Characterization of modifications on bispecific protein using native ion exchange chromatography coupled to mass spectrometry

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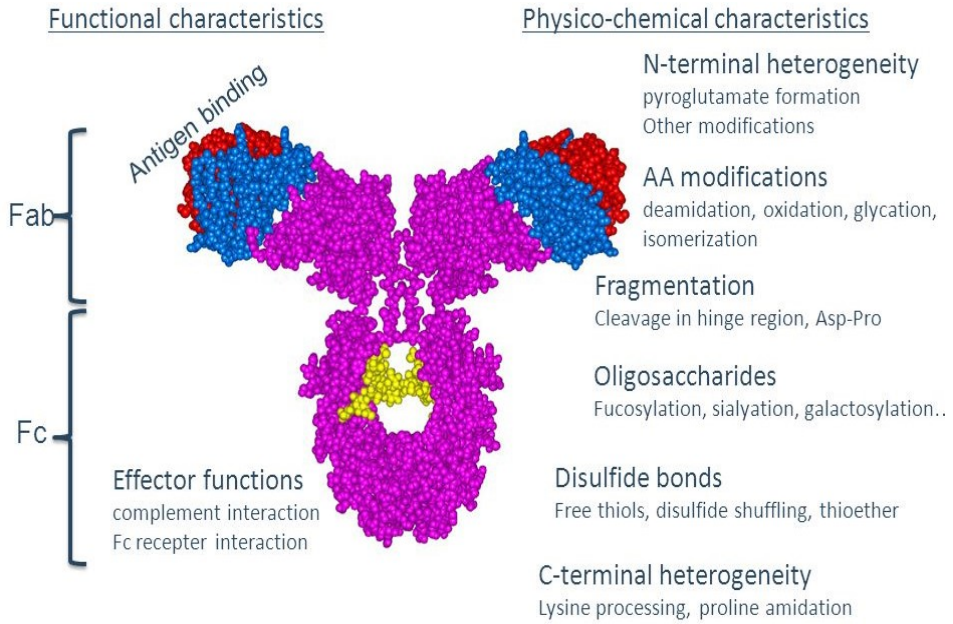
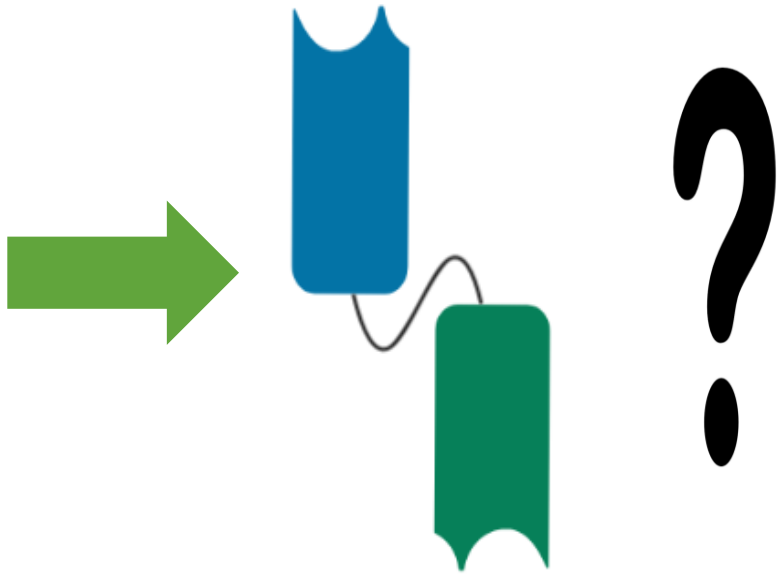
Outline

- Why are modifications important?
  - Current approach in characterization
- Proposed new approach in characterization of modifications
- Results
  - Native
  - Limited Digestion
  - Peptide Mapping
- Conclusions

Why are modifications important ?

- **Modifications on biotherapeutic refers to enzymatic or non-enzymatic modifications that can occur during protein biosynthesis, purification process or accrue during its storage conditions**
- **Modifications can affect potency, pharmacokinetics and may impact immunogenicity**
- **Characterizing modifications of biotherapeutic will help us in better define critical quality attributes (CQAs) related to protein therapeutic**





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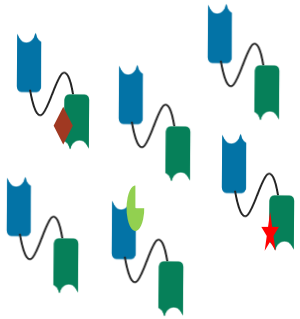
Why study modifications for bispecific protein?



Why study modifications on bispecific protein?

