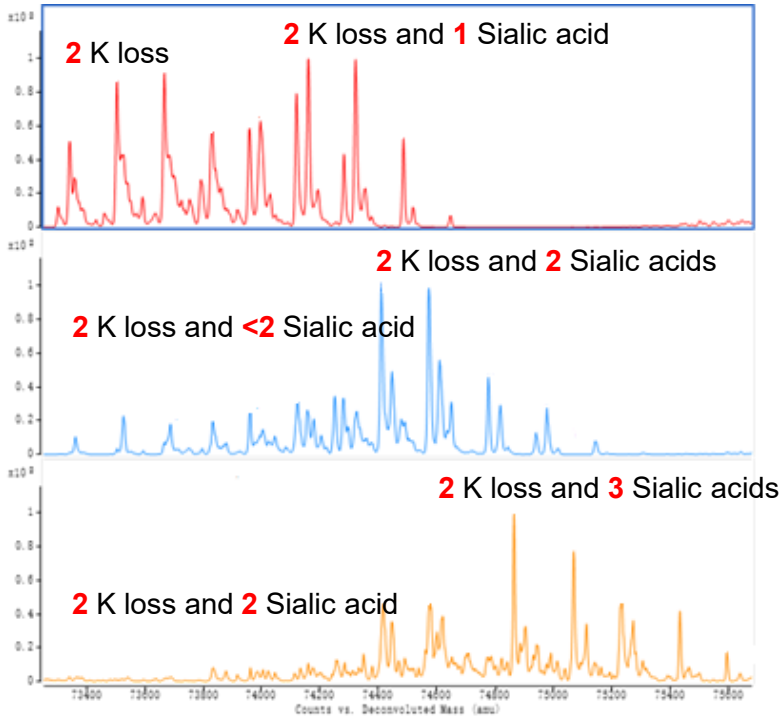


cIEF-MS result



Process Investigation: cIEF-MS to Decipher the ID of Doublet Peaks

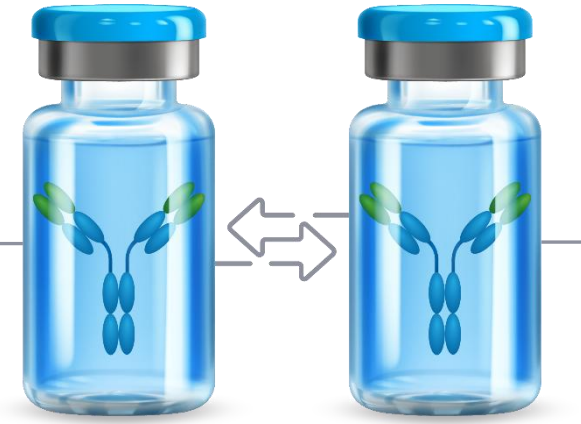




Peak # in cIEF	Modifications
1	Neutral glycan +C-terminal K
2	Neutral glycan +C-terminal K Loss
3	Neutral glycan +2XC-terminal K Loss
4	Acidic glycan (0-1 SA)+2XC-terminal K Loss
5	Acidic glycan (1-3 SA)+2XC-terminal K Loss
6-8	Acidic glycan (>3 SA)+2XC-terminal K Loss

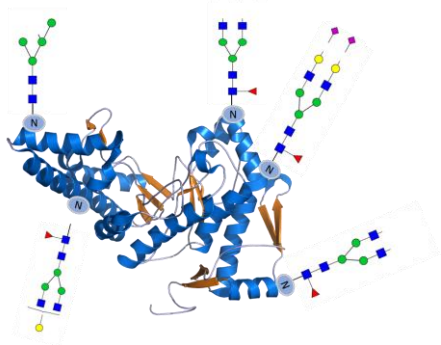
- **The doublet peak is mainly due to different sialyation contents**
- **Both peaks represented product-related variants and can be combined in one pool**

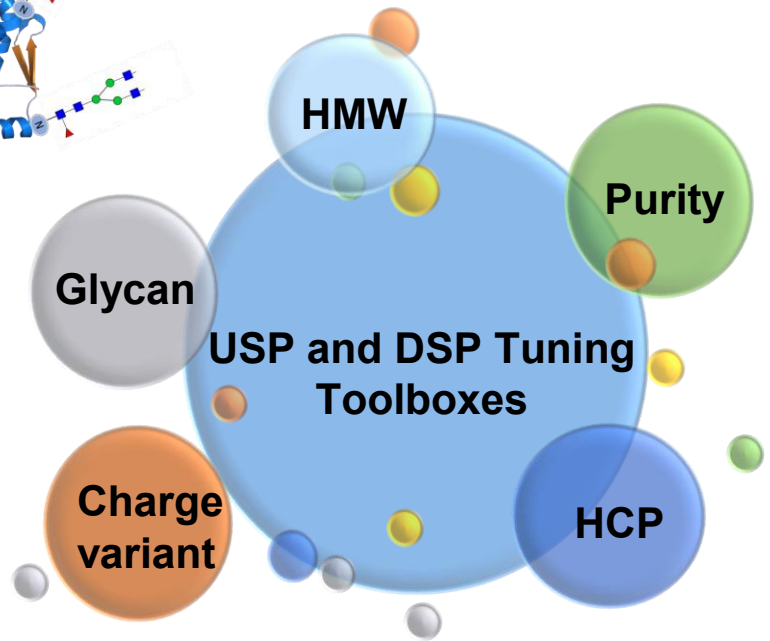
Charge Variant Identification for Biosimilar Program & Post-approval changes for 2nd Generation Product



Biosimilar Program & Post-approval Changes for 2nd Generation Commercial Product

- Meet commercial supply demands with the lowest possible production costs (CoGs)
- Additional optimization and fine-tuning of CQA, especially **charge variants**, are essential during process development to meet comparability/similarity acceptance criteria



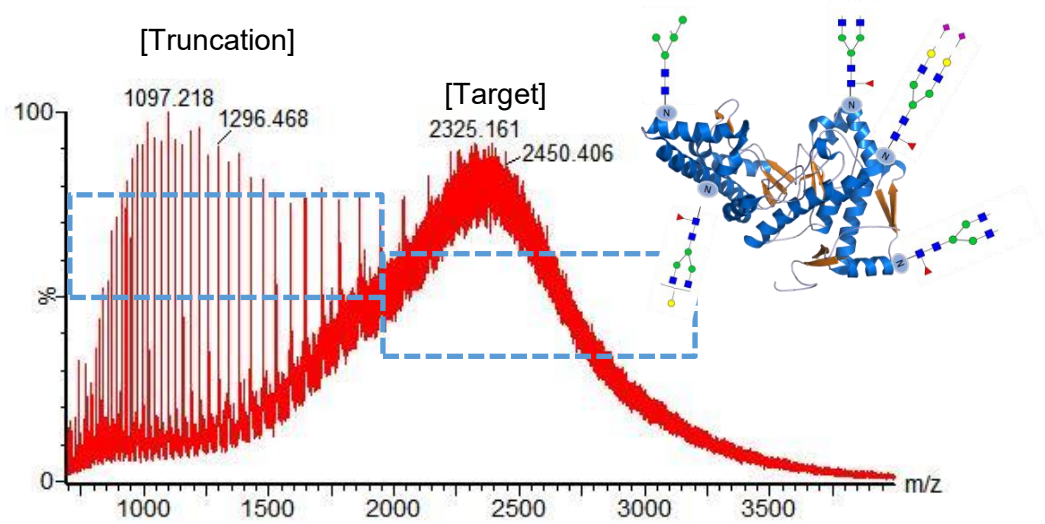


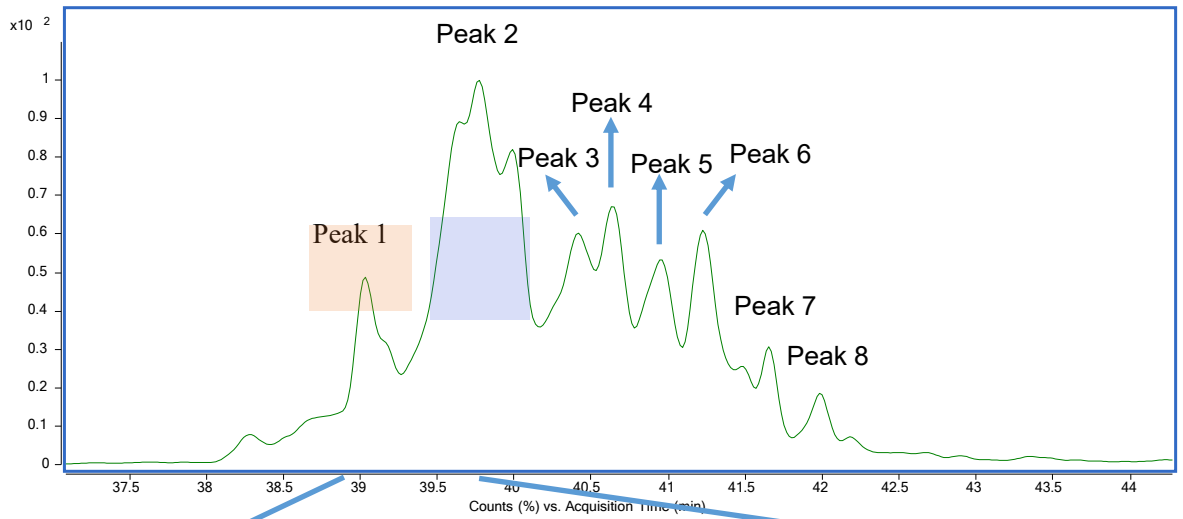
Defining target ranges through preliminary charge variant characterization is essential prior to initiating process development

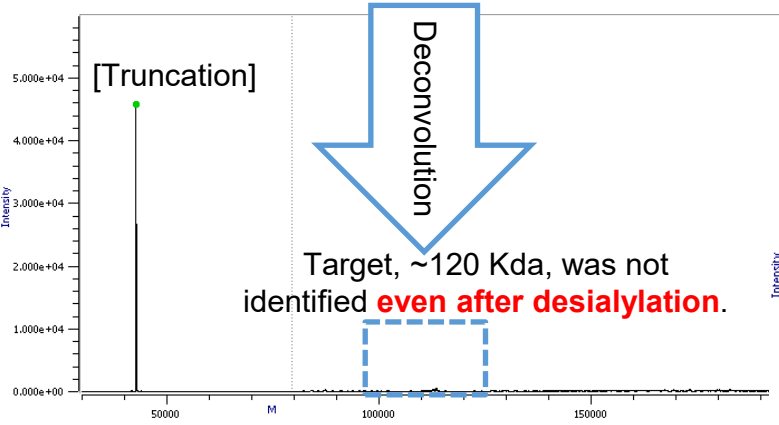
cIEF-MS Based Characterization of Highly *O*-Glycosylated Protein

WuXi Biologics
Global Solution Provider

Complex biotherapeutics – Highly *O*-glycosylated variants identified from cIEF-MS

