MPL

MICROCHEMISTRY, PROTEOMICS, LIPIDOMICS
+ NEXT GENERATION SEQUENCING

Direct Analysis of Heterogeneous Biotherapeutics

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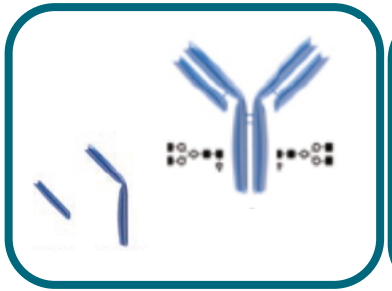
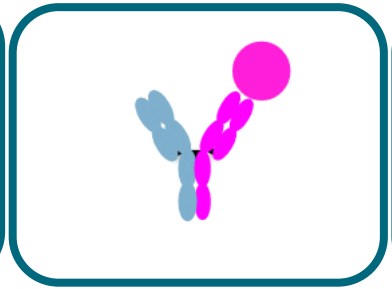
Genentech, Inc.
30 September 2022
CASSS: Thermo Fisher Scientific Lunch Seminar

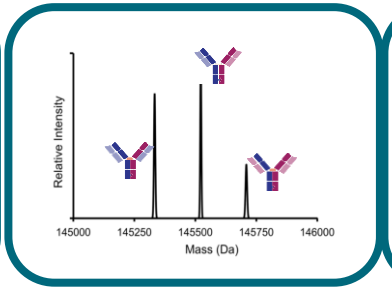
Intact Mass Measurement Approaches for Biotherapeutics



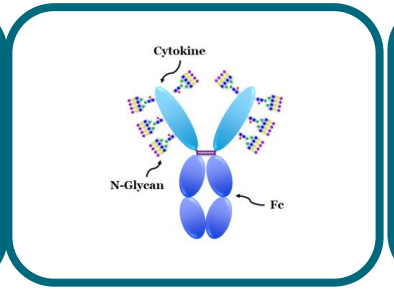
Denaturing MS



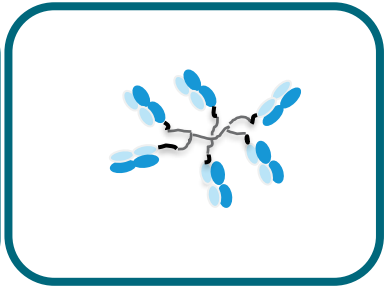
Native MS



Hyphenated nMS



Charge reduction (PTCR)



Charge Detection MS (DMT)

Molecular Complexity

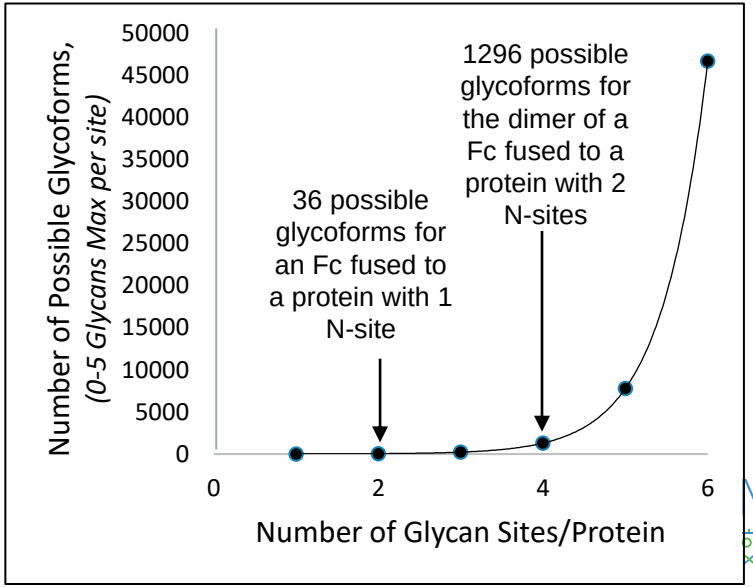
Ease of analysis

Biological representation

Glycoproteins as biotherapeutics

- Glycosylation is present on **>50%** of all human proteins
  - Role of glycosylation in the cell: Fc effector function, protein structure, more…
- **>40% approved biotherapeutics are glycosylated**
  - IgG-based and Fc-fusion biologics
- Glycosylation can affect pharmacological properties of biotherapeutics:
  potency, stability, bioavailability, solubility, and immunogenicity.