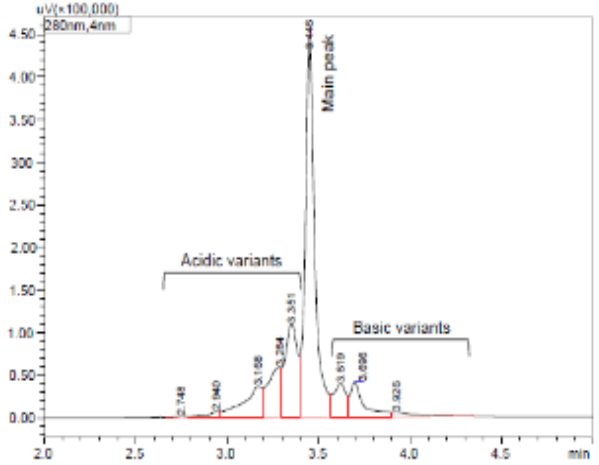
- New modality compared to monoclonal antibody, need to understand impact of biosynthesis of bispecific protein on PTMs
- To understand the impact of purification and storage on modifications to bispecific protein

How to study ?

- Modifications impart different surface charge which can be separated using ion exchange chromatography (IEX)
- Variants are separated as acidic main and basic species

Indicates modifications

Traditional characterization approach



Characterization is performed by fractionation of each variant peak with subsequent analysis to identify attributes associated with each variant

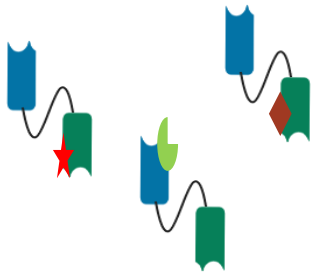
Caveats

- Time consuming and labor intensive process
  - Multiple fraction collection is required for low abundant species
- Substantial sample consumption
- Modifications may be imparted due to sample handling process

