Advancing electron activated dissociation (EAD) in a data-dependent acquisition (DDA) method for improved biotherapeutics characterization

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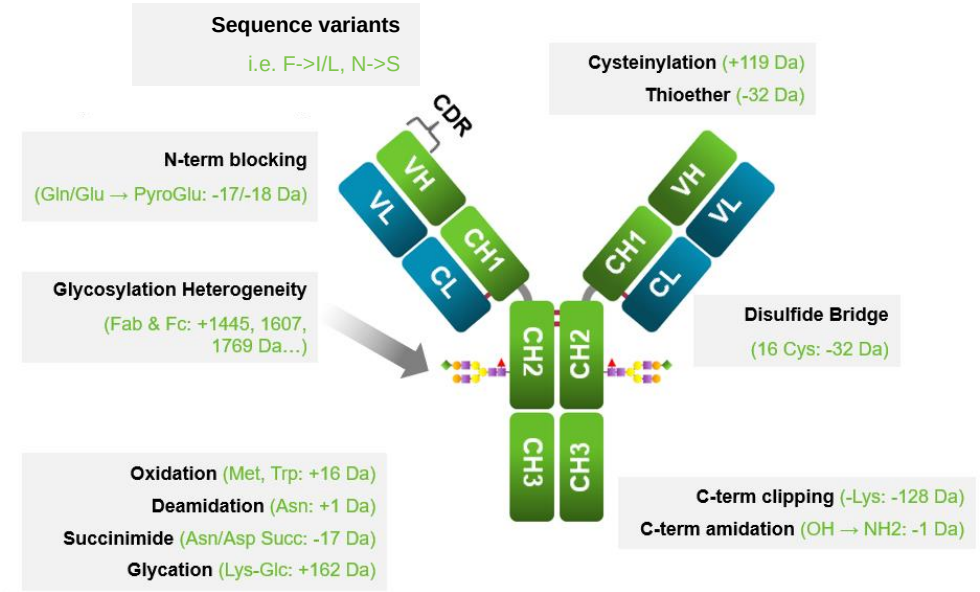
SCIEX
The Power of Precision

Outlines

- Background
- Robust peptide mapping enabled by EAD
- Site-specific intact N/O-linked glycopeptide analysis by EAD
- Amino acid isomer differentiation and its application in sequence variants (SV) analysis
- Conclusions

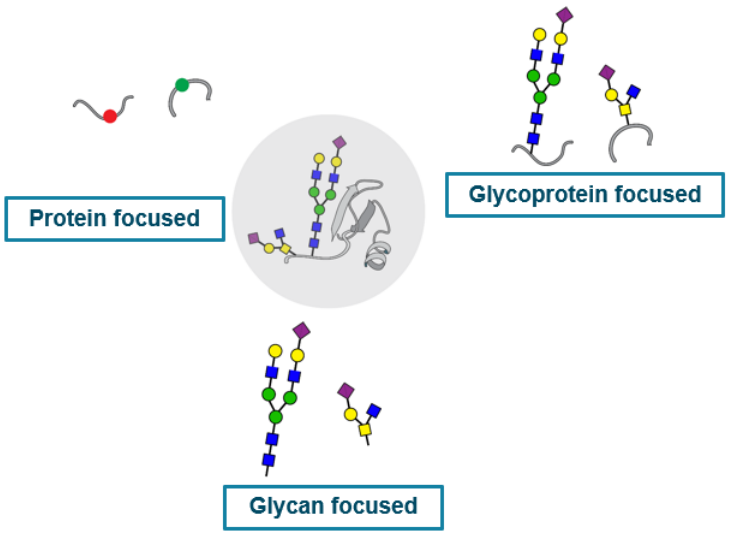
Importance of glycosylation in biotherapeutics development



- **Biotherapeutics**: monoclonal antibodies, fusion proteins and therapeutic replacement enzymes etc.
- **Critical quality attributes (CQA)**
- **Safety and efficacy**
- **Different stages**
  - Discovery
  - Development
  - Quality control

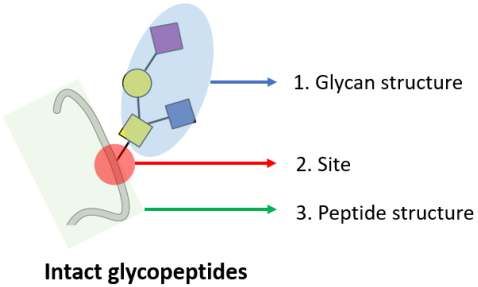
Challenges in glycosylation analysis

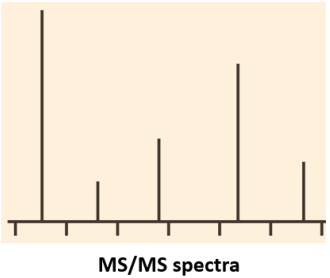
Three MS-based approaches



CID: **C**ollision-**I**nduced **D**issociation
ETD: **E**lectron-**T**ransfer **D**issociation
ECD: **E**lectron-**C**apture **D**issociation