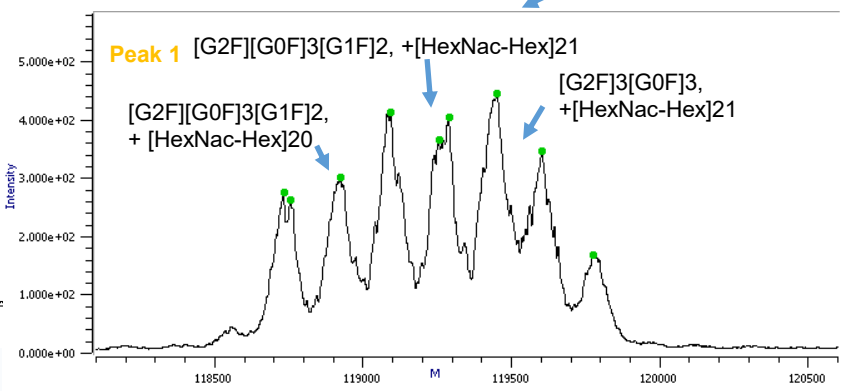




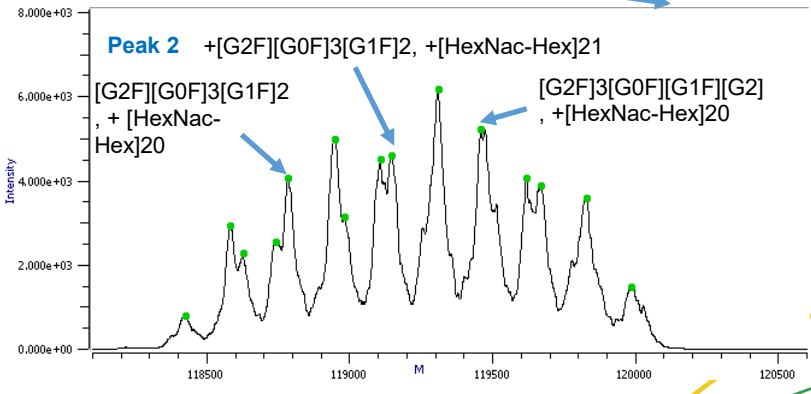


Challenge

No spectrum by LC-MS



Species with 1 C-terminal K

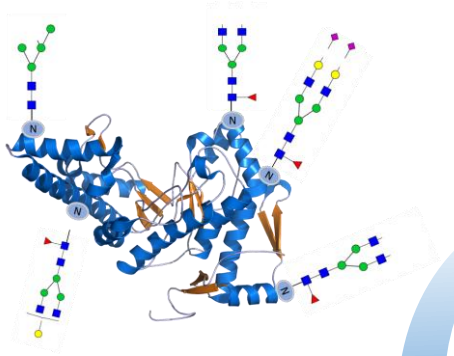


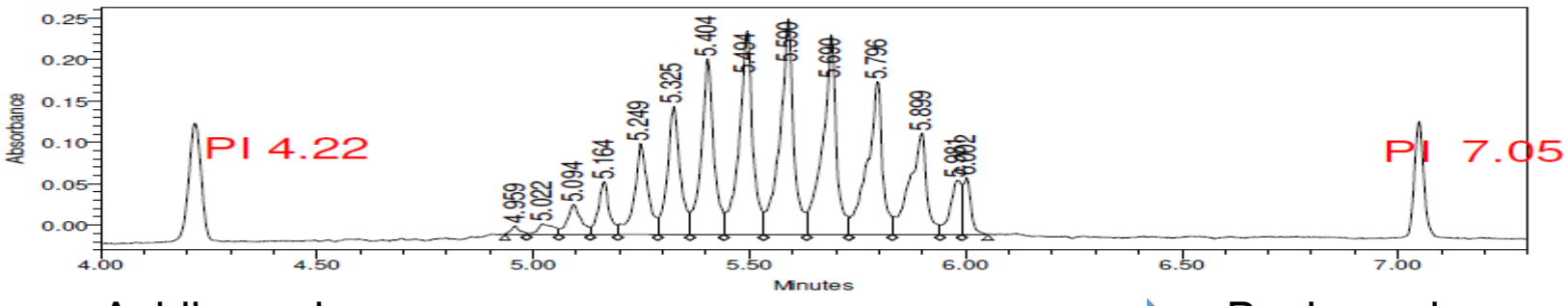
Species without C-terminal K

cIEF-MS Based Characterization of Highly Sialylated Protein

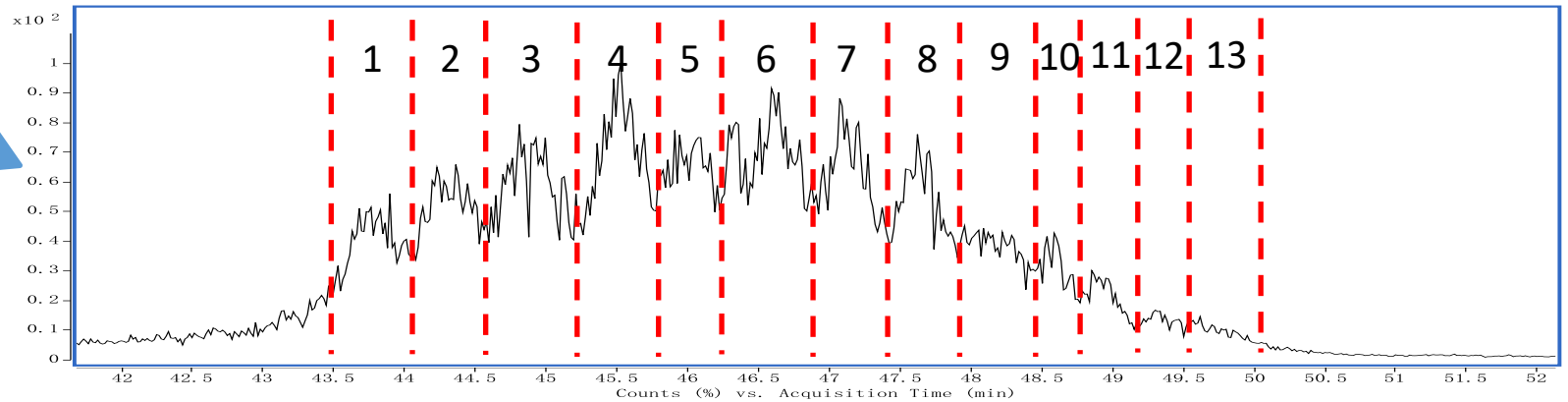
WuXi Biologics
Global Solution Provider

Complex biotherapeutics – Highly glycosylate/sialylated variants identified by cIEF-MS





Acidic peak → Basic peak

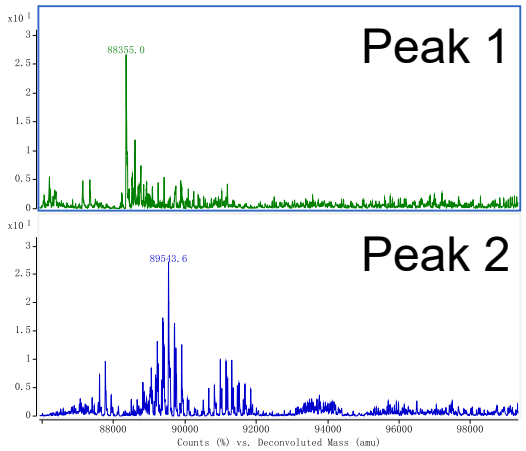


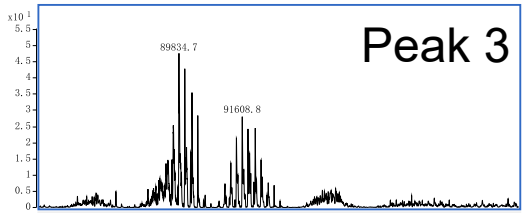
Comparable Basic peak → Acidic peak

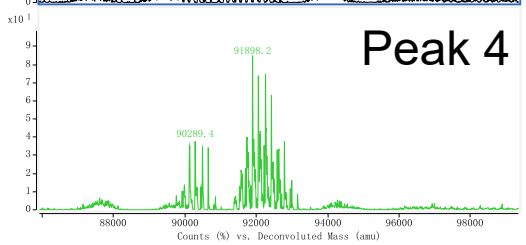
cIEF-MS Based Characterization of Highly Sialylated Protein

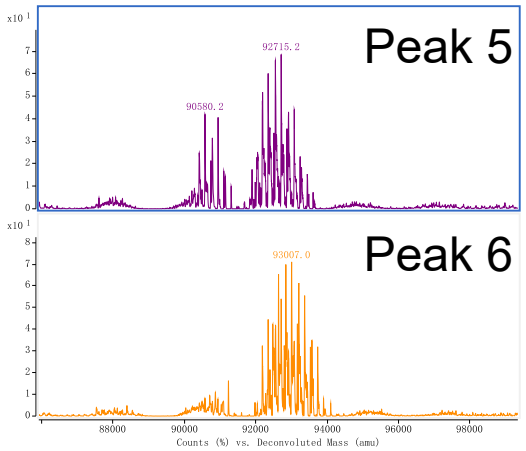
WuXi Biologics
Global Solution Provider

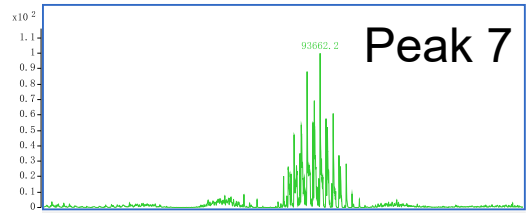
Complex biotherapeutics – Highly glycosylate/sialylated variants identified by cIEF-MS

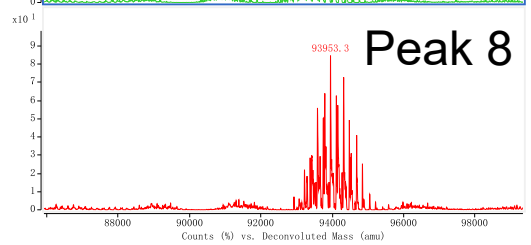


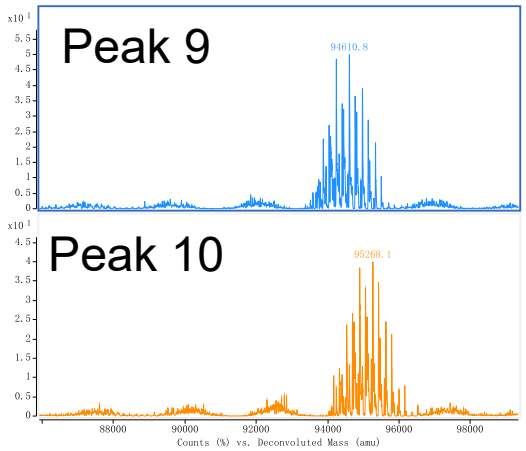


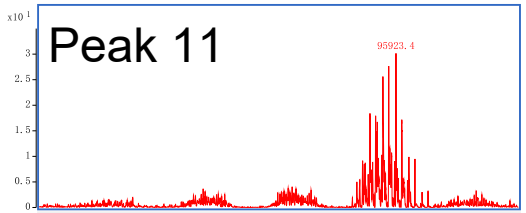


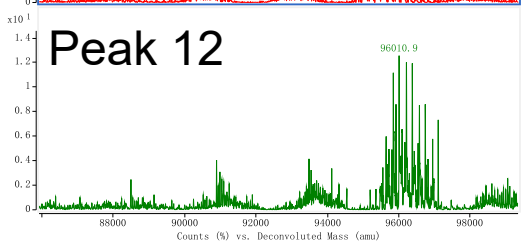


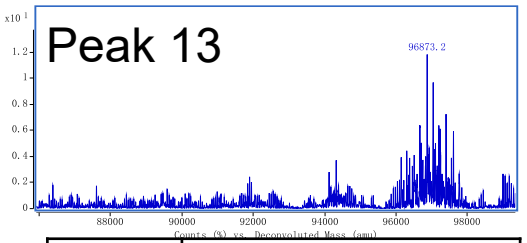








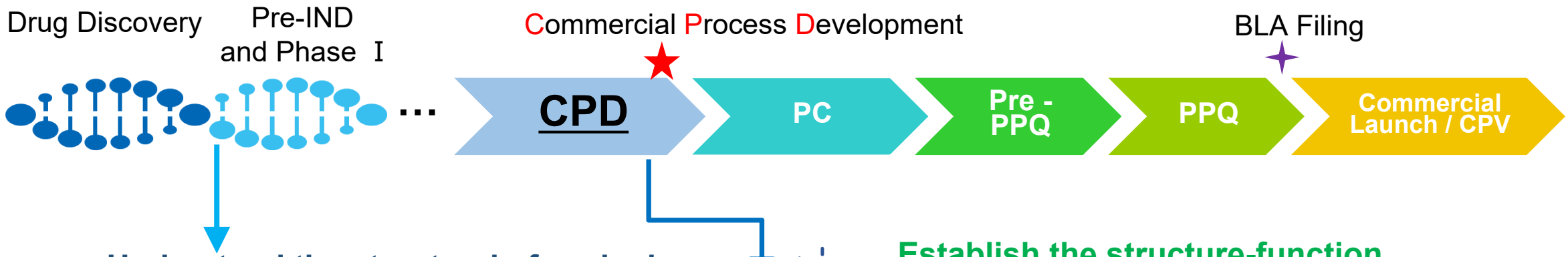




Peak #	Number of Neu5Ac
1	0
2	1
3	2
4	3
5	4
6	5
7	6
8	7
9	8
10	9
11	10
12	11
13	12