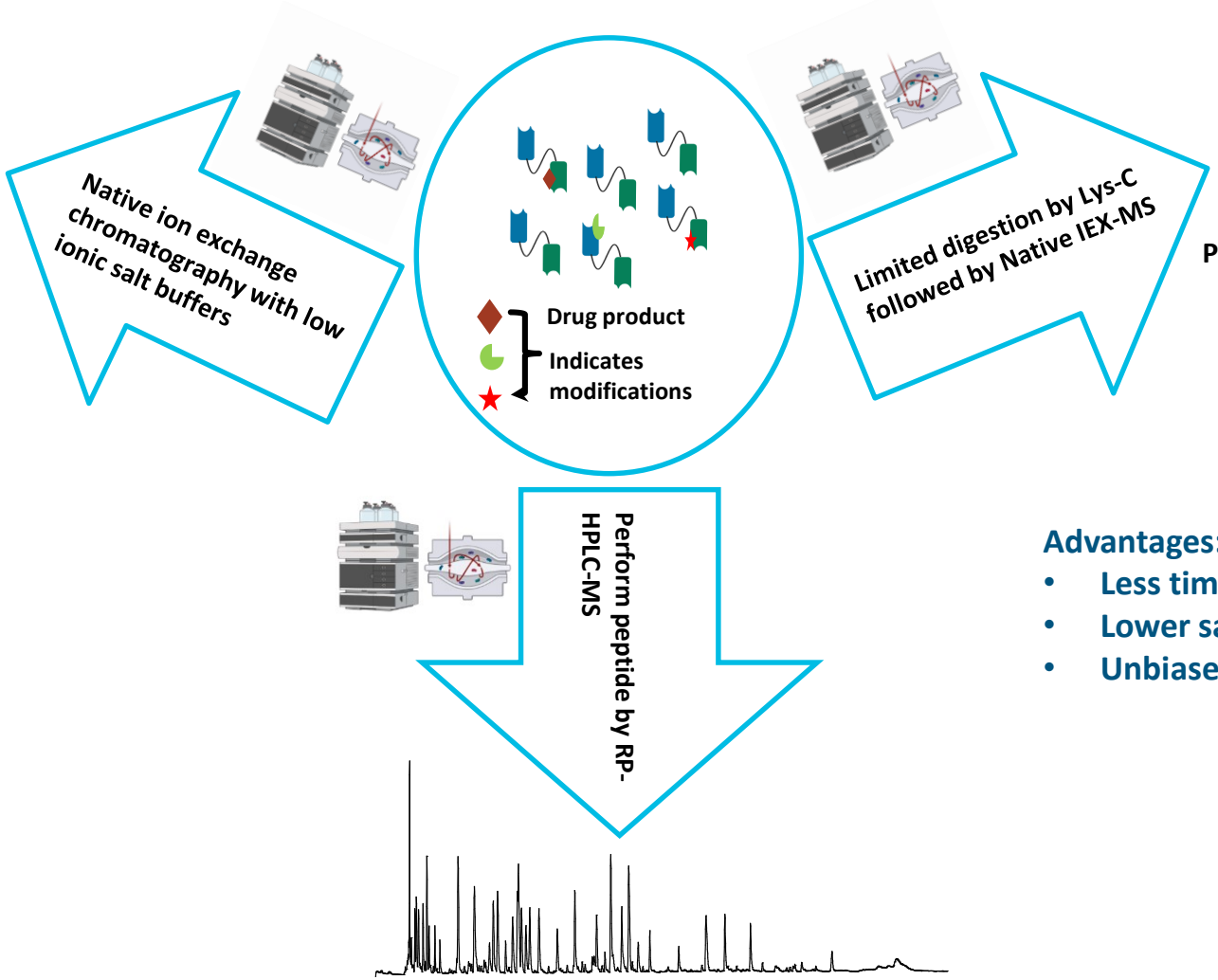
Require

Robust approach to provide quick and unbiased information regarding molecular heterogeneity

Proposed workflow for native characterization of bispecific protein



Predict modifications based on mass difference with main species from ion exchange chromatography under native conditions



Predict modification specific to domains

Advantages:

- Less time consuming than traditional method
- Lower sample consumption
- Unbiased identification

Confirmation of modifications identified by native IEX-HPLC-MS and limited digestion from peptide mapping by RP-HPLC-MS

Method information for IEX-MS

LC method Information

Mobile phase used in native IEX-MS
Solvent A: 50mM Ammonium Acetate, pH 5.8
Solvent B: 150mM Ammonium Acetate, pH 10.2

Mobile phase used in limited digestion (Native IEX-MS)
Solvent A: 20mM Ammonium acetate, pH 4.5
Solvent B: 150mM Ammonium acetate, pH 9.5

Column: YMC Biopro IEX SF, 100 X 4.6mm I.D, 5µM, SF00S05-1046WP (strong cation exchange column)