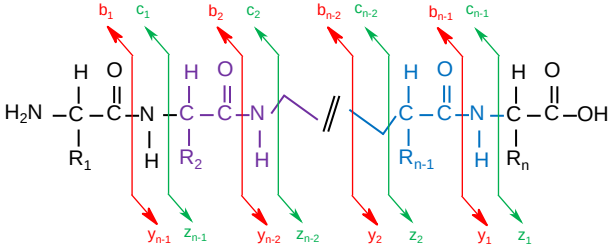
MS/MS fragmentation



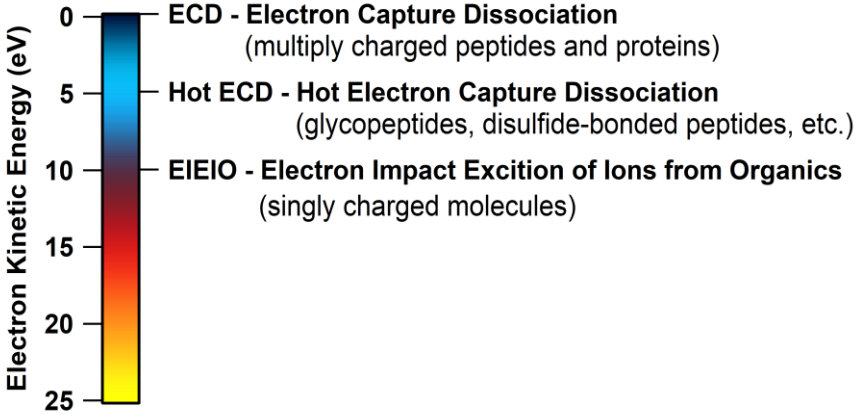


- **CID:**
  - Dissociation of labile covalent PTMs
- **ETD:**
  - Slow reaction
  - Poor MS2 data quality ETnoD
  - Prefer higher charge states
- **ECD**
  - Flow-through devices with poor efficiency and software integration

Electron activated dissociation (EAD)



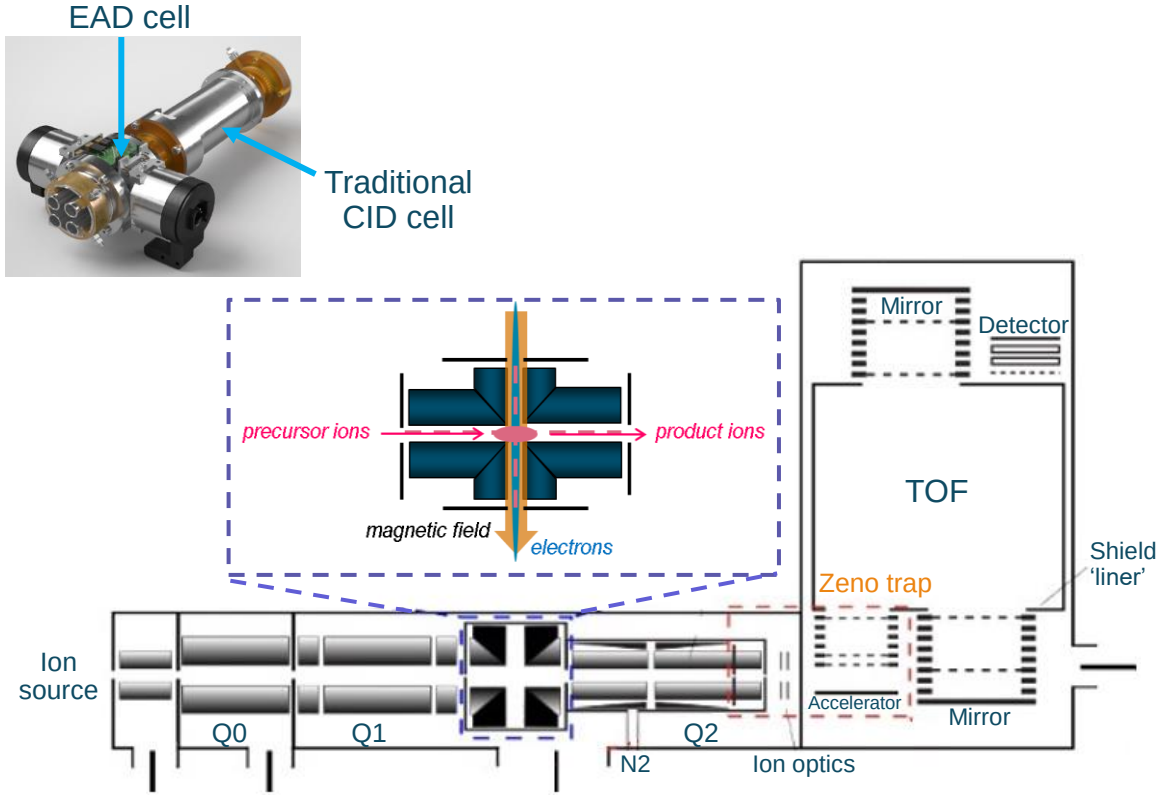
- Free electrons are captured by ions and form a radical state which then fragments
- Electrons introduced with different energies will induce fragmentation in different molecule types
- EAD cell enables you to perform ECD, EAD (Hot ECD) and EIEIO in one instrument



Baba, Takashi, et al. Analytical chemistry 87.1 (2015): 785-792.
Baba, Takashi, et al. Journal of the American Society for Mass Spectrometry (2021).

EAD implemented in Q-TOF system

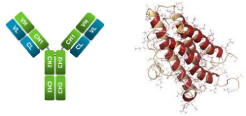
ZenoTOF 7600 System

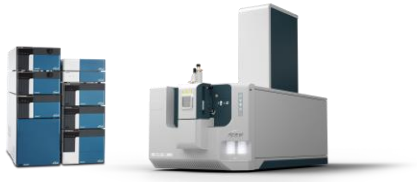


Key features of EAD

- Speed of fragmentation and acquisition
  - Electron capture reaction times ~ 10-30ms
  - DDA on LC time scale up to 20 Hz
- Reagent-free, electron capture dissociation
- Tunable energy
- No need for further activation of charged reduced species by CID

LC-MS workflow





Biologics Explorer software

Sample preparation → **Data generation IDA** → **Database search**

- **Samples:**
  - mAbs
  - glycoprotein standards
- **Reduced peptide mapping:**
  - Denaturation
  - Reduction
  - Alkylation
  - Enzyme digestion
  - Quench reaction

LC condition	Values
LC system	ExionLC system (SCIEX)
Mobile phase	0.1% FA H_2O/ACN
Gradient	35 min to 45% B
Column	C18 1.7 µm; 2.1 x 150 mm
Column temp.	50ºC
Flow rate	0.25 mL/min