- A single glycosylation site on a protein can produce **vast molecular heterogeneity** that precludes direct analysis by mass spectrometry (MS), the predominant analytical tool for glycoprotein characterization.



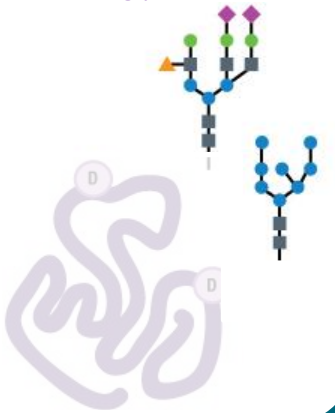
Strategies to investigate glycoproteins by MS

Ease of analysis

Biological representation

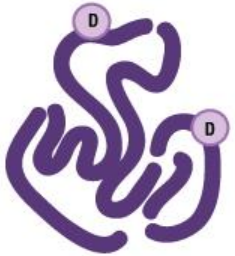
Glycan Comp

Released glycans
Total glycoforms



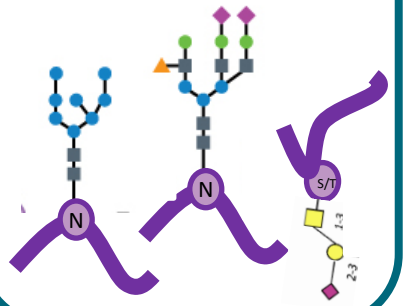
Site Occupancy

Percentage PTM for each
N-motif position



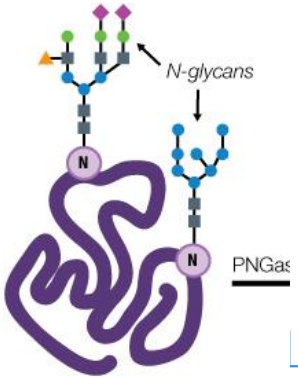
Site Mapping

Which glycoforms are
present on what sites

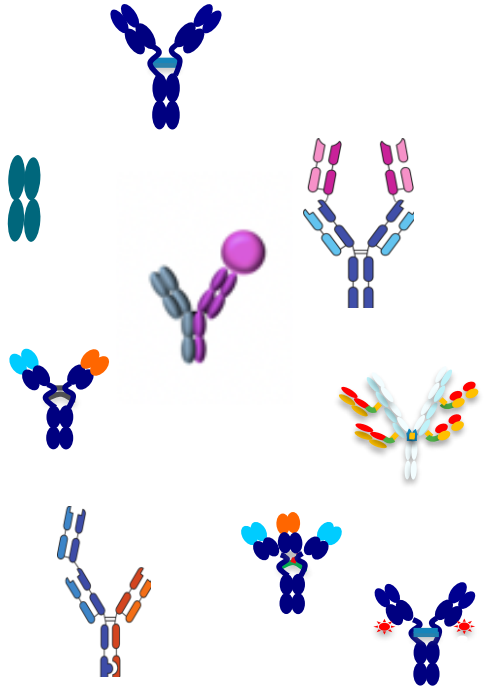


Intact MS

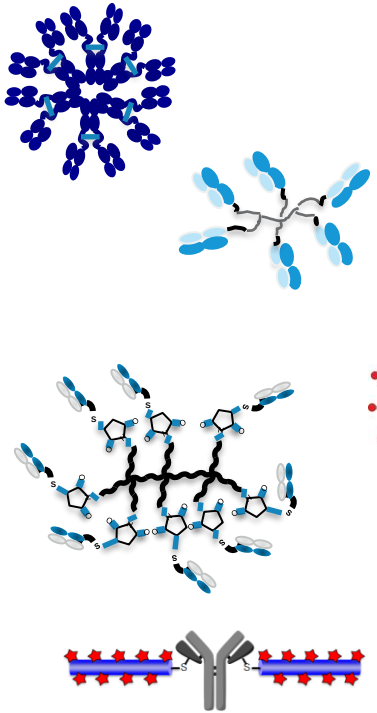
Nature of PTMs on molecule and
complexity