Flow rate: 0.5ml/min

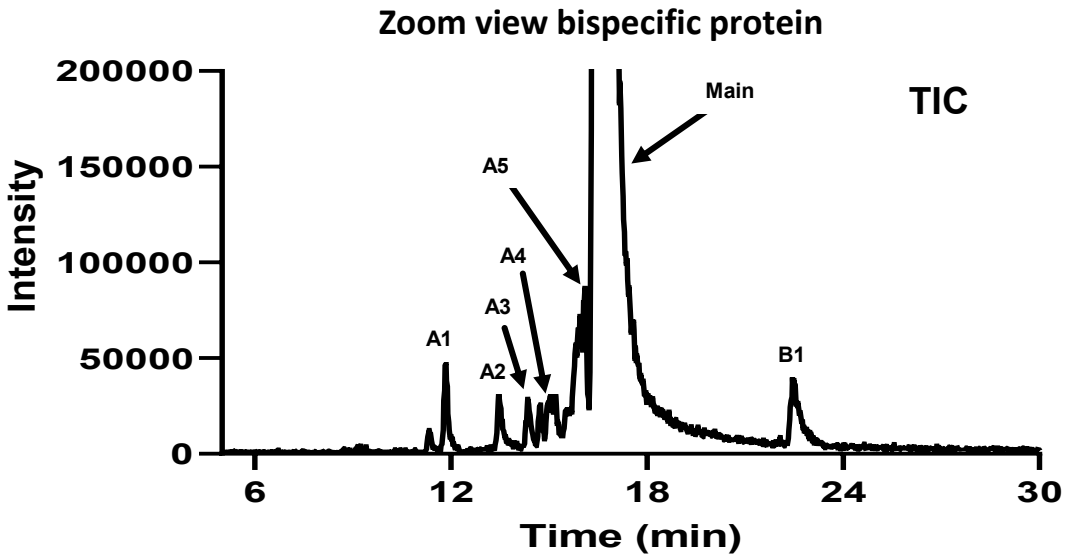
Gradient change: 2% change B/min

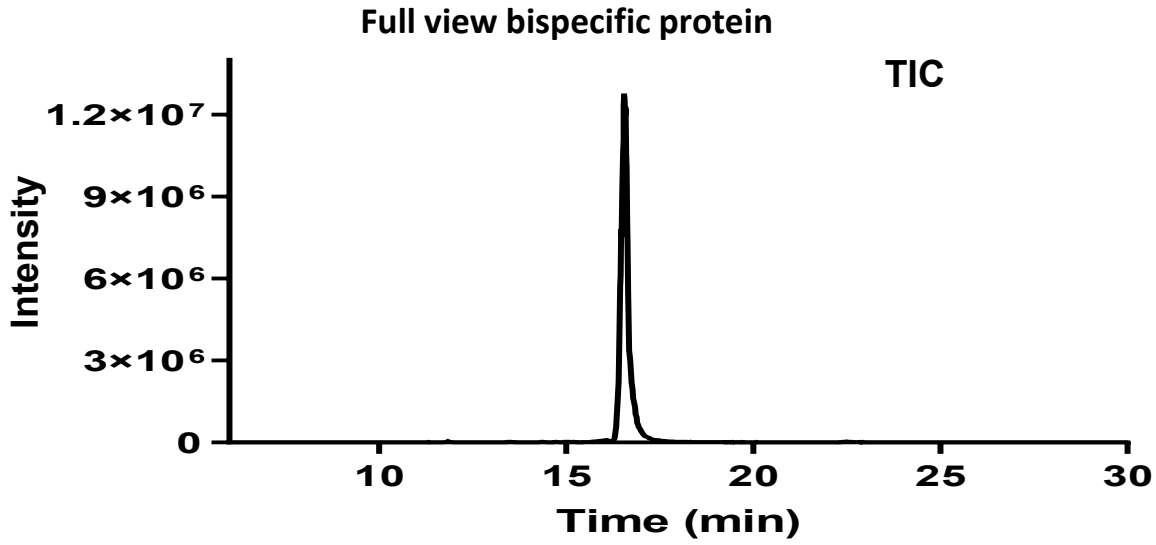
Run time: 63 min

MS instrument: Thermo QE+ Biopharma

Parameter	Condition
In-source CID	100 eV
Resolution	17500
AGC	3e6
Max IT	200ms
Spray voltage	3.8kV
S-lens	150
Capillary temp	300^{0}C
Source heat	275^{0}C
HMR mode	On
Trapping gas	1.3
M/Z range	2100-6800
Aux heat temp	220^{o}C

Prediction of modification on drug substance

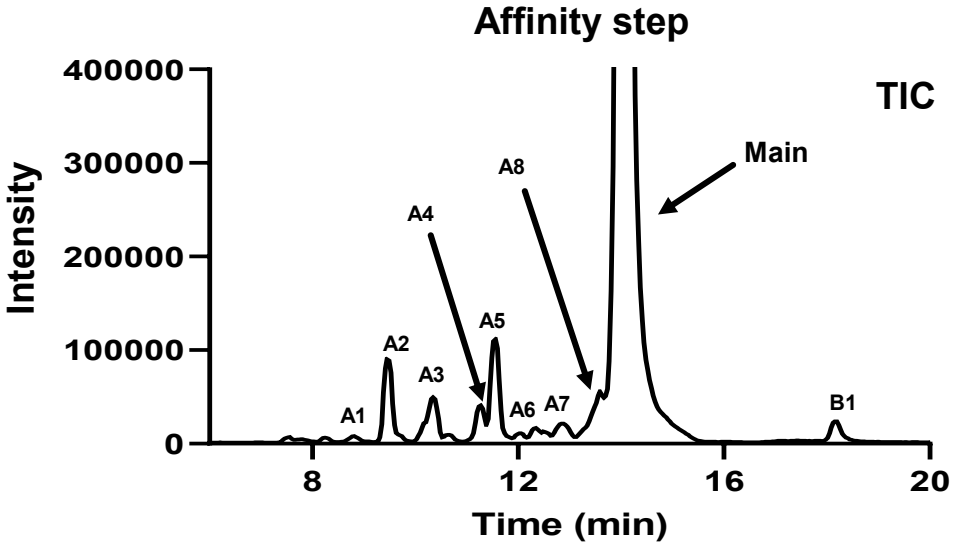




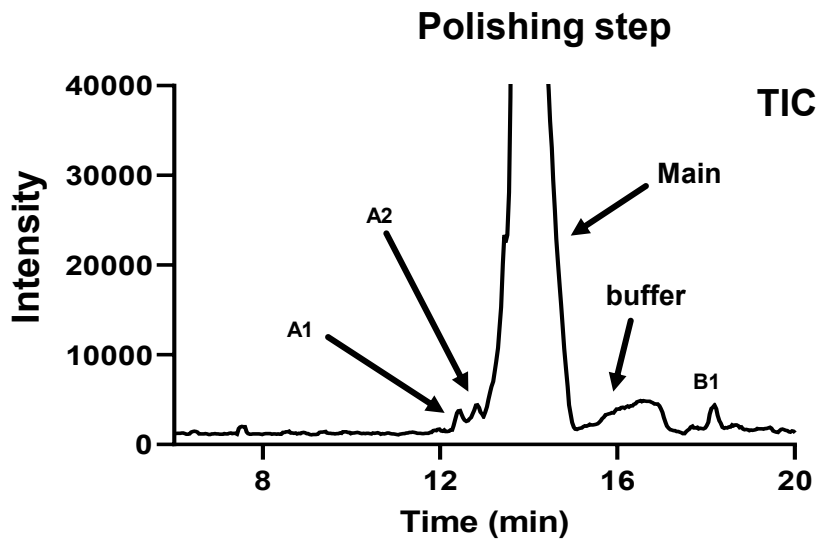
Species	Difference with Main peak	Predicted modifications
A1	1	Deamidation
A2	1.5	
A3	995.2	O-glycan
A4	915.7	
A5	58.7	Amino Acid Substitution (Gly-Asp)
Main	0	
B1	2X	Dimer