03 Charge Variant Characterization by Offline ‘Discovery’ Assisted Fractionation

Characterization of Charge Variants at Different Drug Discovery and CMC Development Stages



Understand the structural of each charge variant; limited functional testing

Establish the structure-function relationship of each charge variant

1. Regulatory requirement: BLA filling **S.3.1 Elucidation of Structure**
2. Deep understanding of product degradation pathways and CQA risk assessment
3. Support justification of specification

-ICH Q8: *"A greater understanding of the product … can create a basis for more flexible regulatory approaches."*

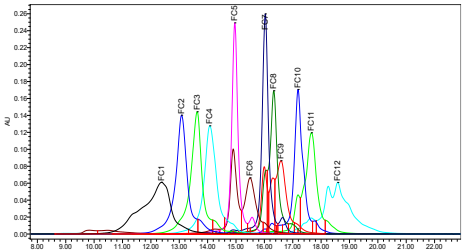
Offline Charge Variant Characterization by Fractionation -Traditional Strategy

WuXi Biologics
Global Solution Provider

Scale up to semi-preparative column and check performance

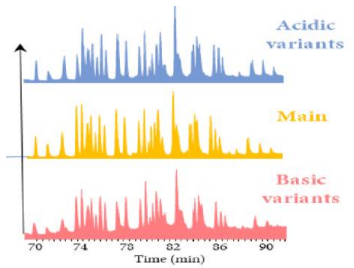


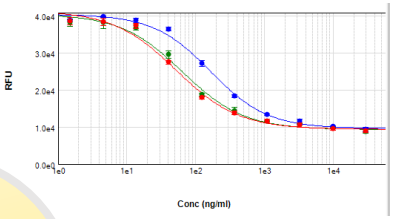
iCIEF and intact MS analysis to determine fraction pooling



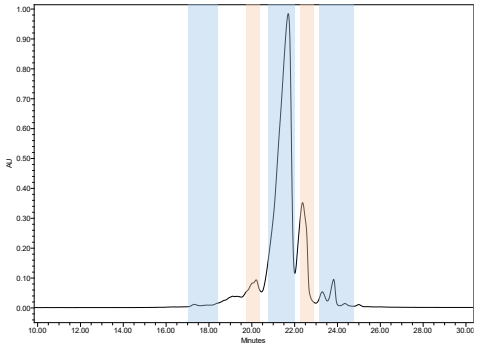
Purity Confirmation

Structure/function characterization

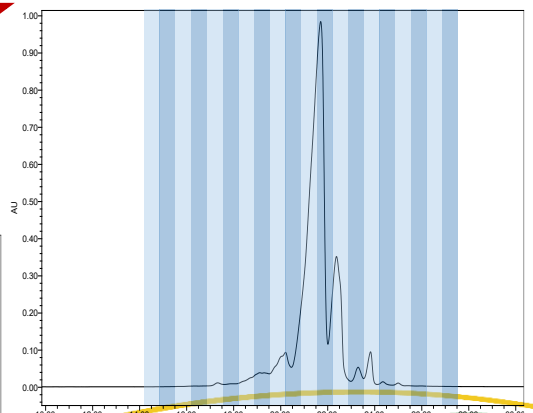




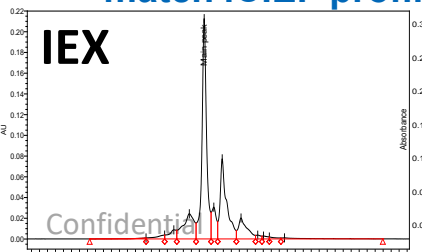
Formal fractionation collection

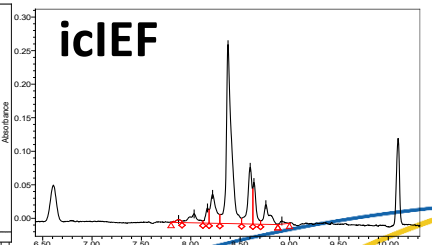


Pre-study before fractionation



IEX method development on analytical column to match iCIEF profile





Control Strategy

Define CQA/non-CQA

Challenge 1: IEX Method Development and Scale up

WuXi Biologics
Global Solution Provider

✓ **IEX Development Process:**

Method development on analytical column

↓

Scale up of analytical method

$$F2=F1 \times \frac{d2^2}{d1^2}$$

$$M2=M1 \times \frac{L2}{L1} \times \frac{d2^2}{d1^2}$$

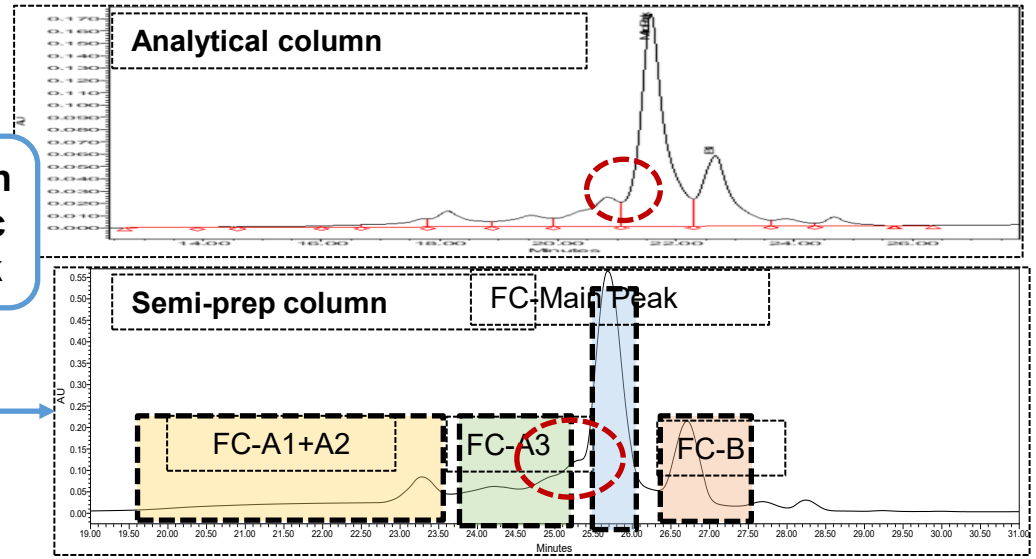
↓

Optimization of Flow rate and loading amount

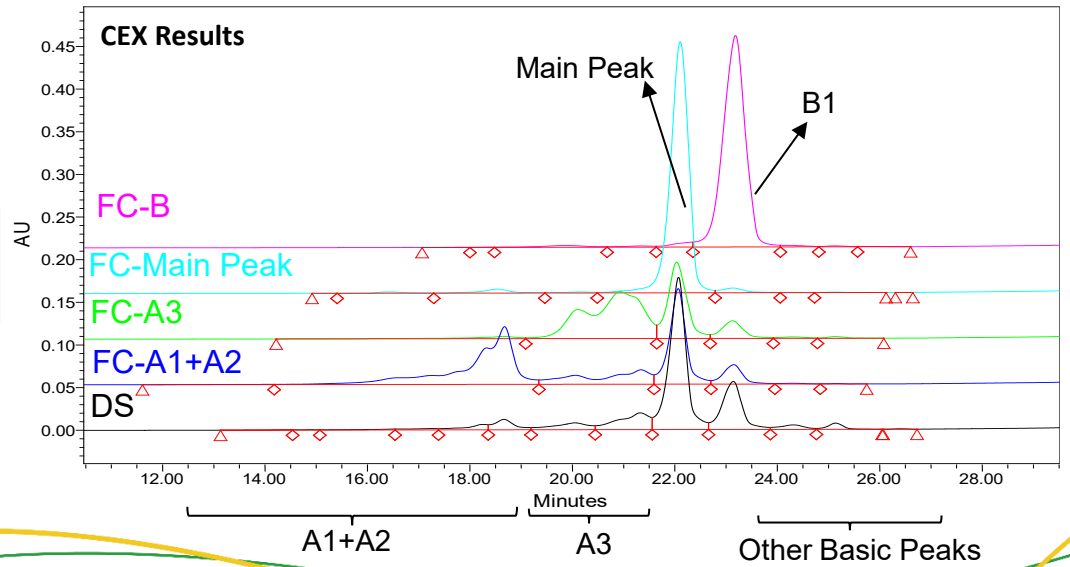
↓

CEX Purity Analysis for Fractions

Poor resolution between acidic and main peak



Low CEX purity of acidic fractions



Challenge2: Pre-study to Determine Peak Collection Window and Fraction Pooling Strategy