Increasingly complex modalities require adaptive analytical strategies

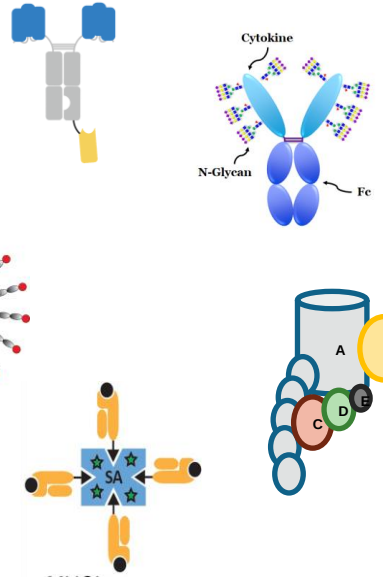


Antibody/Fab Derivatives



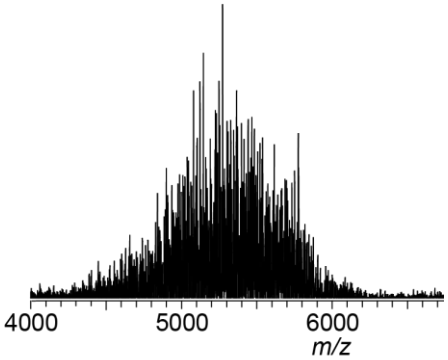
Conjugated Formats

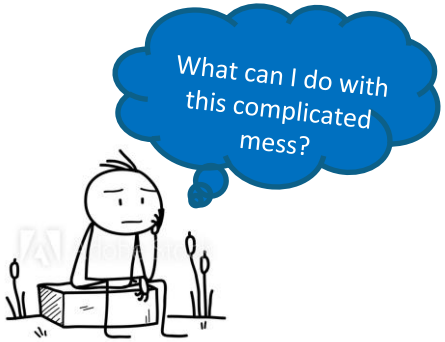
Fc Fusion proteins

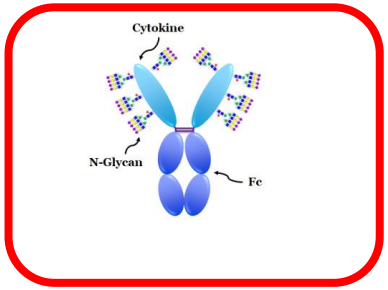


Non-covalent complexes/membrane proteins

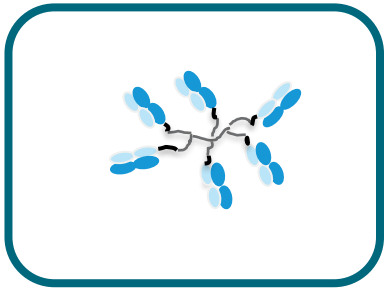
Intact Mass Measurement Approaches for Biotherapeutics





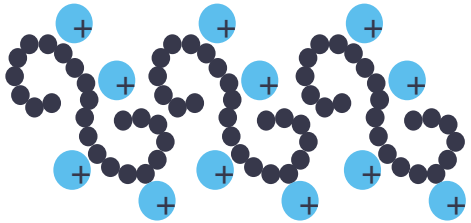


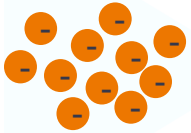
Charge reduction (PTCR)

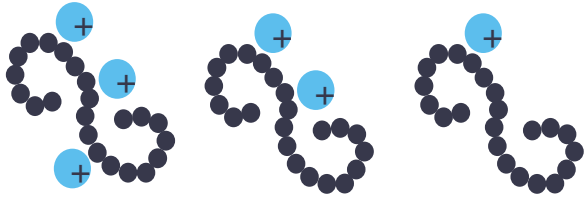


Single Charge Detection (DMT)

Proton Transfer Charge Reduction (PTCR)