Main peak was observed at mass error of 16ppm

Impact of process on modifications

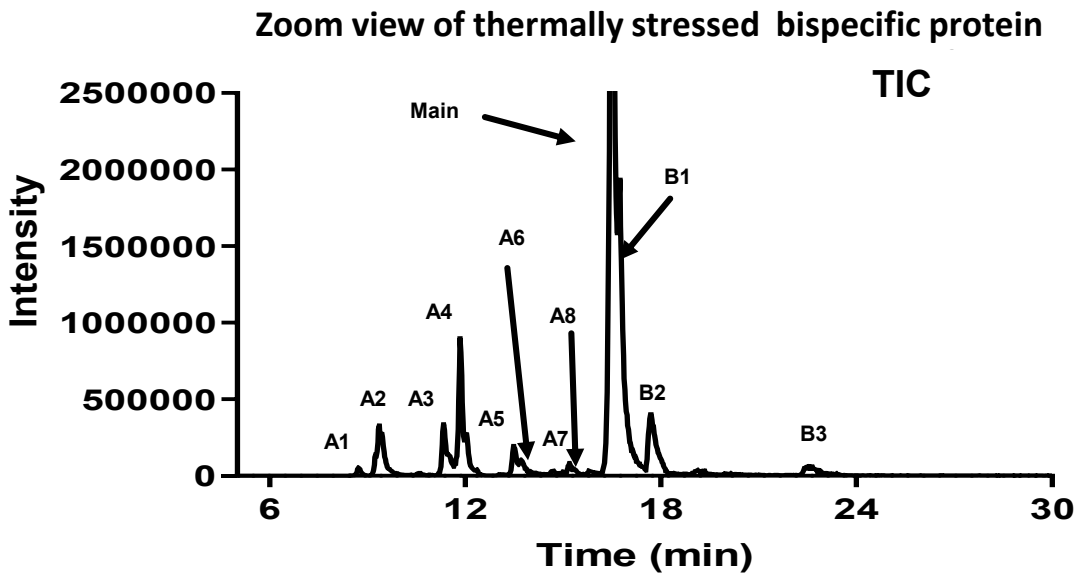


Species	Difference with Main peak	Predicted modifications
A1	NA	Antigen binding domain fragment (G/G linker)
A2	2351	N-linked glycan
A3	2060	
A4	-0.5	Deamidation
A5	0.5	
A6	995.3	O-glycan
A7	916	
A8	58.5, 161	Amino acid substitution, Glycation
Main	0	
B1	2X	Dimer



Species	Difference with main peak	Predicted modifications
A1	57, 915	Amino acid substitution, O-glycan
A2	16	Hydroxylation, oxidation
Main Peak	0	
B1	2X	Dimer

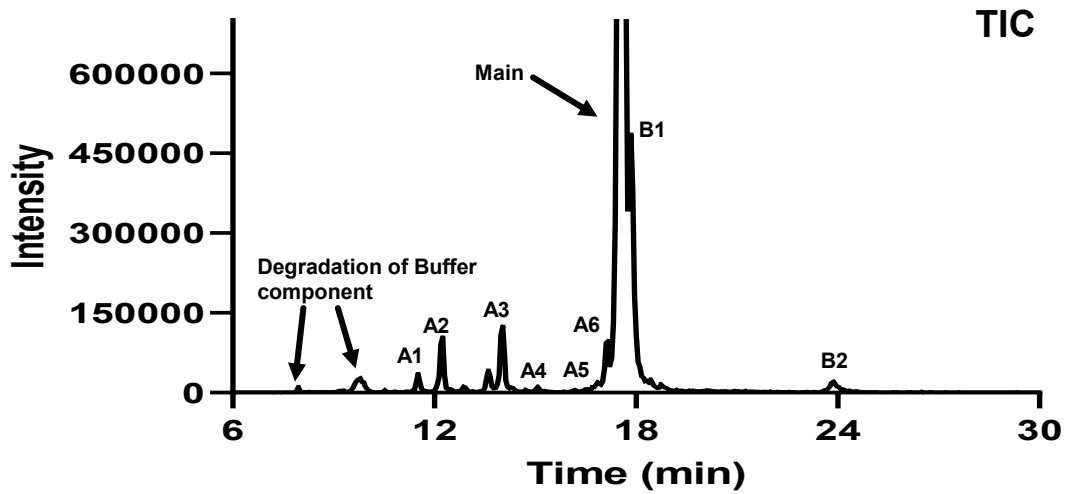
Modification observed for thermal stress material