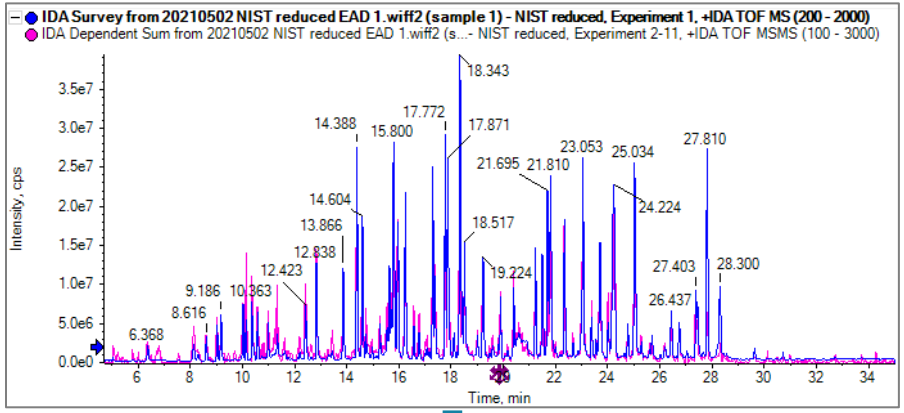
MS condition	Values
MS system	ZenoTOF 7600 system
Experiment type	DDA with top10 and dynamic exclusion
Cycle time	1.25 s
Fragmentation	EAD with 7eV, 10 ms reaction time
Zeno trap	ON

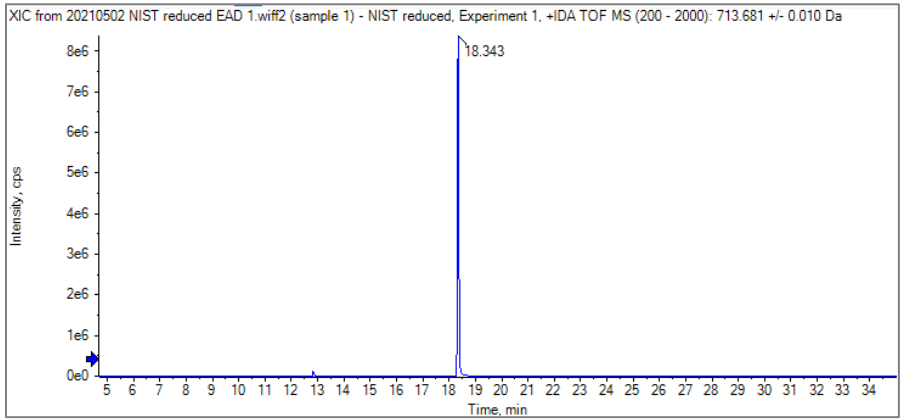
Posters: Mon. #96, 284; Tue. #148,156, 290; Wed. #245; Thur. #005

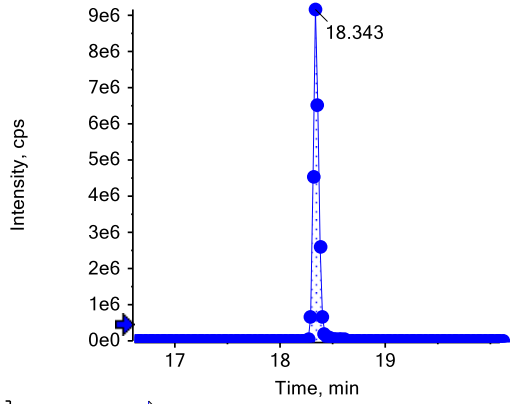
EAD in a DDA acquisition of NISTmAb peptide digest