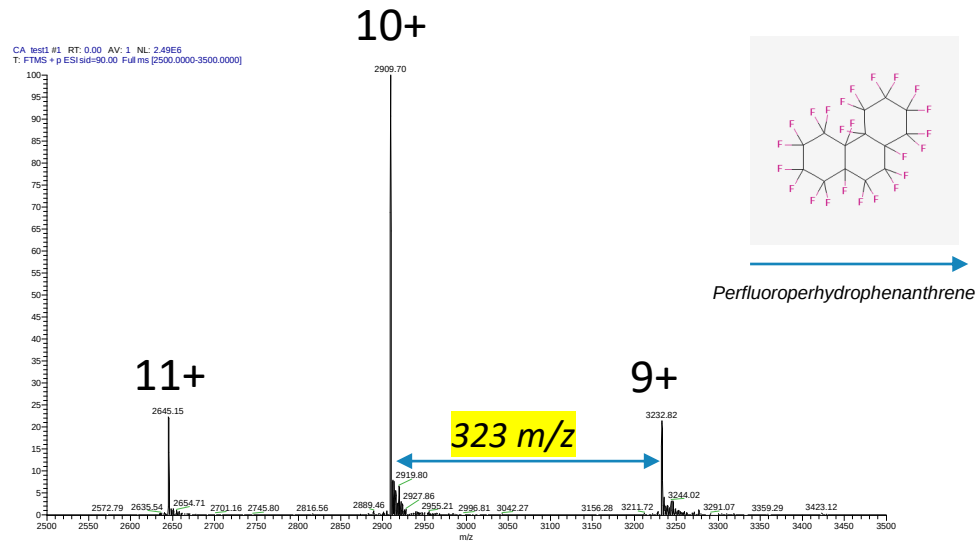
$C_{14}F_{24}$

m/z = 623.9 Th

- Charge (z) is determined based on the spacing between the adjacent charge states.
- PTR may ***create*** charge state distributions

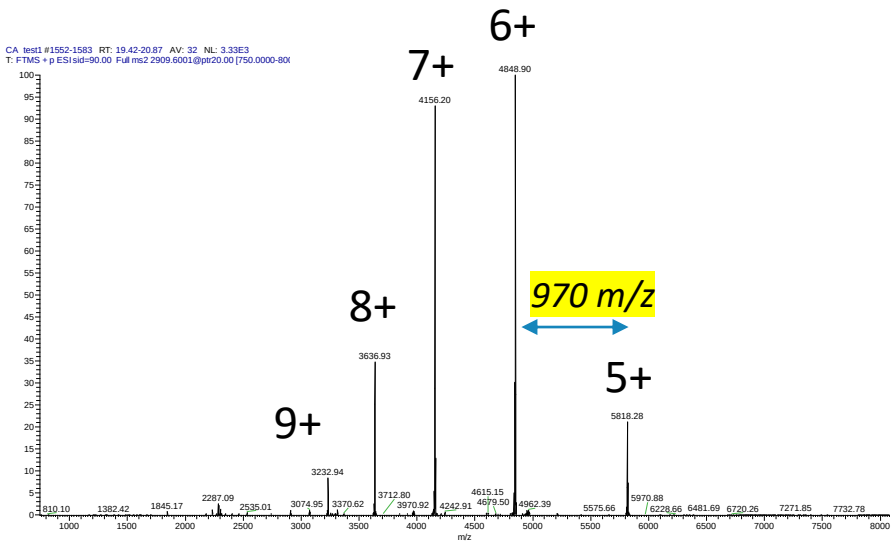
Proton Transfer Charge Reduction (PTCR) on resolved charge states