Species	Difference with Main peak	Predicted modfications
A1 (Clips)	NA	Antigen binding domain 1 and antigen binding domain -2 fragments
A2	2	Deamidation
A3	0.5	
A4	0.7	
A5	1.5	
A6	995.7	O-glycan
A7	913.7	
A8	58.7	Amino Acid substitution
Main	0	
B1	-0.3	Isomerization
B2	-19.5	Succinimide formation
B3	2X	Dimer

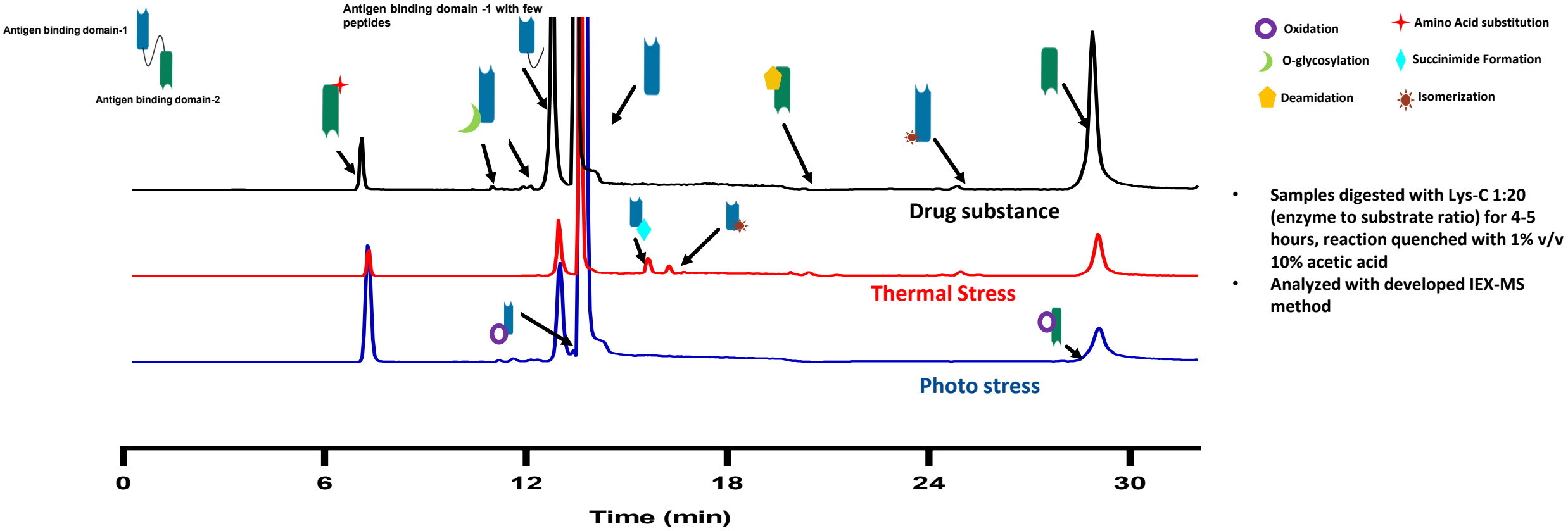
Modification observed for photo stressed material

Zoom view of photo stressed bispecific protein



peak Name	Difference	Predicted modification
A1	1.3	Deamidation
A2	1.1	
A3	1.2	
A4	995.1	O-glycan
A5	59.8	AA substitution
A6	32.3	Double oxidation
Main	0,16	Main and single oxidation
B1	0.1	Isomerization
B2	2X	Dimer

Limited digestion for bispecific protein



- Samples digested with Lys-C 1:20 (enzyme to substrate ratio) for 4-5 hours, reaction quenched with 1% v/v 10% acetic acid
- Analyzed with developed IEX-MS method

- Antigen binding domain-1 showed presence of O-glycosylation and isomerization while antigen binding domain-2 showed presence of deamidation and amino acid substitution
- Under thermal stress 2 species were observed which showed presence of succinimide formation and isomerization on antigen domain-1
- Photo stressed material showed presence of oxidation on antigen binding domain and antigen binding domain-2 domain

Peptide mapping procedure

Digestion Kit: Promega low pH digestion kit (catalog # VA1040) (uses rLys-C and modified trypsin based peptide digestion)