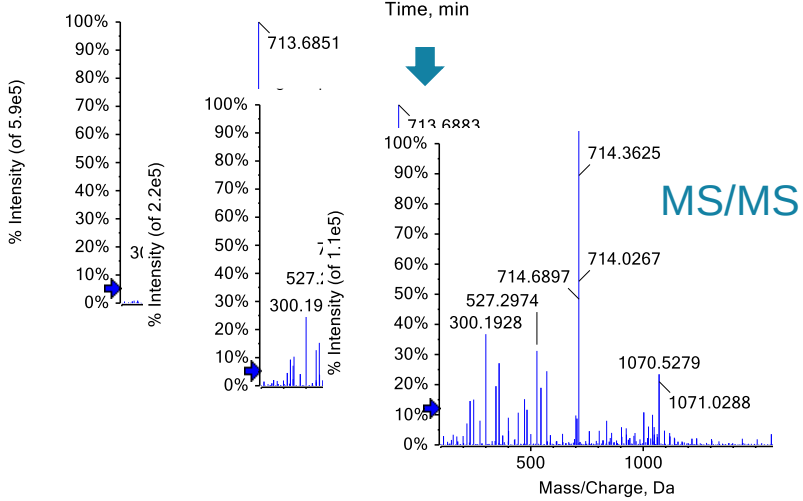
TIC

XIC

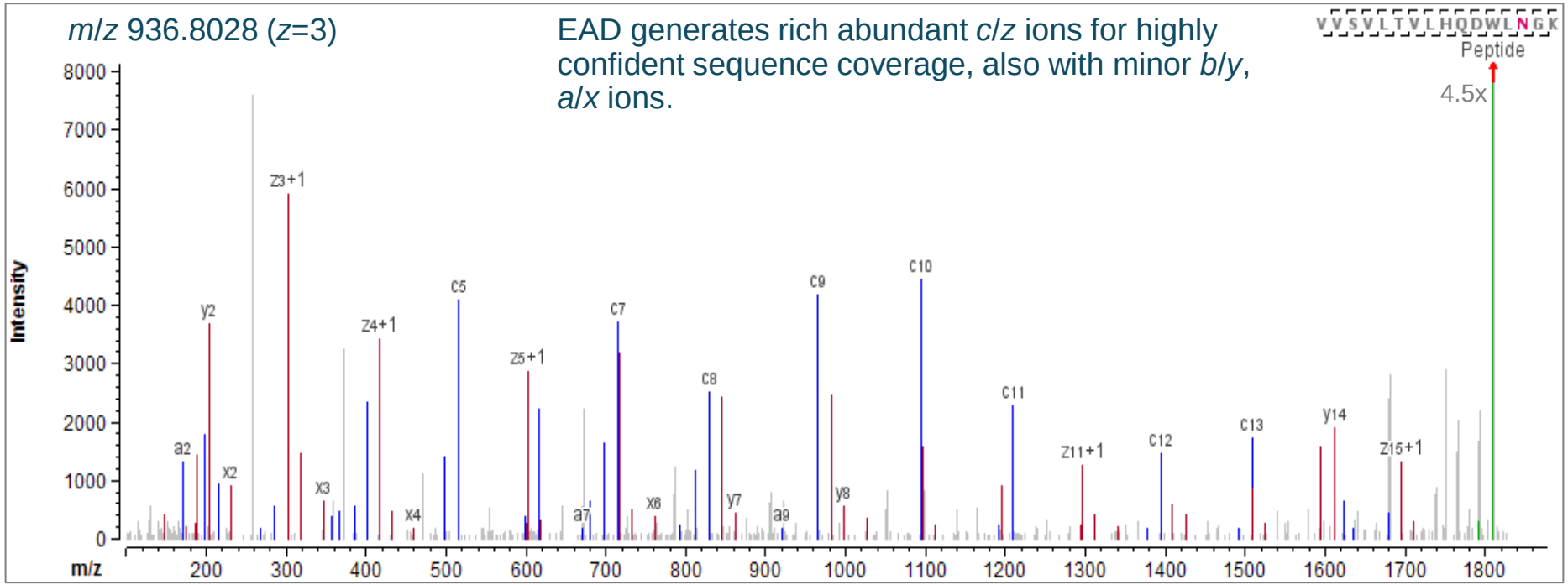




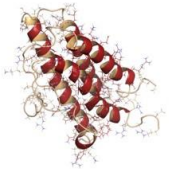




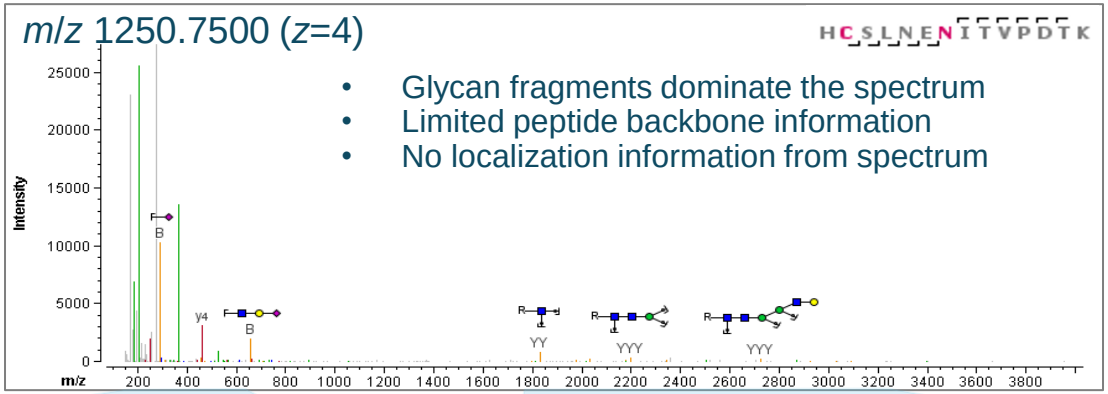
EAD provides exceptional peptide sequencing



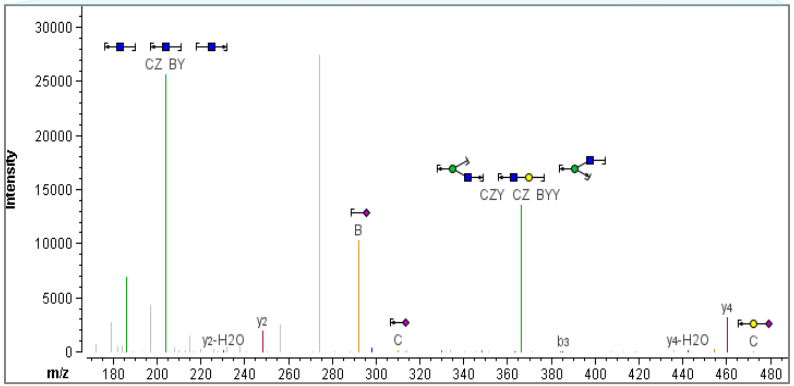
Limitations of CID for site-specific N-glycopeptide analysis

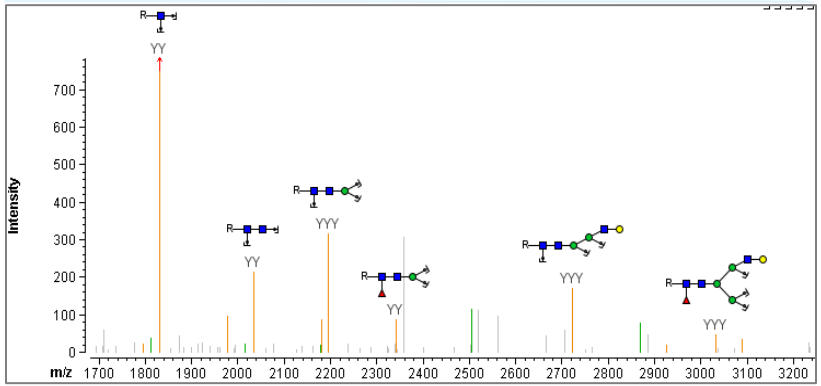


~21 kDa
erythropoietin

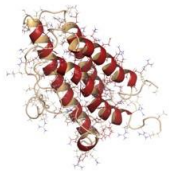


- Glycan fragments dominate the spectrum
- Limited peptide backbone information
- No localization information from spectrum