WuXi Biologics
Global Solution Provider

Fractionation Windows Determine Workflow

Testing each vial

MS analysis

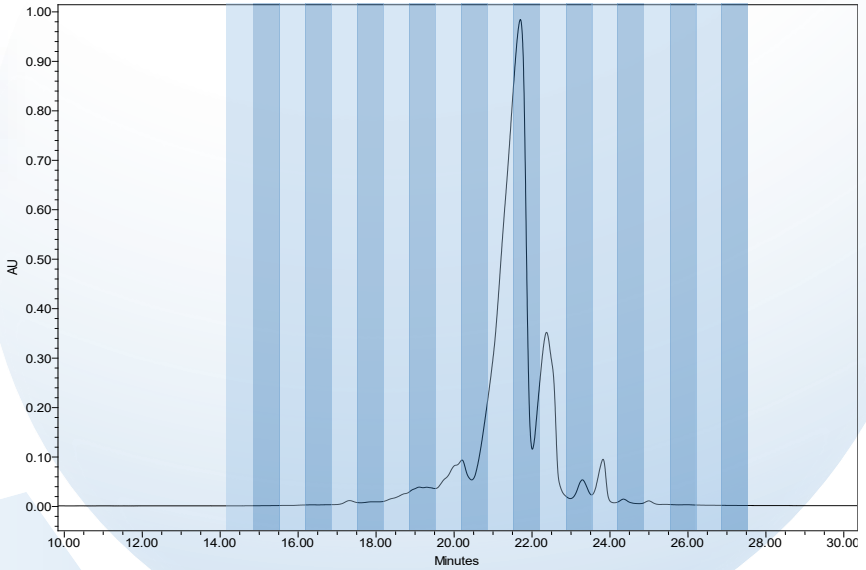
Repeat pre-study

Collect all fractions from first peak to last peak

Confirm IEX and iCEF matching performance

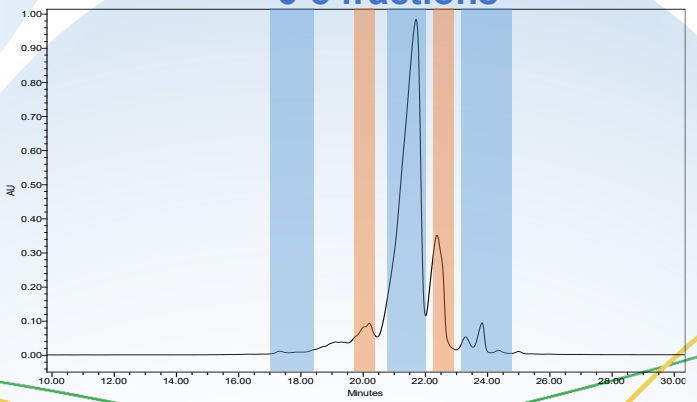
Adjust fraction pooling strategy

Determine the final collection window



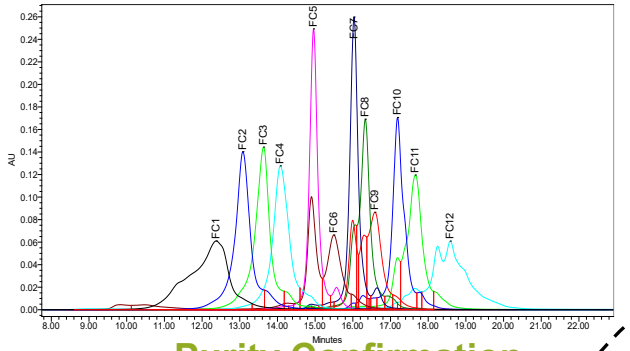
Before pooling
20-30 fractions

After pooling
6-8 fractions



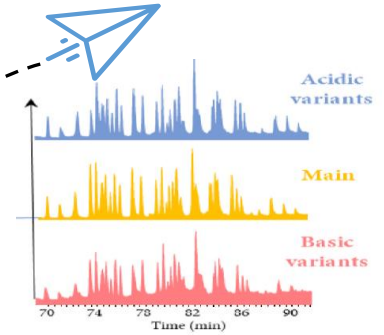
Offline Charge Variant Characterization by Fractionation -'Discovery' Assisted Strategy

WuXi Biologics
Global Solution Provider

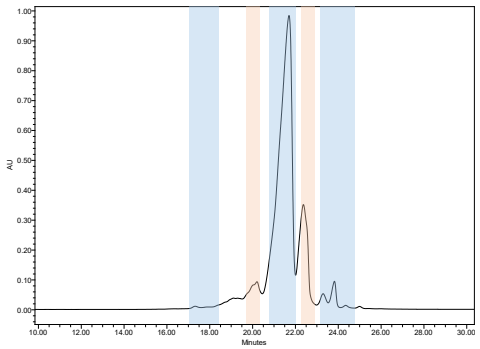


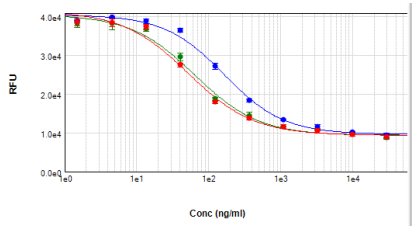
Purity Confirmation

Structure/function characterization



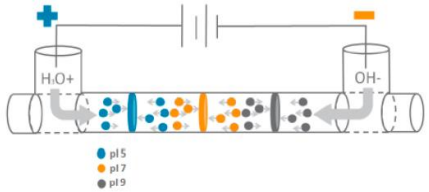
Formal Fraction Collection





IEX Method development by GoSilico™

cIEF-MS determine fraction pooling strategy



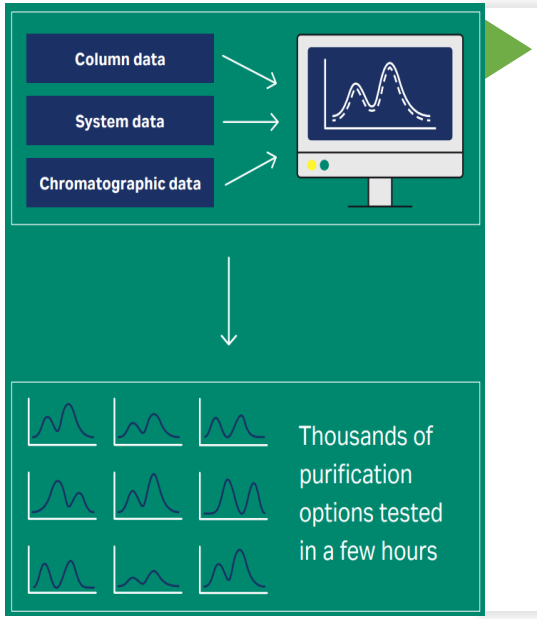
MS

Control Strategy

Define CQA/non-CQA

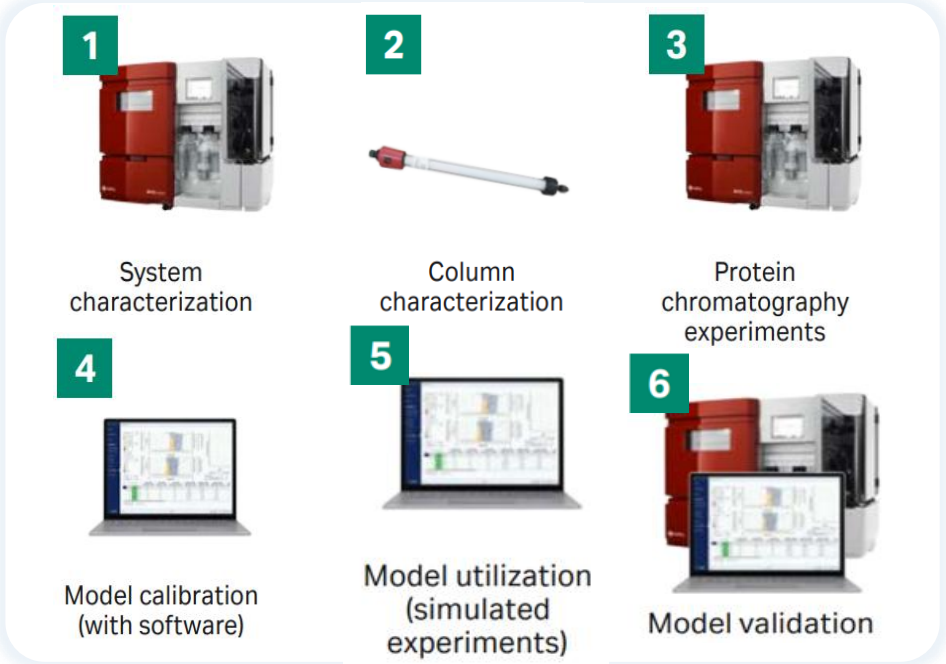
IEX Method Development –Gosilico™ Modelling

Significantly reduced rounds of experiments through application of mechanistic models using computer simulations (GoSilico™ Chromatography Modeling Software).



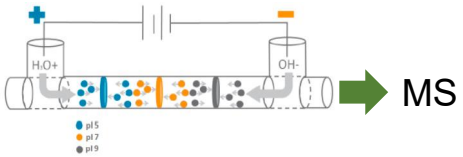
A variety of models available and keep updating

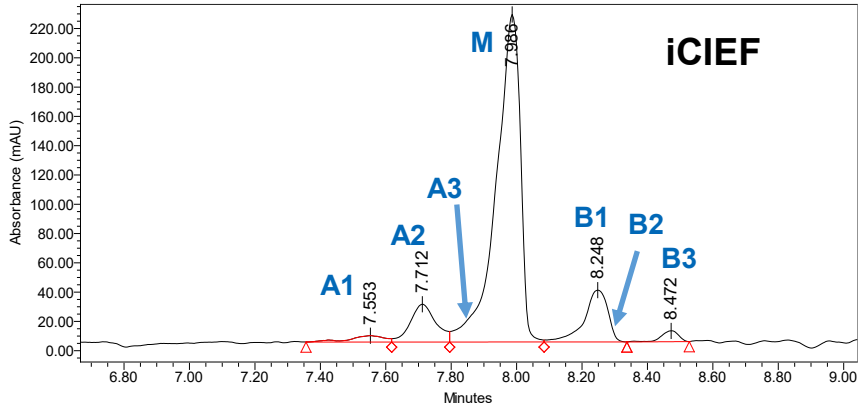
$K'_{kin,i}\frac{\partial q_i}{\partial t} = k_{eq,i,i}\cdot q_{max,i}\cdot\left(1-\sum_{j=1}^{N}\frac{q_j}{q_{max,j}}\right)\cdot c_{p,i} - q_i$	Langmuir ("Langmuir"), cf. Schmidt-Traub *et al.*, 2012, [ISBN:978-3-527-64930-3]
$K'_{kin,i}\frac{\partial q_i}{\partial t} = k_{eq,i,i}\cdot q_{max,i}\cdot\left(1-\sum_{j=1}^{N}\frac{q_j}{q_{max,j}}\right)\cdot c_m^{\sigma_i}\cdot c_{p,i} - q_i$	Langmuir with Modifier ("Langmuir with Modifier")
$K'_{kin,i}\frac{\partial q_i}{\partial t} = k_{eq,i,i}\cdot\bar{q}_{salt}^{\,\nu_i}\cdot c_{p,i} - q_i\cdot c_{p,salt}^{\,\nu_i}$ with $\bar{q}_{salt} = \Lambda_{IEC} - \sum_{j=1}^{N}\frac{\nu_j+\sigma_j}{f_j}\cdot q_j$	Steric mass action ("SMA"), cf.Schmidt-Traub *et al.*, 2012, [ISBN:978-3-527-64930-3]
$k_{kin,i}\frac{\partial q_i}{\partial t} = k_{eq,i}\cdot\bar{q}_{salt}^{\,\nu_i}\exp\left(K_{salt,i}*c_{p,salt}+K_{protein,i}\cdot c_{p,i}\right)\cdot c_{p,i} - q_i\cdot c_{p,salt}^{\,\nu_i}$	Generalized Ion Exchange isotherm ("IEC 2008") by Mollerup *et al.*, 2008, [DOI:10.1002/ceat.200800082]
$k_{kin,i}\frac{\partial q_i}{\partial t} = k_{eq,i}\exp\left(k_{eq2,i}\cdot pH + k_{eq2,i}\cdot pH^2\right)\cdot\bar{q}_{salt}^{\,\nu_i(pH)}\cdot c_{p,i} - q_i\cdot c_{p,salt}^{\,\nu_i(pH)}$ with $\nu\,(pH) = \nu_0 + \nu_1\cdot pH$	pH-extended SMA ("SMA With pH 2017) adapted from Hunt *et al.*, 2017, [DOI:10.1002/9781119031116.ch13]
$k_{kin,i}\frac{\partial q_i}{\partial t} = \exp(-G\nu_i + \nu_i G z)\bar{q}_{salt}^{\,\nu_i}\cdot c_{p,i} - q_i\cdot c_{p,salt}^{\,\nu_i}$ with $\nu_i = \frac{-N_{Cter}}{1+10^{pKa_{Cter}-pH}} + \frac{-N_{Tyr}}{1+10^{pKa_{Tyr}-pH}} + \frac{-N_{Cys}}{1+10^{pKa_{Cys}-pH}} + \frac{N_{Arg}}{1+10^{pH-pKa_{Arg}}} + \frac{N_{Lys}}{1+10^{pH-pKa_{Lys}}} + \frac{N_{His}}{1+10^{pH-pKa_{His}}} + \frac{N_{NTerm}}{1+10^{pH-pKa_{NTerm}}} + \frac{N_{NTerm}}{1+10^{pH-pKa_{NTerm}}}$	pH-dependent Ion Exchange isotherm ("IEC 2014") by Schmidt *et al.*, 2014, [DOI:10.1002/jssc.201301007]