Mobile Phase

A: 0.1% Formic acid in water
B: 0.1% Formic acid in acetonitrile

Flow rate: 0.25 mL/min

Instrument: UPLC (waters Acquity) binary pump system

Column: Waters, peptide BEH C18 column, 130A, 1.7µM, 2.1 X 150mm
Load : 20 µL

Gradient: 0.3% change B/min
Total run time: 123min

Instrument	Thermo QE+
MS Settings	
Full MS	
Resolution	140,000
AGC target	2.00E+06
Max It	200
Scan range	200-2000 m/z
dd-MS^2	
Resolution	35000
AGC target	1.00E06
Max IT	250
loop count	5
Isolation Window	2.5 m/z
(N) CE	31
Dynamic exclusion	7 sec
Tune settings	
Spray voltage	3.8 kV
Capillary temperature	300^0C
Probe heater temperature	250^oC
S-lens	60
Polarity	Positive

Peptide map result for drug substance

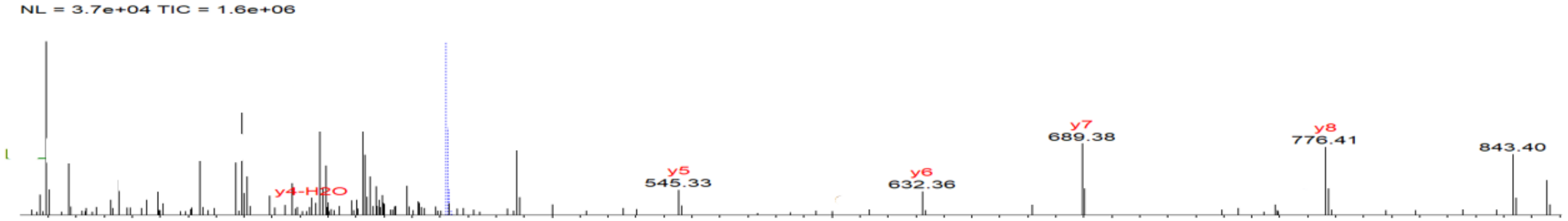
sample type	Modifications	Amino Acid residue	Domain
DS	Deamidaion	N360 and N363	Antigen binding domain-2
	Amino Acid Substitution	G467	Antigen binding domain-2
	Isomerization	D54	Antigen binding domain-1
Thermal Stress	Deamidation	N360, N363, N84, N313	Antigen binding domain-1 and 2
	Succinimide	D172	antigen binding domain
	Isomerization	D54	Antigen binding domain
Light stress	Oxidation (Double oxidation of W)	W47, W52, W490, W495	Antigen binding domain 1 and 2
	Deamidation	N360 and N363	Antigen binding domain 2
	Isomerization	D54	Antigen binding domain-1

Note : Indicates major degradation pathway observed

Observation for modification correlates with findings from limited digestion and native IEX-MS indicating observed modification are present at native level

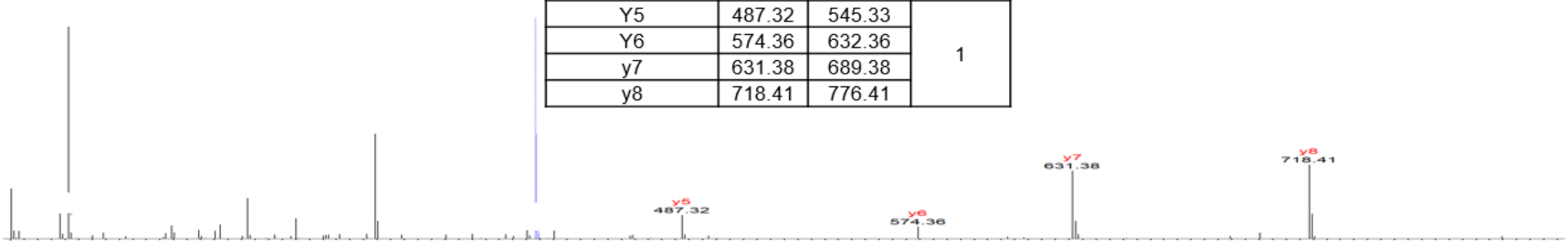
MS2 conformation for amino acid substitution