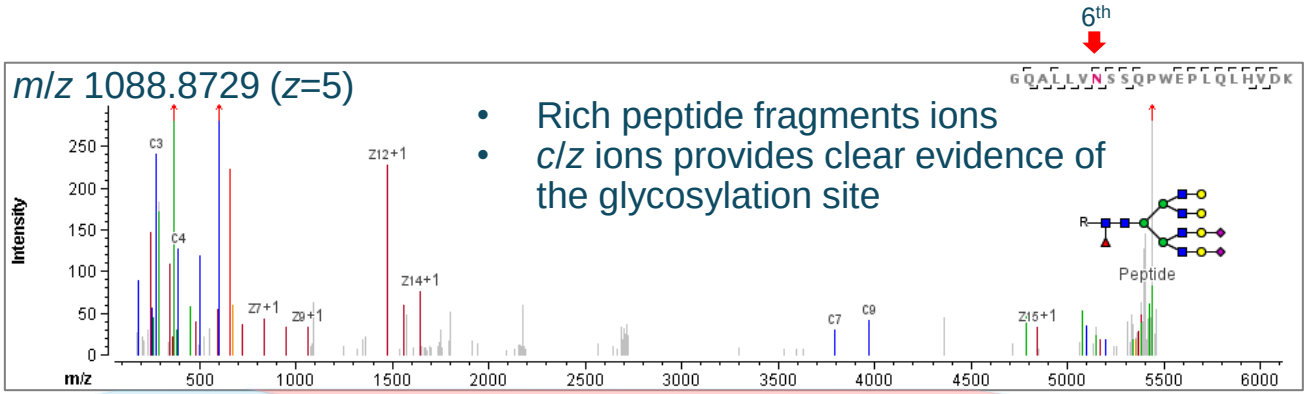




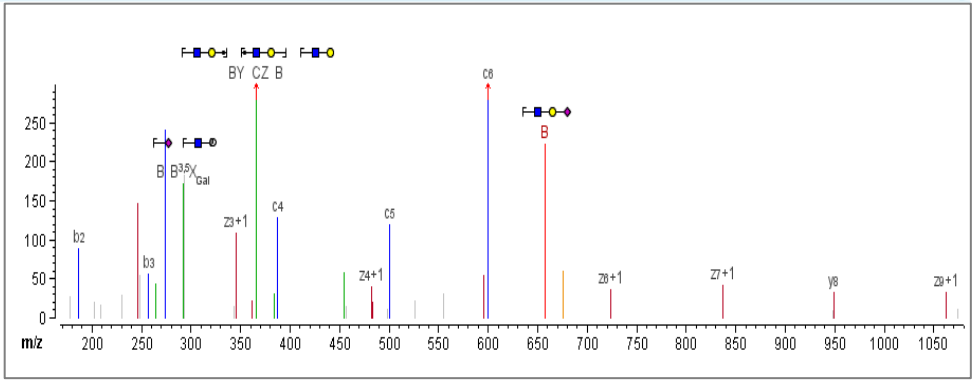
Benefits of EAD for site-specific N-glycopeptide analysis

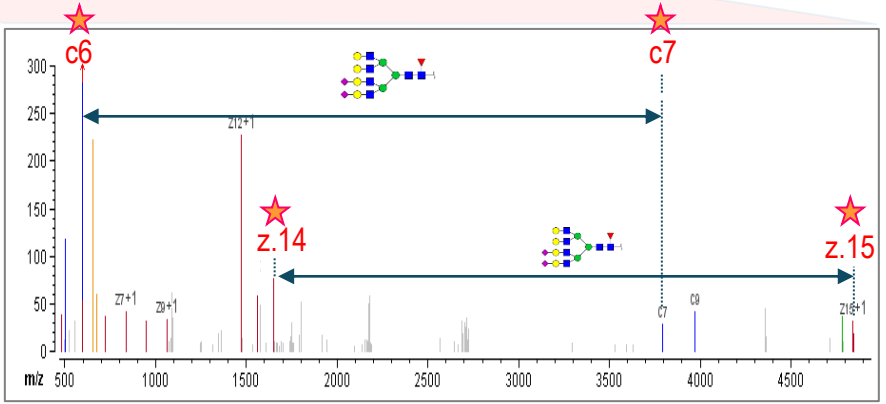


~21 kDa
erythropoietin

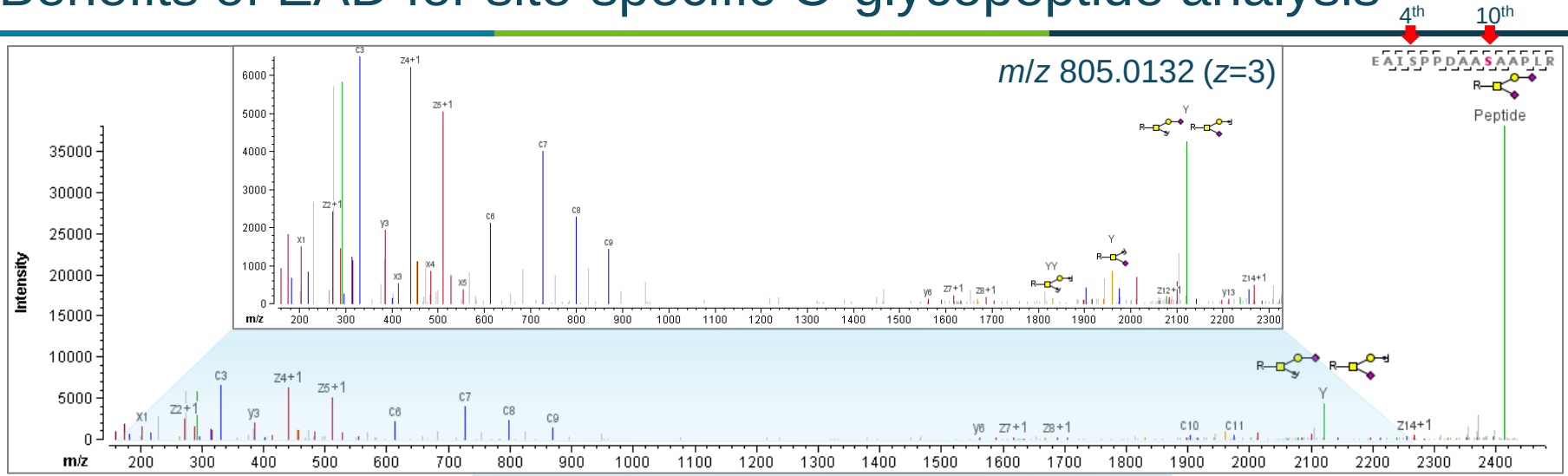


- Rich peptide fragments ions
- *c*/*z* ions provides clear evidence of the glycosylation site