Before PTCR

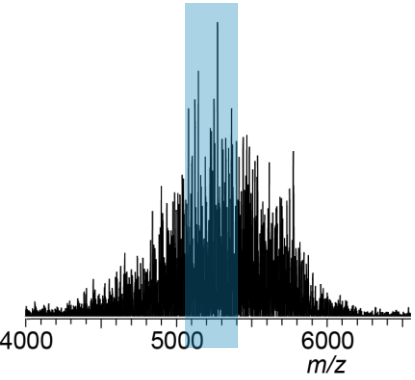


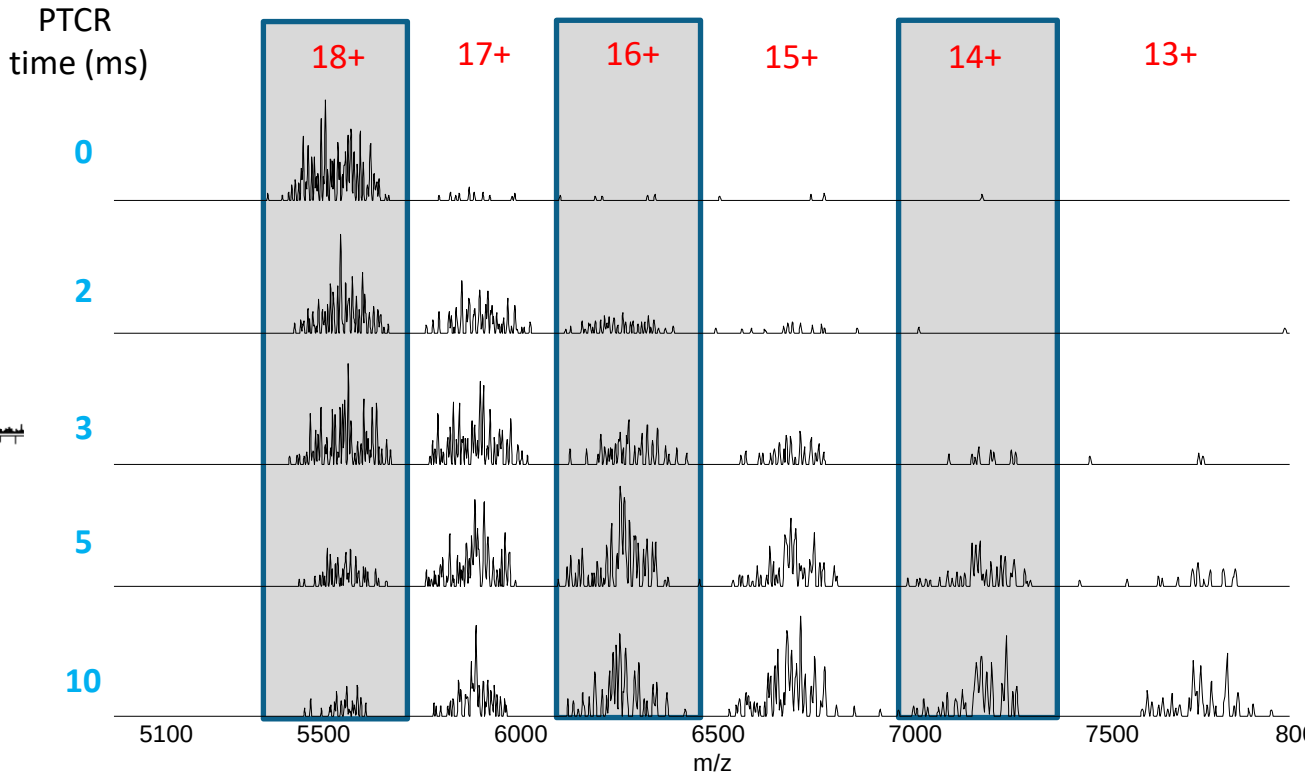
Perfluoroperhydrophenanthrene

After PTCR



PTCR on unresolved charge states

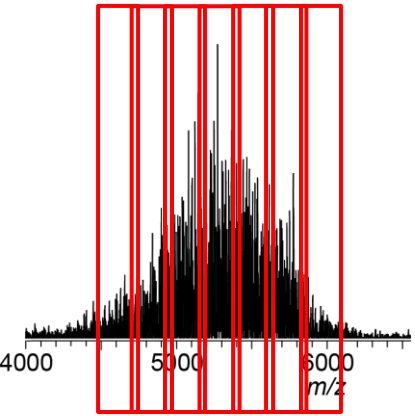


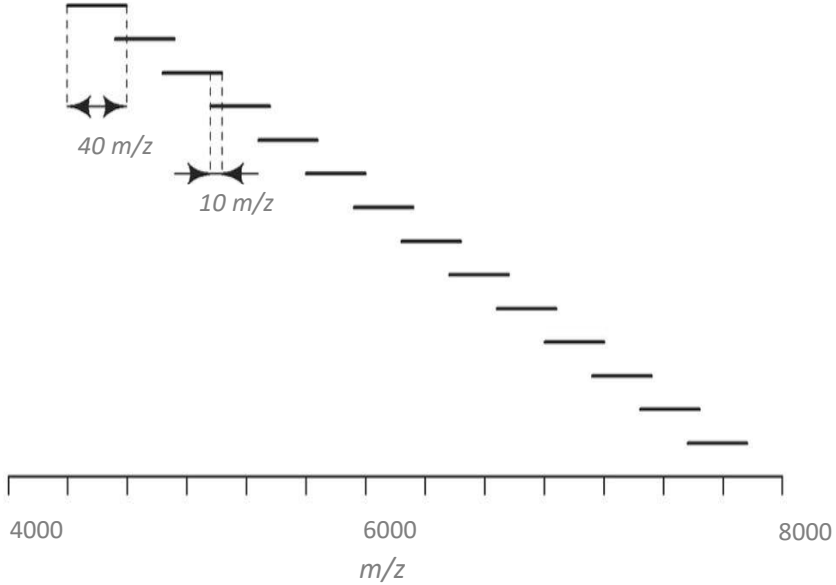


*ASMS 2019: Orbitrap Eclipse with extended mass range (8k) and PTCR

UNIGLAMS: UNiversal Intact GLycoprotein Analysis by Mass Spectrometry

Overlapping windows of PTCR spectra are acquired and stitched together for deconvolution