X X X X X X **D** X X ⟵ Modified peptide



X X X X X X **G** X X ⟵ Native peptide

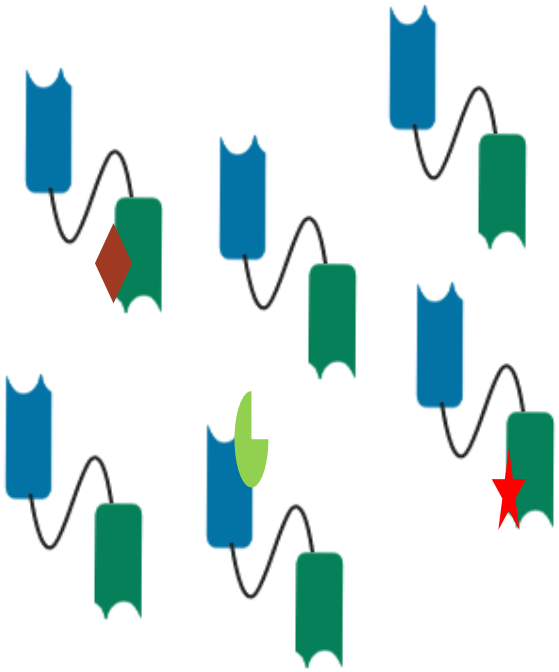
Fragment Ion type	Native	Modified	Charge
Y5	487.32	545.33	1
Y6	574.36	632.36	
y7	631.38	689.38	
y8	718.41	776.41	



Conclusions

Native IEX-MS

- Successfully developed Ion exchange gradient for separation of bispecific protein variants
- Optimized MS conditions for native mass-spectrometry observations
- Successfully predicted modification at native level
  - Predicted modifications for drug substance and different forced degradation conditions (Thermal stress and photo stress conditions)
  - Predicted modification that were cleared away by purification of bispecific protein



Limited digestion

- Successfully predicted modifications related to specific domain

Peptide map

- Identified and correlated modification observed by peptide map to modification predicted a native and limited digestion level

Workflow

Application of this workflow for different protein modality will aid in time saving and provide unbiased information regarding modifications

THANK YOU

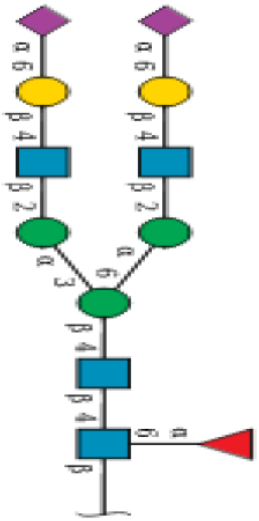
Dr. John Harrahy
Dr. Weidong Cui

Prof. Alexander Ivanov and lab members

BACKUP

Predicted glycan

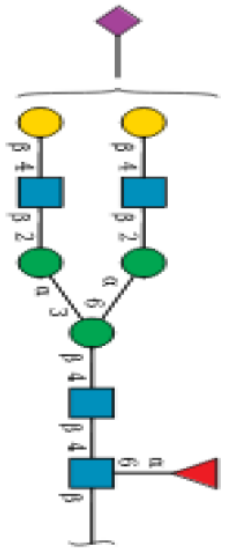
N-linked Glycan observed for affinity purified column



Neu?c(a2-6)Gal(b1-4)GlcNAc(b1-2)Man(a1-3)[Neu?c(a2-6)Gal(b1-4)GlcNAc(b1-2)Man(a1-6)]Man(b1-4)GlcNAc(b1-4)[Fuc(a1-6)]GlcNA

Observed difference: +2351

Average mass : 2352.1

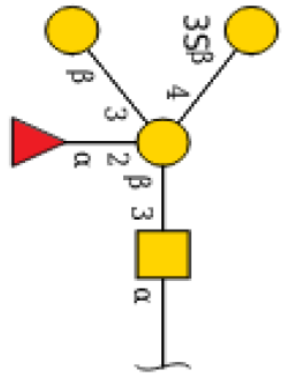


Gal(b1-4)GlcNAc(b1-2)Man(a1-3)[Gal(b1-4)GlcNAc(b1-2)Man(a1-6)]Man(b1-4)GlcNAc(b1-4)[Fuc(a1-6)]GlcNAc+"+ NeuAc

Observed difference: +2060

Average mass : 2059.7

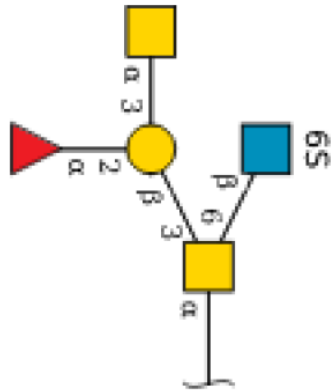
O-linked glycan observed for DS and in-process purification sample



HSO3(-3)Gal(b1-4)[Fuc(a1-2)][Gal(b1-3)]Gal(b1-3)GalNA

Observed difference: +915

Average mass : 915.8



Fuc(a1-2)[GalNAc(a1-3)]Gal(b1-3)[HSO3(-6)GlcNAc(b1-6)]GalNAc

Observed difference: +995

Average mass : 995.8

Source: unimod