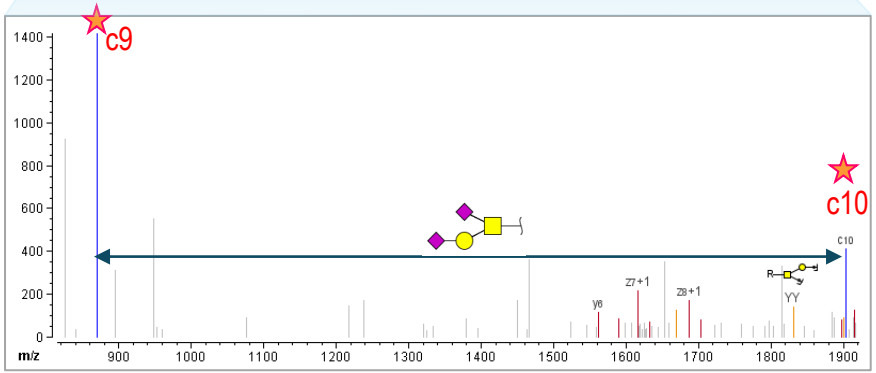




Benefits of EAD for site-specific O-glycopeptide analysis



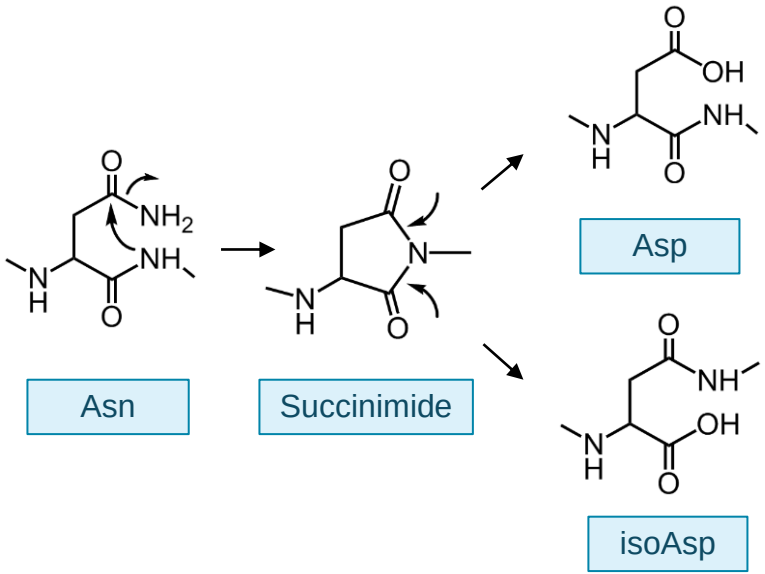
Fragment	Peptide	Modifications
c2	EA	
c3	EAI	
c6	EAISPP	
c7	EAISPPD	
c8	EAISPPDA	
c9	EAISPPDAA	
c10	EAISPPDAAS	Core1_S2 [S10]
c11	EAISPPDAASA	Core1_S2 [S10]
c13	EAISPPDAASAAP	Core1_S2 [S10]
c14	EAISPPDAASAAPL	Core1_S2 [S10]



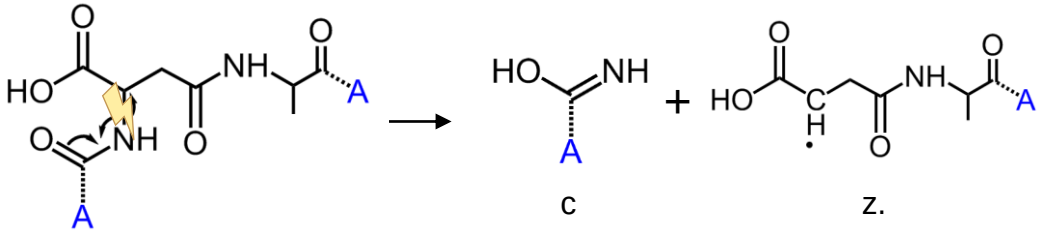
- 4th Ser and 10th Ser
- *c*9 and *c*10 $\Delta m/z$ = 1034.45
- Confident O-glycan localization at 10th Ser