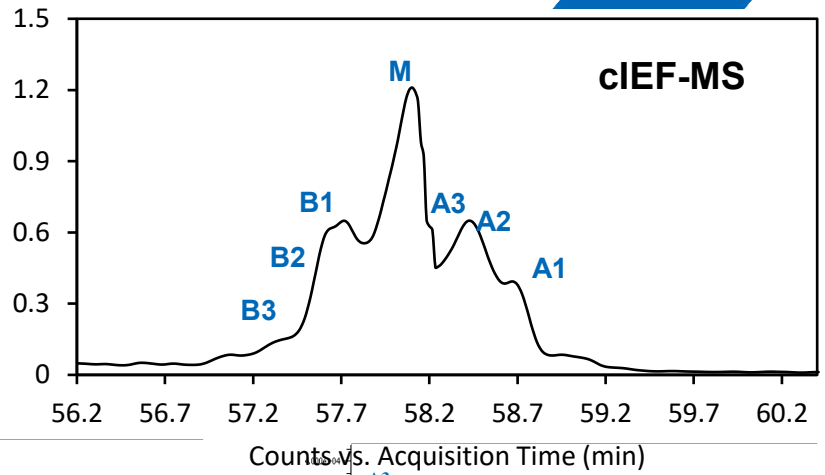


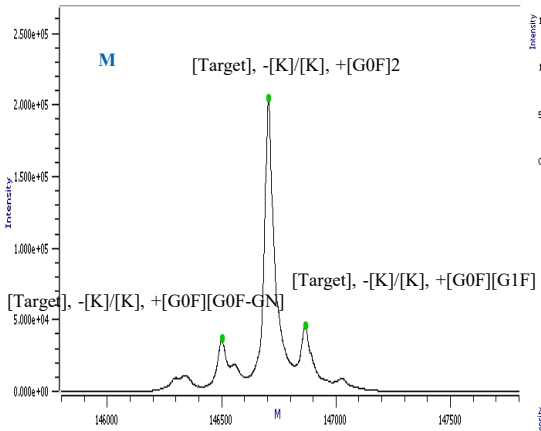
Molecule Type	Timeline-1	Timeline-2	Timeline-3	Timeline-4	Total Timeline
mAb	2-3 days	2 days	1 day	1 day	< 1 weeks with significantly reduced Timeline
Complex Molecule		2 days	2 days	1 day	

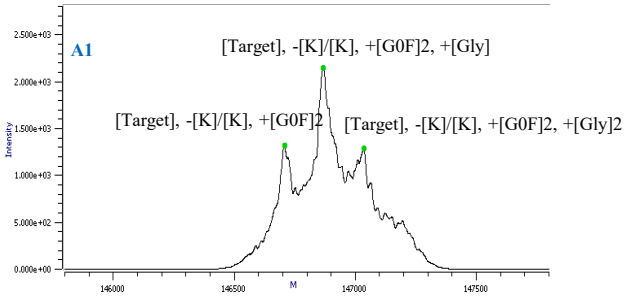
cIEF-MS Determine Peak Boundary and Pooling Strategy



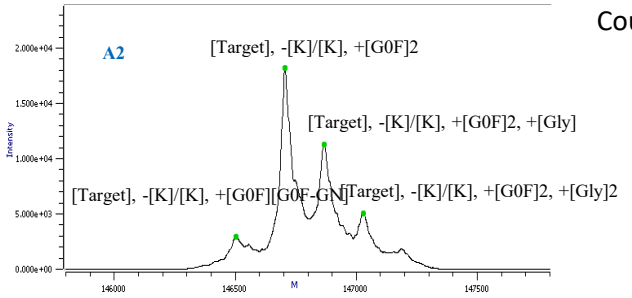




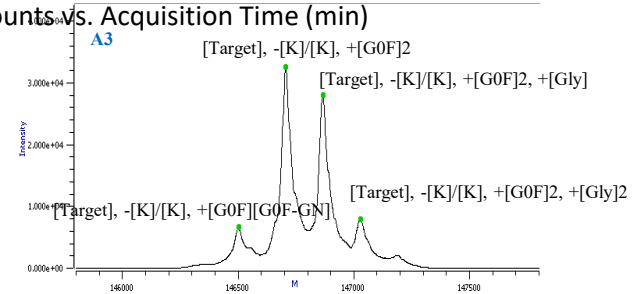




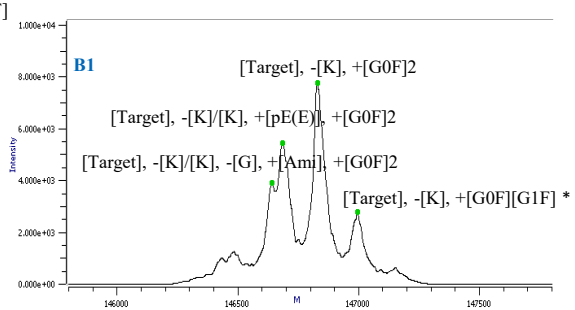
Peak #A1: Glycation ?



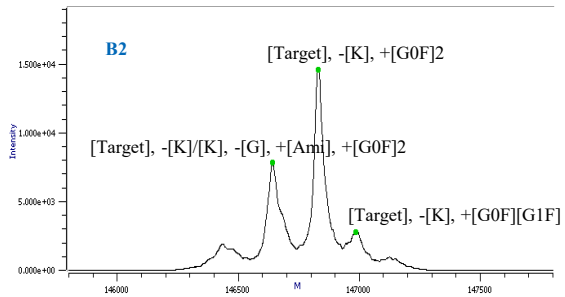
Peak #A2: deamidation/glycation



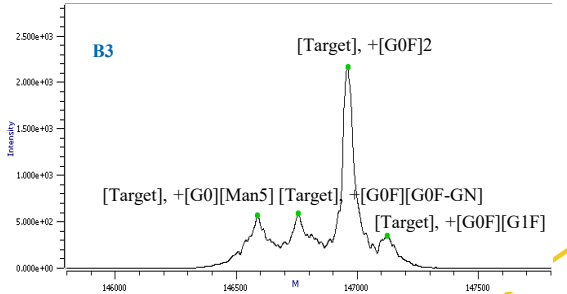
Peak #A3: Glycation



Peak #B1: pyroGlu(E)&Lys



Peak #B2: Lys



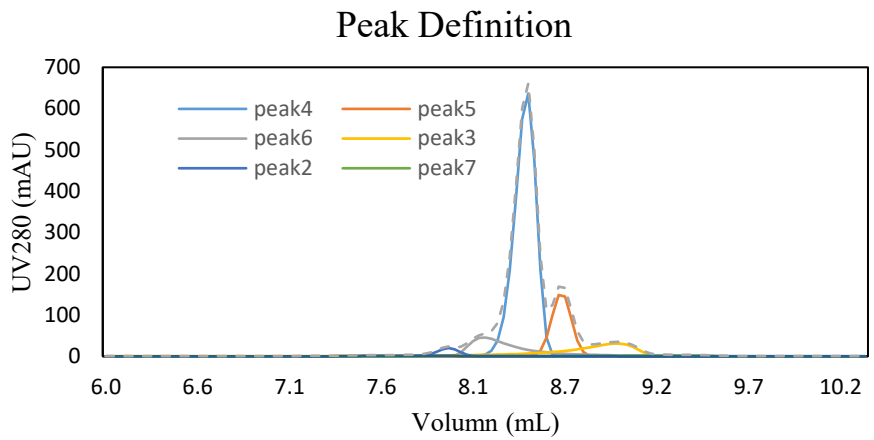
Peak #B3: 2Lys

IEX (HPLC) Method Development –Gosilico™ Modelling

Calibration run

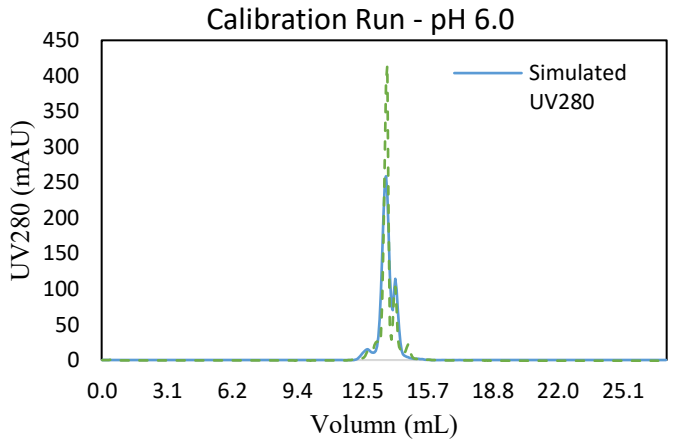
Experiment run	Load density (g/L resin)	Elution length	pH
Gradient 1	1	0~100% 10 CV	6.0
Gradient 2	1	0~100% 20 CV	6.0
Gradient 3	1	0~100% 30 CV	6.0
Gradient 4	1	0~100% 20 CV	5.5
Gradient 5	1	0~100% 20 CV	7.0

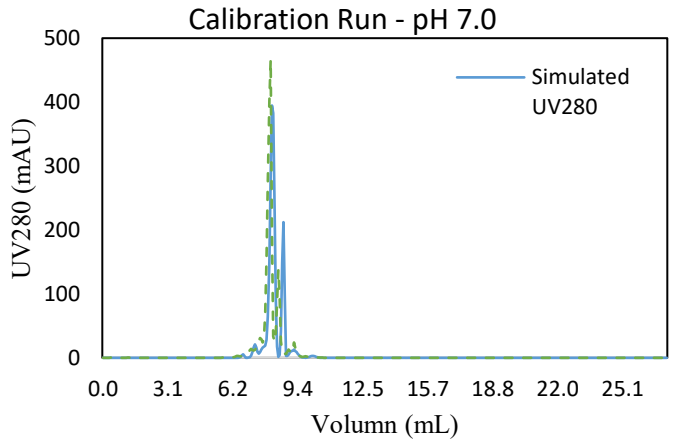
Define Peak Component: 7 peaks based on cIEF-MS identification



Model Selection and Calibration in GoSilico

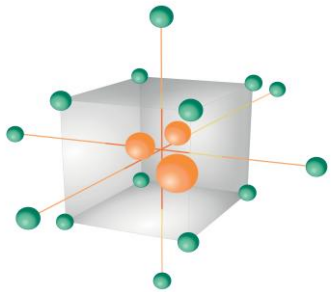
Column Model	Equilibrium Dispersive
Pore Model	No Pore Model
Adsorption Model	SMA with pH 2017

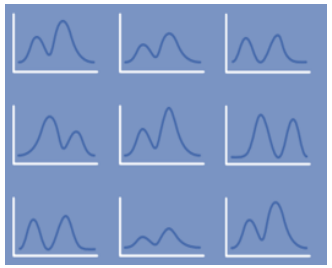


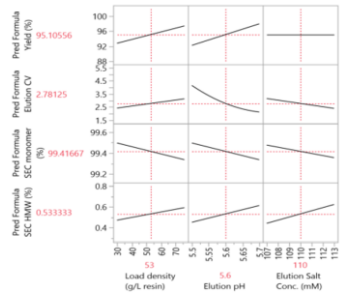


IEX (HPLC) Method Development –Gosilico Simulation

Model Utilization and Simulated Chromatography



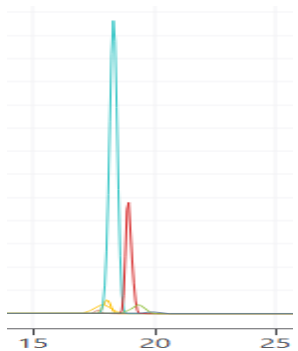




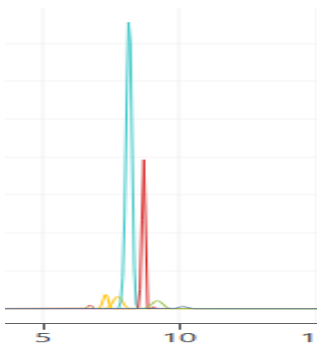
- Good resolution
- Appropriate RT
- Good peak response