Example: m/z 4000-7000, 30Th step, 60Th isolation, 10Th overlap, 100uscans, PTCR4

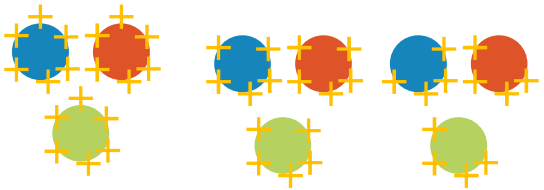
UNIGLAMS Workflow

SOURCE

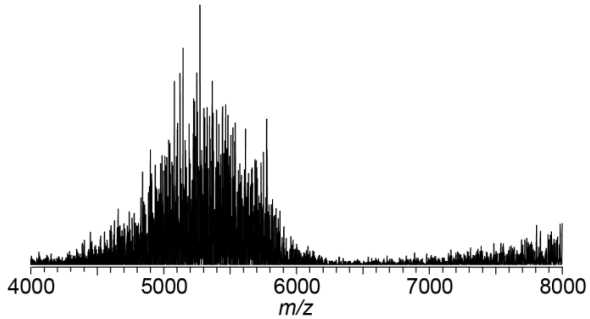
ISOLATION

PCR

ORBITRAP MS



Spectrum



Luis Schachner

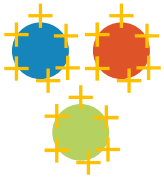
UNIGLAMS Workflow

SOURCE

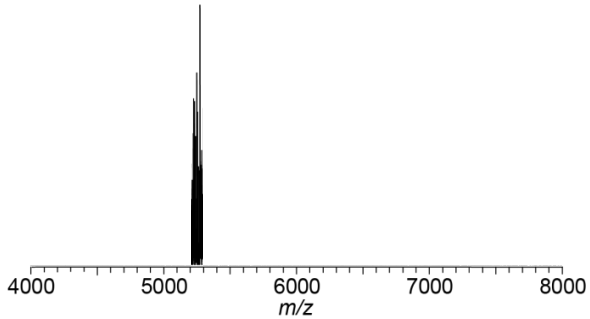
ISOLATION

PTCR

ORBITRAP MS



Spectrum



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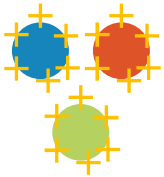
UNIGLAMS Workflow