EAD generates diagnostic ions for isoAsp

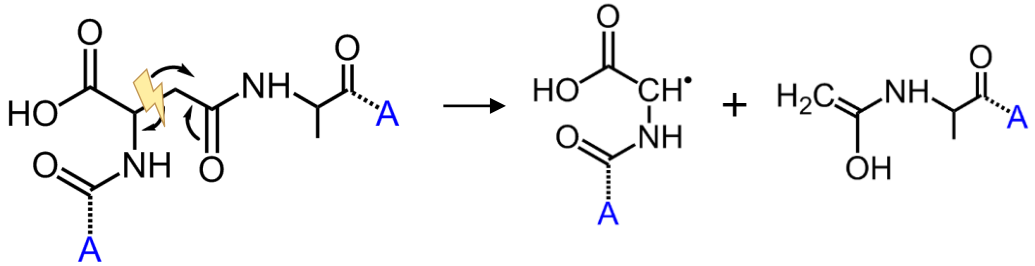
Asp deamidation process



isoAsp with EAD



isoAsp diagnostic ions generation pathway

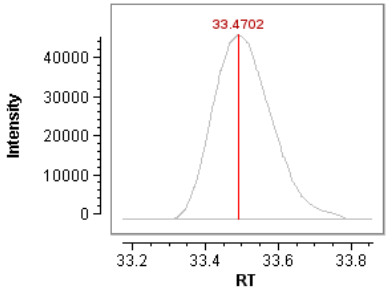


c + 57 Da

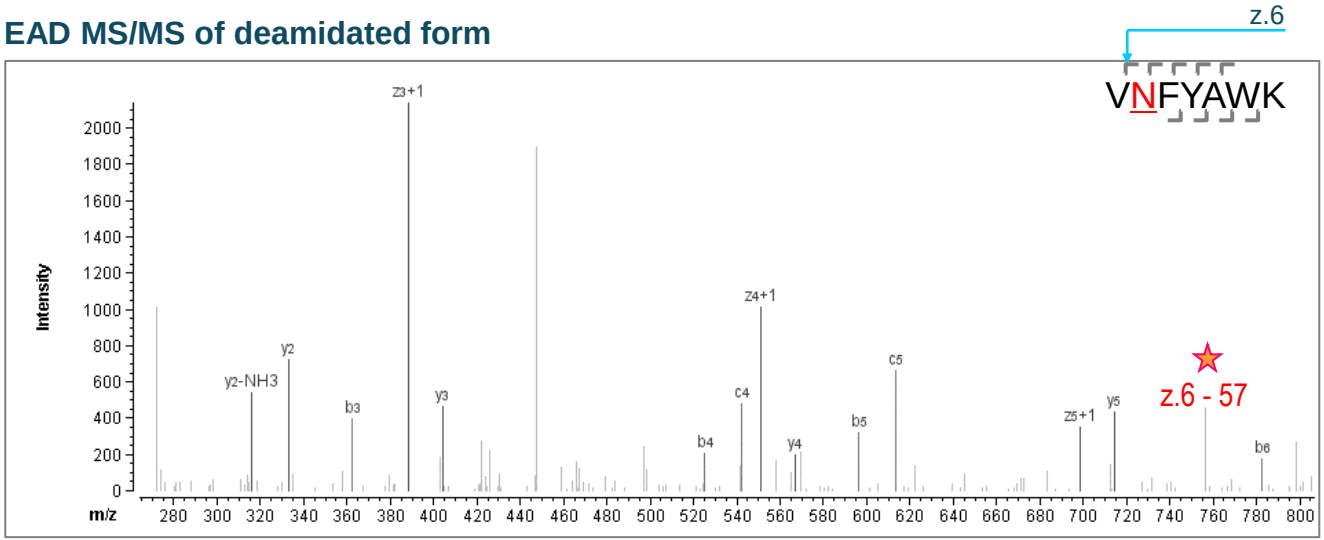
z. - 57 Da

isoAsp signature ion *z*.6-57 was observed in EAD spectra

XIC of deamidated form

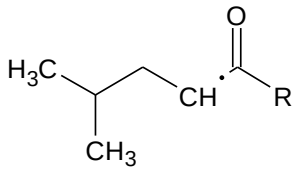


EAD MS/MS of deamidated form



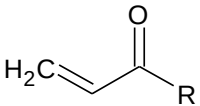
EAD generates diagnostic ions for Leu and isoLeu

Leu



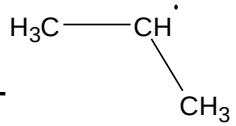
z

EAD



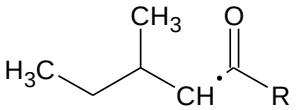
w

\+



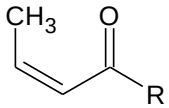
- 43 Da

isoLeu



z

EAD



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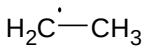
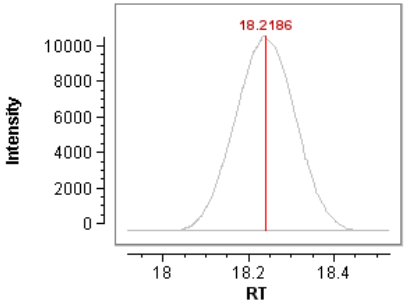