Model Recommended Process Parameters and Simulated Chromatography

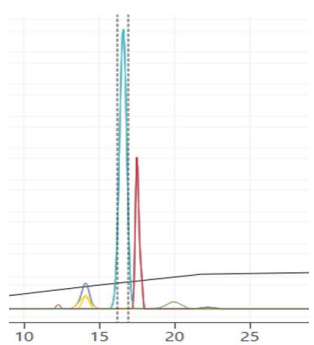
Process parameters	Parameters range
Buffer A pH	6.0-7.0
Buffer B pH	6.0-7.0
Buffer B NaCl conc. (mM)	300-600
Stage 1 (0-20 min) A% gradient	85-95
Stage 2 (20-52 min) A% gradient	50-85



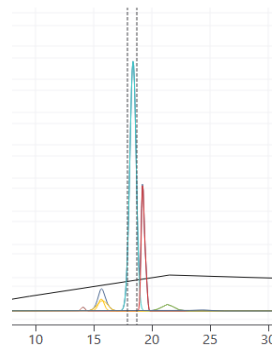
pH=5.5;500mm NaCl Condition1



pH=7.0; 500mm NaCl Condition2



pH=7.0; 500mm NaCl Condition3

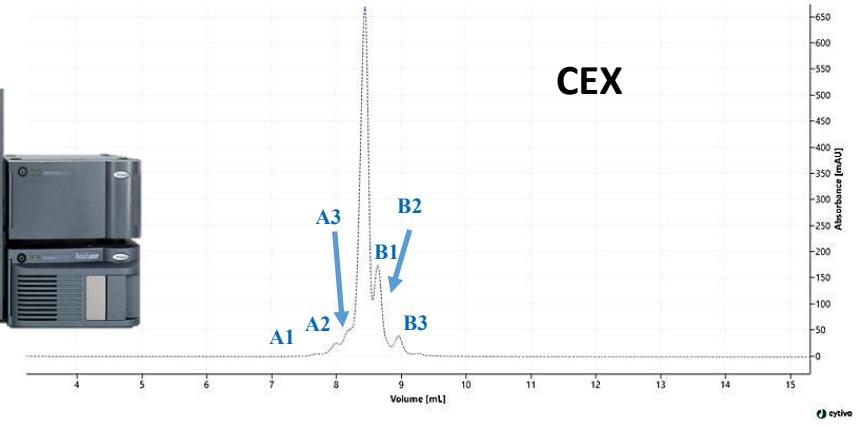


pH=6.8; 300mm NaCl Condition4

IEX (HPLC) Method Development –Validation

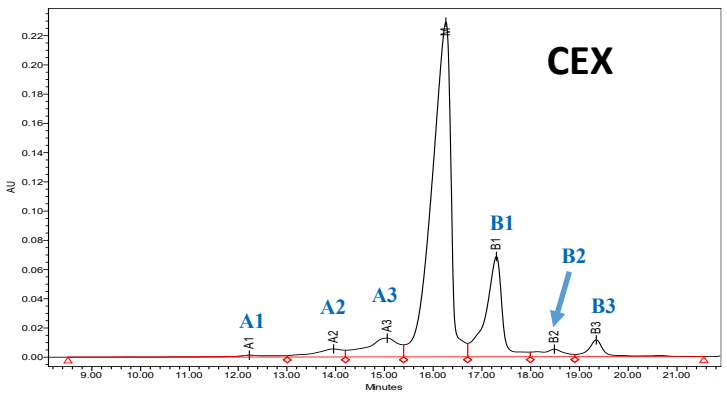
Model Calibration Input and Original Chromatography

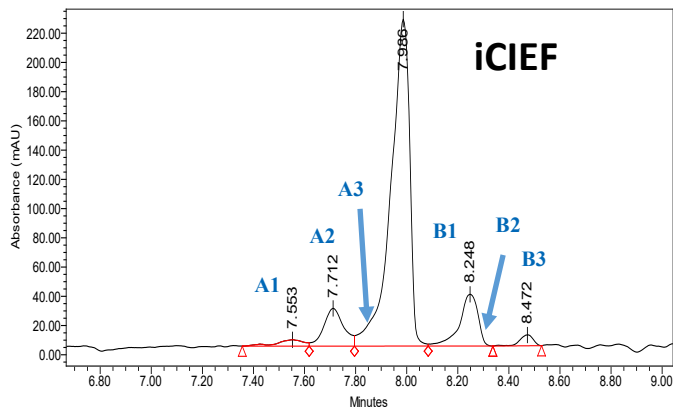
Process parameters	Parameters range
Buffer A pH	6.0-7.0
Buffer B pH	6.0-7.0
Buffer B NaCl conc. (mM)	300-600
Stage 1 (0-20 min) A% gradient	85-95
Stage 2 (20-52 min) A% gradient	50-85



Model Recommended Process Parameters and Chromatography of Validation Run

Buffer A	pH=**7.0**			
Buffer B	**500** mm NaCl, pH=**7.0**			
Inject amount	100 μg			
Stage	Time	Flow rate (mL/min)	A%	B%
1	0	1	100	**0**
2	20	1	90	**10**
3	52	1	88	**12**



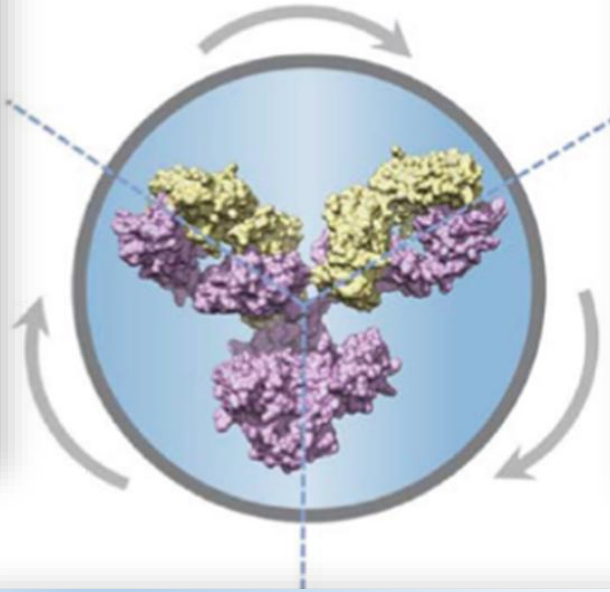


Comprehensive Structure & Function Characterization After Fractionation

WuXi Biologics
Global Solution Provider

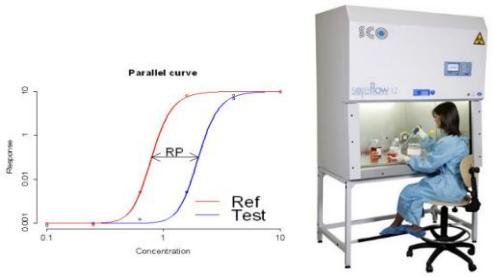
Structural Attributes :

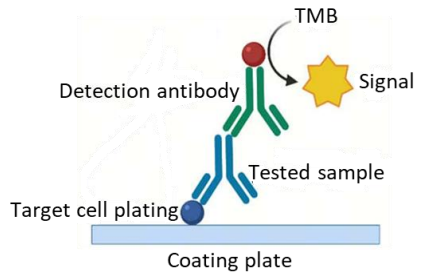
- *Intact Mass*
- *Peptide Mapping*
- *Disulfide Mapping*
- *N-/O-Glycan Analysis*



Functional Attributes:

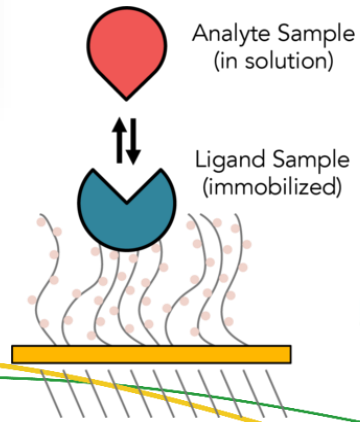
- *Ag-Ab Binding*
- *Biological Activity*
  - *Cell cytotoxicity*
  - *Neutralization*
  - *Cytokine release*
  - *Blockade bioassay*

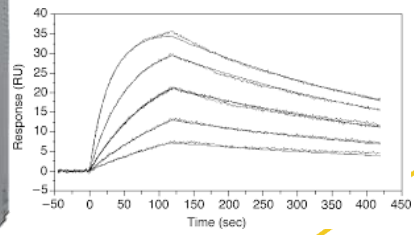




Analysis of effector functions:

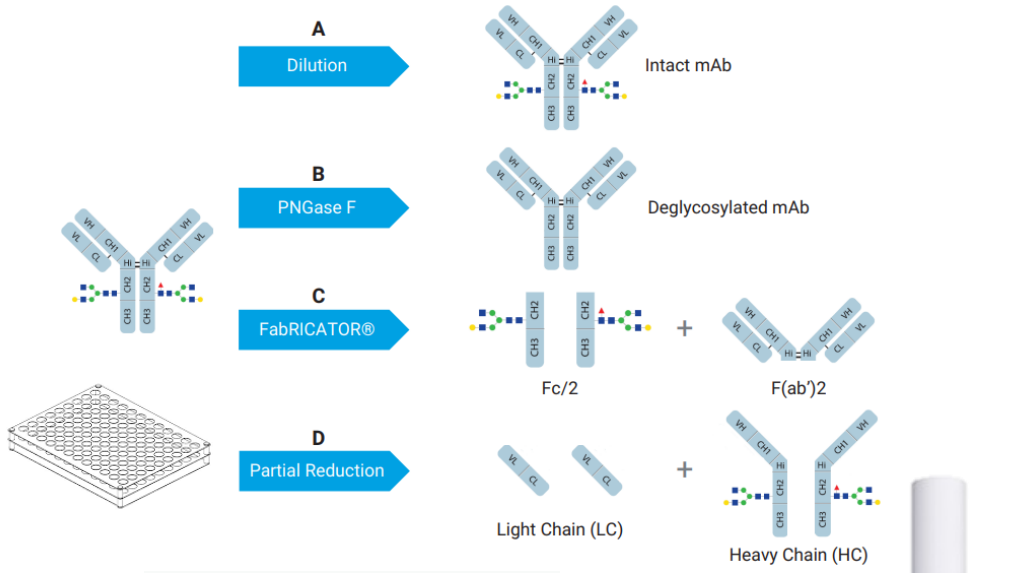
- *ADCC, ADCP, CDC, FcRn*

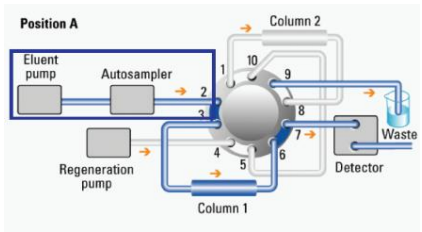


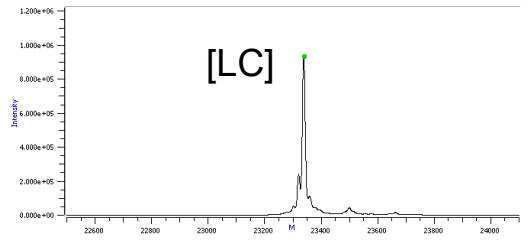


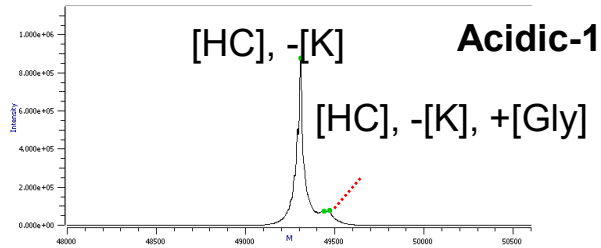
In-depth Charge Variant Characterization After Fractionation -De-glycosylated Reduced Intact Mass