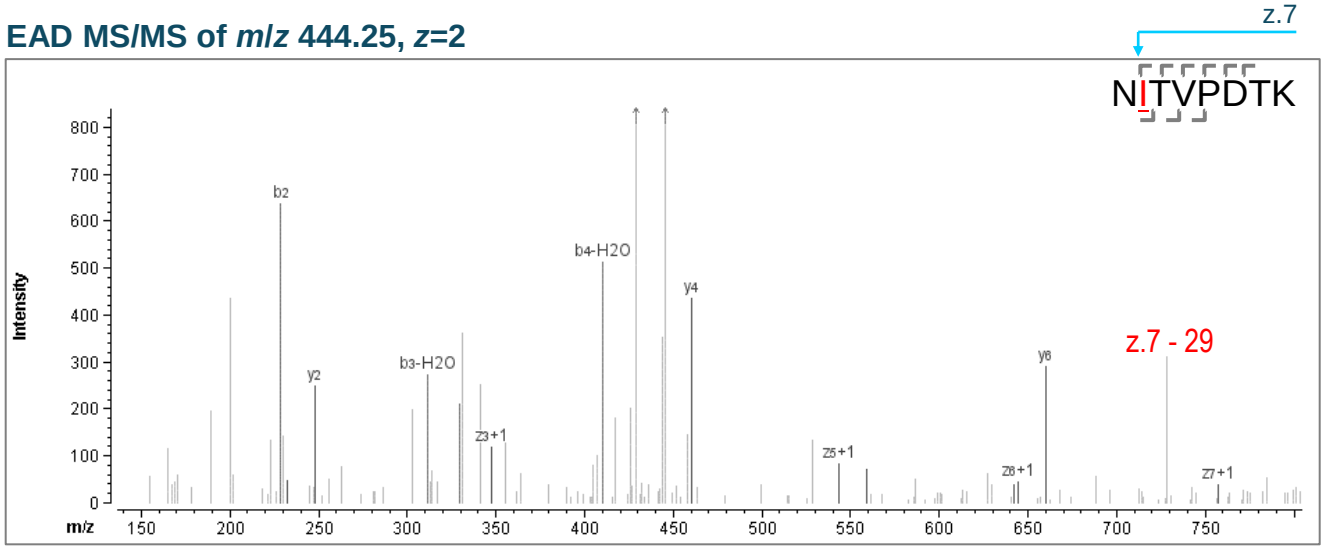
- 29 Da

isoLeu signature ion *z*.7-29 was observed in EAD spectra

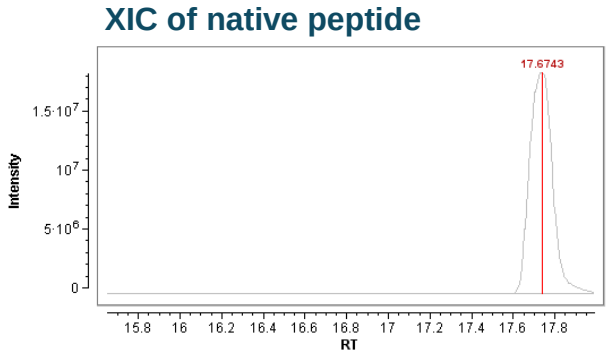
XIC of *m/z* 444.25, *z*=2

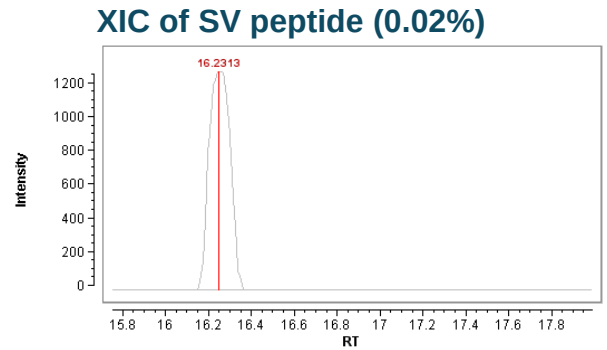


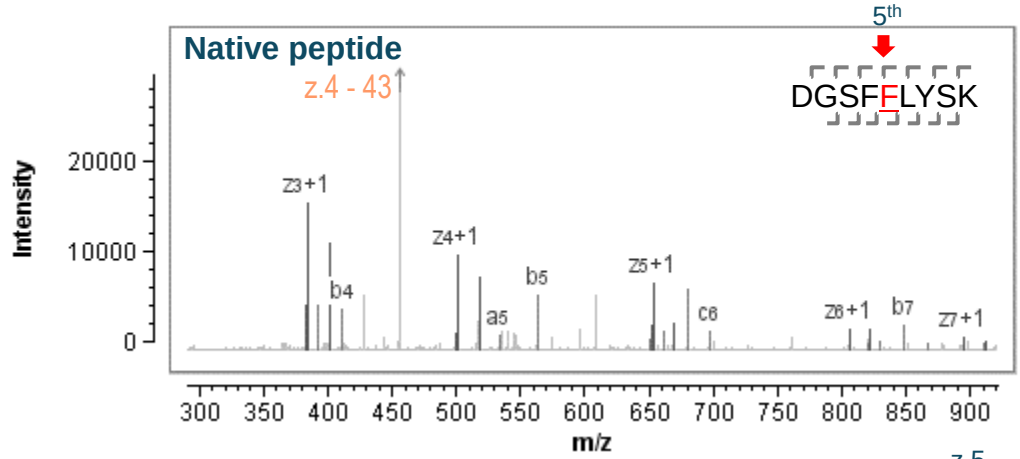
EAD MS/MS of *m/z* 444.25, *z*=2

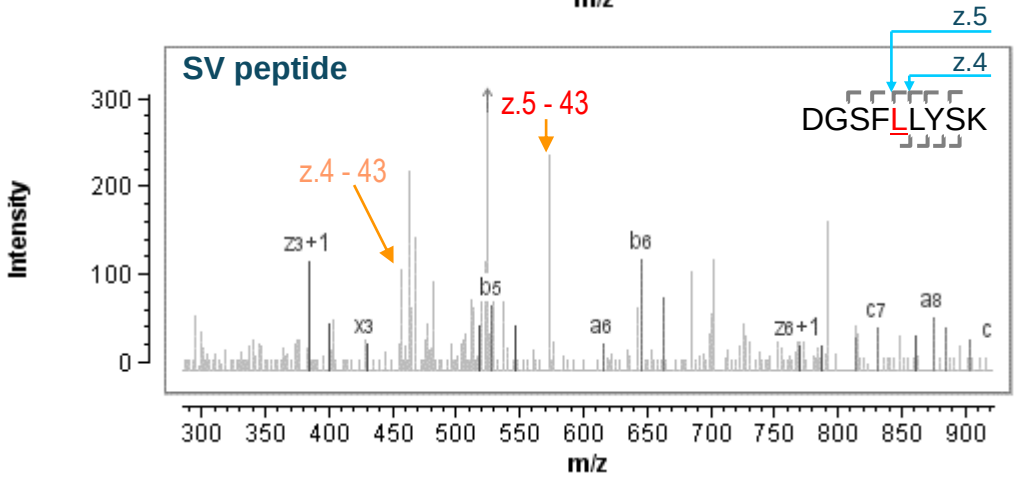


Sequence variants (SV) analysis – F to I/L









Conclusions

- ❖ EAD enables improved biotherapeutics characterization.
- Superior sequence coverage for mAb analysis.
  - EAD performed in a DDA experiment enables high-throughput and highly effective electron-based fragmentation of large number of peptides from protein digests
  - The reagent-free EAD equipped with adjustable electron energy provides highly efficient fragmentation to generate a wealth of *c*/*z*, *b*/*y*, *a*/*x* ions for exceptional sequence coverage for peptide mapping
- EAD preserves labile PTMs (i.e. glycosylation).
  - enabling unambiguous site localization for both N-glycopeptides and O-glycopeptides with multiple (potential) glycosylation sites
- EAD provides the capability for amino acid isomer differentiation
  - Can readily applied for sequence variants analysis for isomer differentiation (i.e. F->I/L)

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