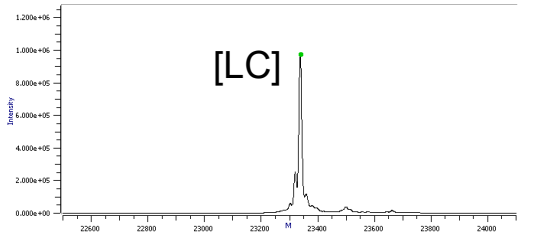
From the results of De-glycosylated Reduced Mass, higher abundance of **glycation** was identified in heavy chain of **Acidic Peak 2 and 3.**

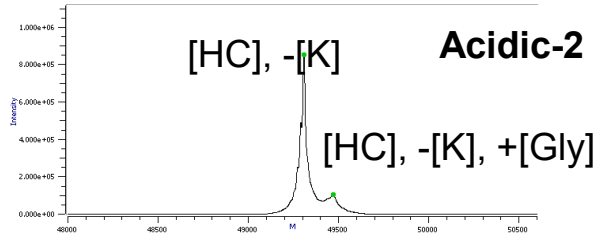


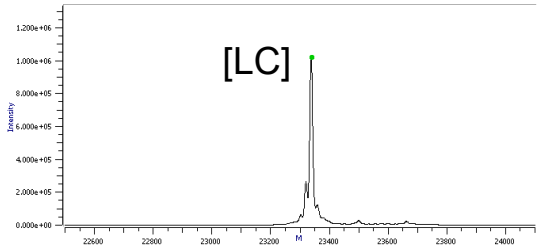


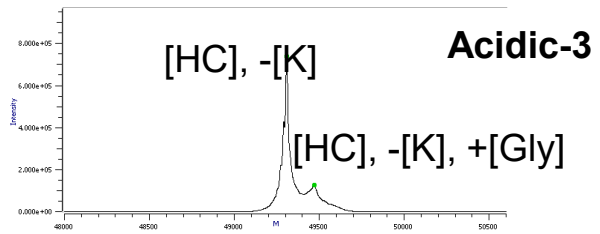


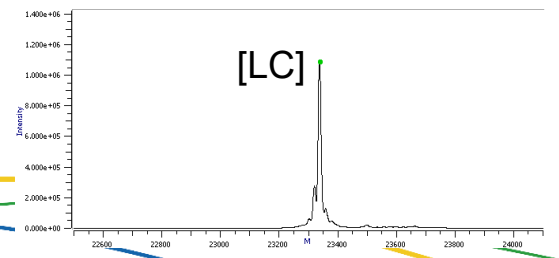


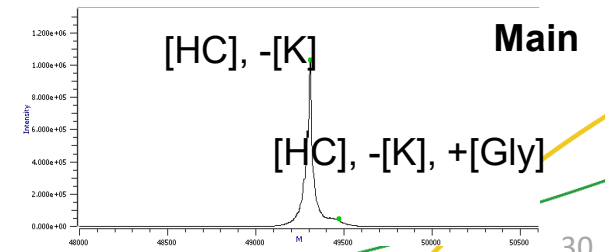








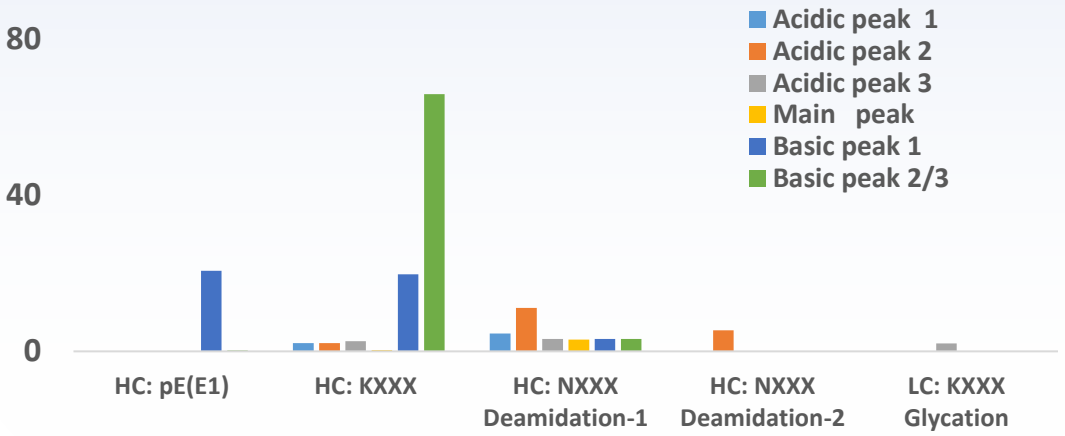




In-depth Charge Variant Characterization After Fractionation -Peptide Mapping and Glycan Analysis

WuXi Biologics
Global Solution Provider

Peptide Mapping for CEX Fractions



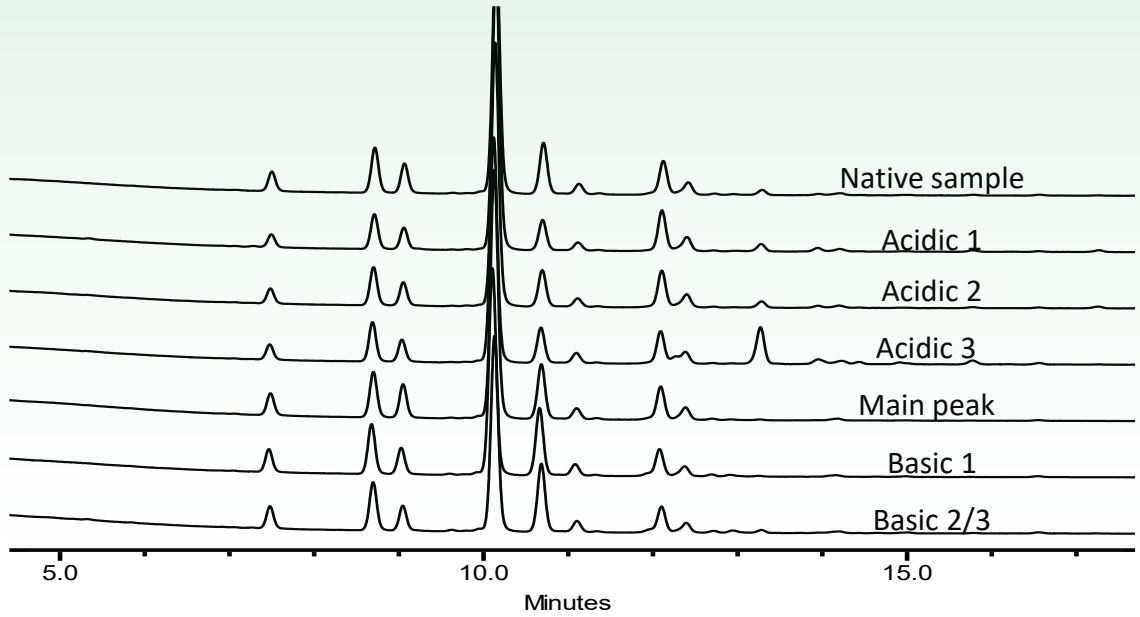
Source of acidic variants

- ❑ Confirm the higher deamidation site-1 NXXX and site-2 in Acidic peak 2.
- ❑ One glycation hotspot in Acidic peak 3

Source of basic variants

- ❑ Confirm lower ratio of glutamine cyclization-pE (Q1)
- ❑ K450 loss in Basic Peak 1 and 2/3.

N-glycan Profile for CEX Fractions

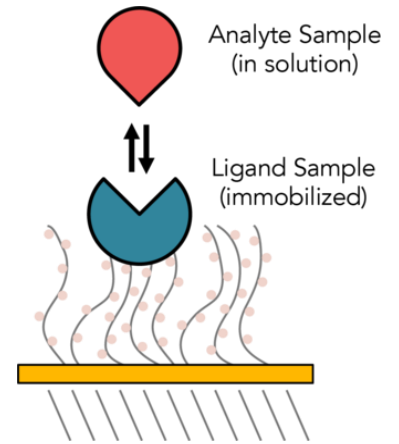


Source of acidic variants

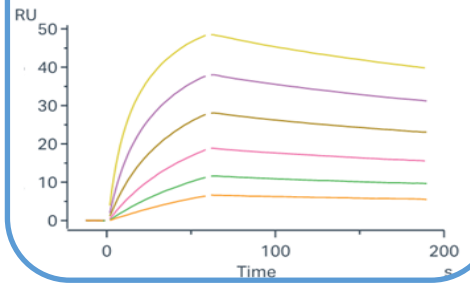
- ❑ Higher sialic acid level (G1FS1-GN) in Acidic peak 3

In-depth Charge Variant Characterization After Fractionation -Effector Function by SPR

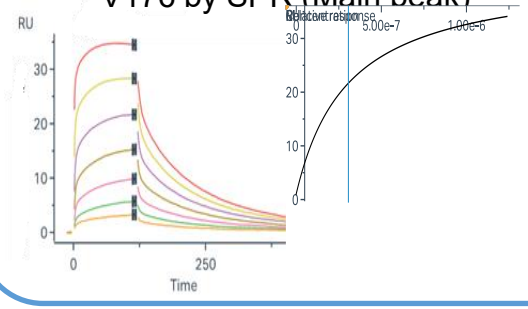
Compared with main peak, acidic peaks and basic peaks (fraction Acidic peak 1/2/3, Basic peak 1/2/3) showed comparable binding affinity to different human FcγR receptors



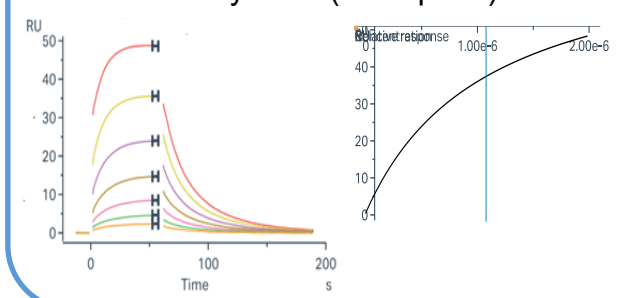
Binding of mAb to FcγRI by SPR (Main peak)



Binding of mAb to FcγRIIIa V176 by SPR (Main peak)



Binding of mAb to FcγRIIIa F176 by SPR (Main peak)



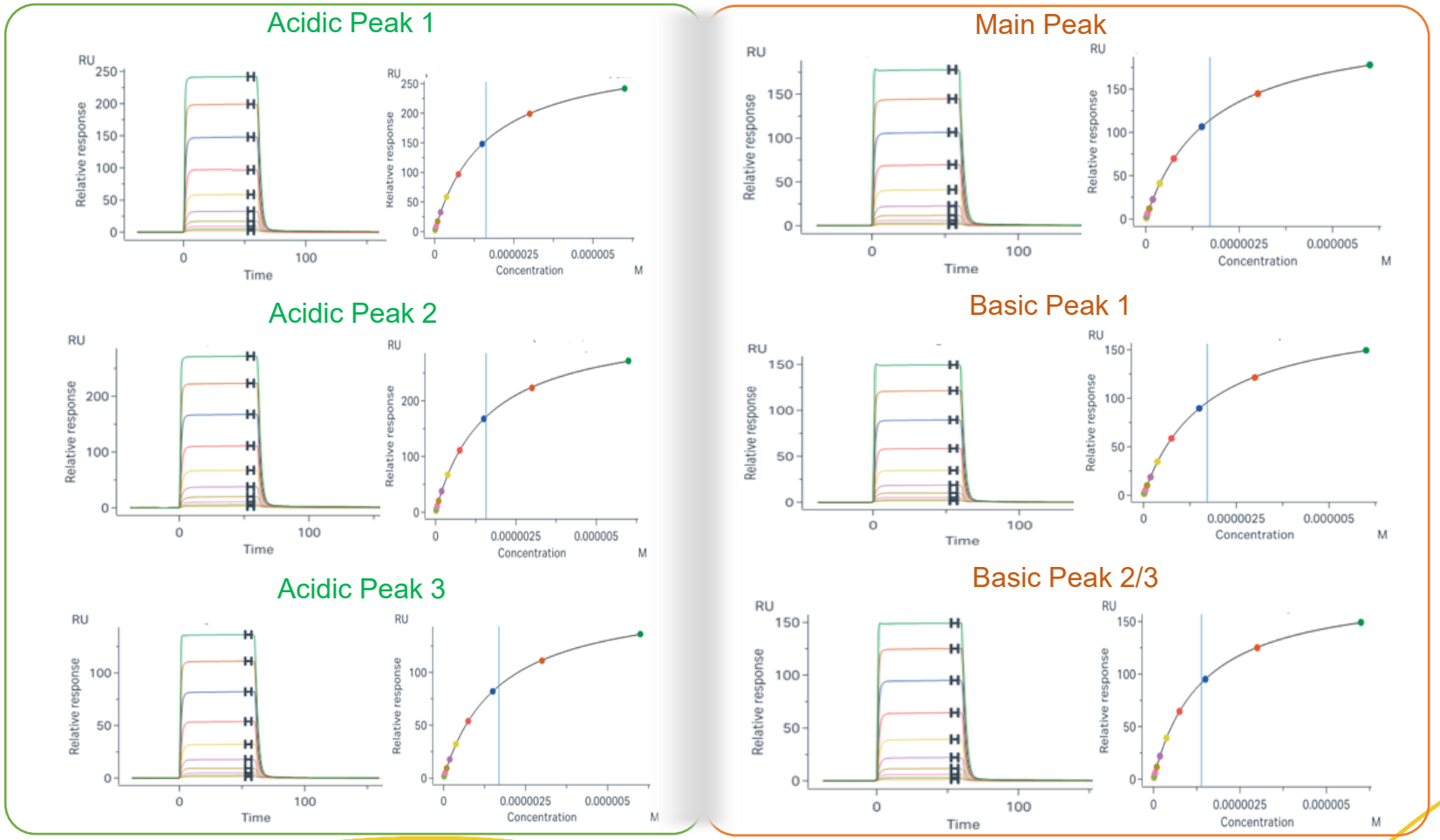
KD (M)	FcγRI	FcγRIIa R167	FcγRIIa H167	FcγRIIIa V176	FcγRIIIa F176
Acidic peak 1	3.5E-09 M	6.8E-06 M	2.0E-06 M	3.0E-06 M	1.1E-06 M
Acidic peak 2	3.6E-09 M	6.9E-06 M	1.8E-06 M	3.2E-06 M	1.2E-06 M
Acidic peak 3	3.2E-09 M	7.0E-06 M	1.9E-06 M	3.3E-06 M	1.0E-06 M
Main peak	3.8 E-09 M	6.9E-06 M	1.9E-06 M	3.1E-07 M	1.1E-06 M
Basic peak 1	3.7E-09 M	6.9E-06 M	2.1E-06 M	2.9E-07 M	1.2E-06 M
Basic peak 2/3	3.9E-09 M	7.3E-06 M	1.7E-06 M	3.6E-07 M	1.3E-06 M

In-depth Charge Variant Characterization After Fractionation -Effector Function by SPR

WuXi Biologics
Global Solution Provider

Compared with main peak, acidic peaks and basic peaks (fraction Acidic peak 1/2/3, Basic peak 1/2/3) showed comparable binding affinity to human FcRn.

FcRn by SPR	KD (M)
Acidic peak 1	1.6E-06
Acidic peak 2	1.6E-06
Acidic peak 3	1.7E-06
Main peak	1.7E-06
Basic peak 1	1.7E-06
Basic peak 2/3	1.7E-06



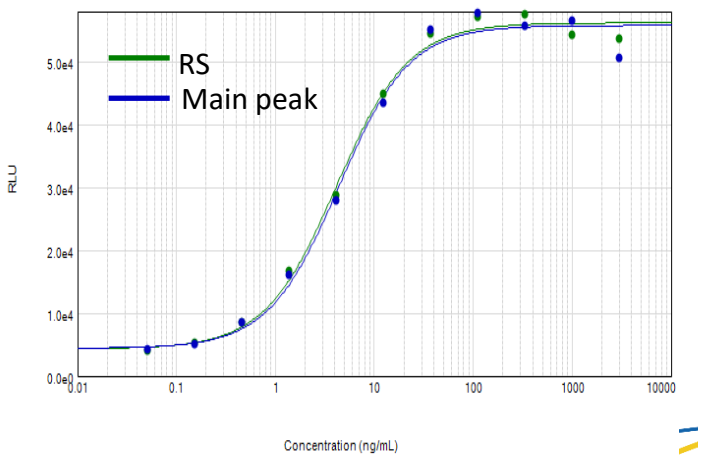
In-depth Charge Variant Characterization After Fractionation -Effector Function by Cell-based Bioassay

WuXi Biologics
Global Solution Provider

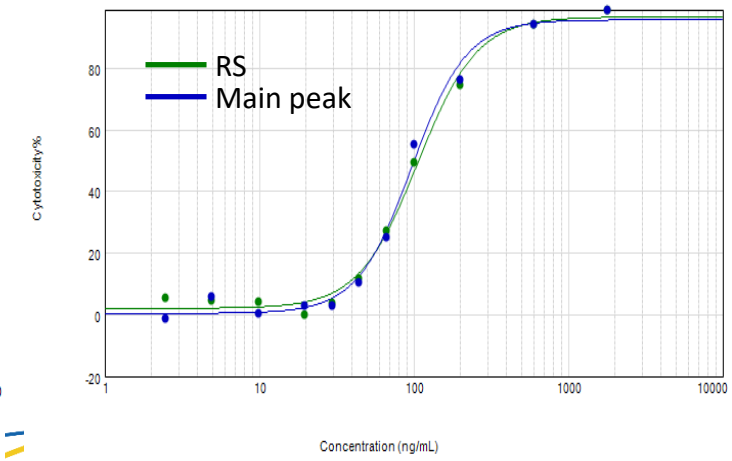
Compared with main peak, acidic peaks and basic peaks (fraction Acidic peak 1/2/3, Basic peak 1/2/3) showed comparable activity in cell based ADCC, CDC, FcRn binding assay

Relative potency	ADCC	CDC	FcRn Binding
Acidic peak 1	92%	99%	107%
Acidic peak 2	103%	106%	102%
Acidic peak 3	89%	101%	108%
Main peak	96%	104%	103%
Basic peak 1	98%	103%	104%
Basic peak 2/3	103%	103%	102%

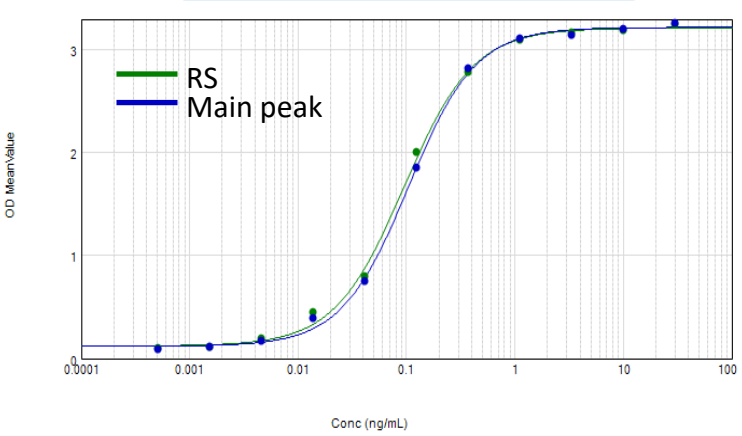
ADCC (Main peak)



CDC (Main peak)



Cell-based Competitive Binding Assay for FcRn (Main peak)

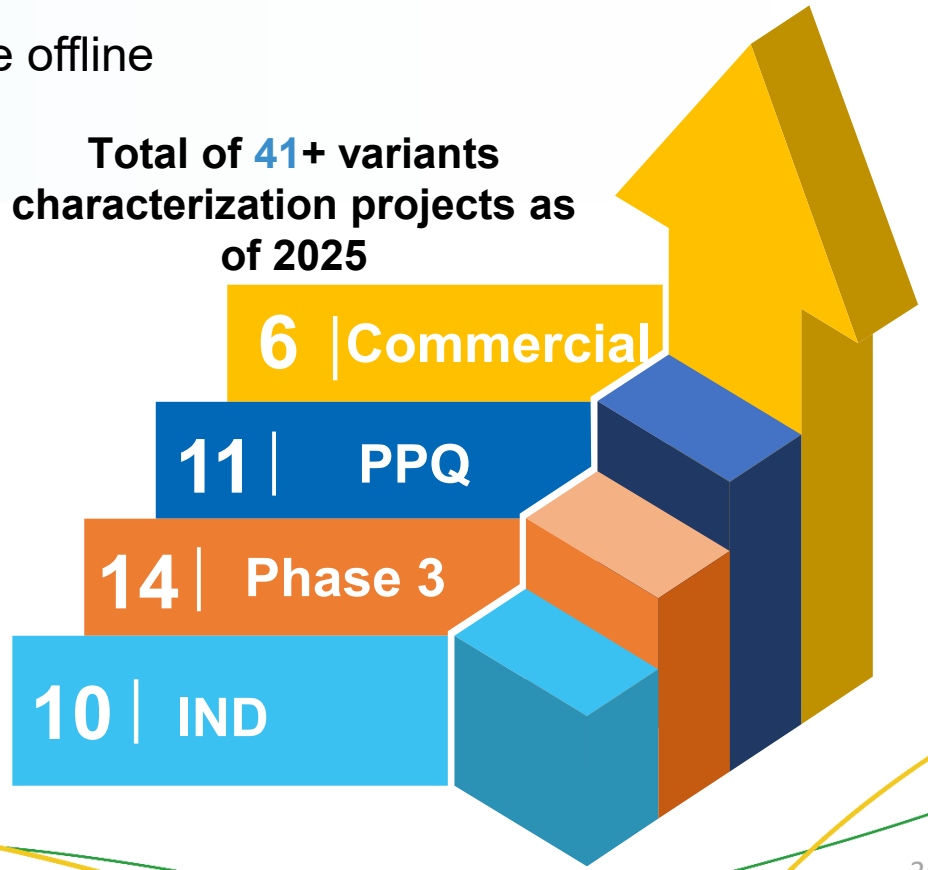


Summary 04

Summary

cIEF-MS has become essential tool for charge variant characterization, which has been routinely applied for analyzing different modalities including monoclonal antibody, bispecific and fusion proteins.

The ‘Discovery’ assisted approach significantly accelerated the offline fractionation workflow. Generally, 2–3 mg within 2 weeks.



Acknowledgements

Wuxi Biologics

- BioDev Wuxi Process Development

Cytiva GoSilico™

- Dong Zhang
- Yijia Guo

CMP Scientific

- James Xia

CASSS Mass Spec Organizers and Session Chairs

Agilent technologies

- Clara Chen (China team)
- Jun Li (China team)

Audiences and Attendees

WuXi Biologics
Global Solution Provider

Thank You

CRDMO
EVERY BIOLOGIC CAN BE MADE