SOURCE

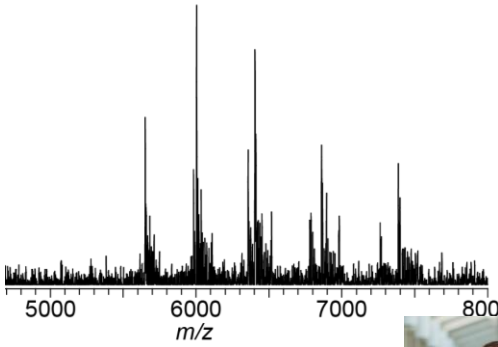
ISOLATION

PTCR

ORBITRAP MS

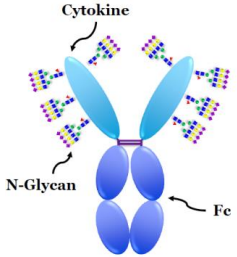


Spectrum

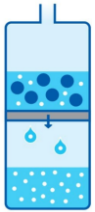


Luis Schächner

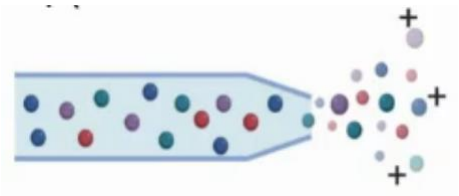
UNIGLAMS workflow



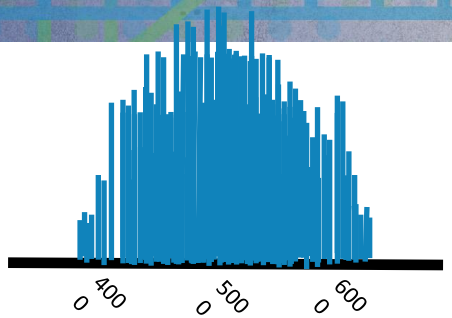
Glycoprotein



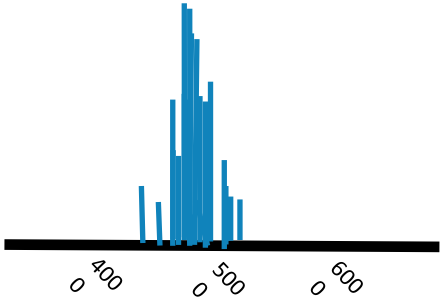
Desalt & buffer exchange to 100mM Ammonium acetate



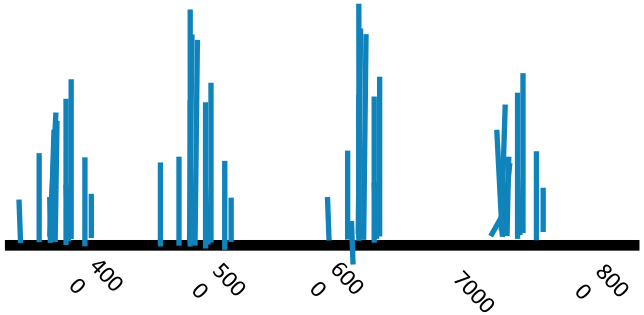
Nano capillary infusion to mass spectrometer



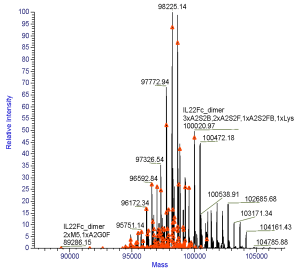
MS^1 to determine m/z envelope range for acquisition and optimum SID



Narrow MS^2 isolation at apex to confirm ion signal



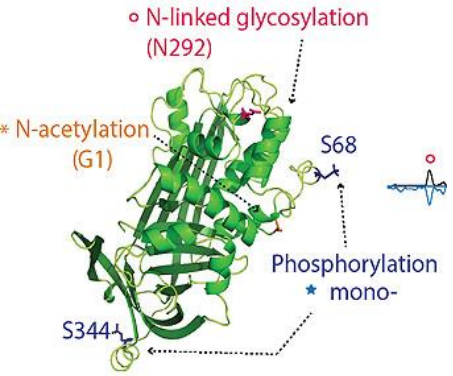
Increase PTCR in MS^2 isolation range to establish appropriate duration

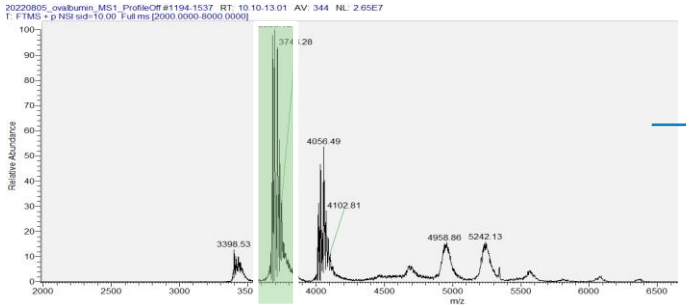


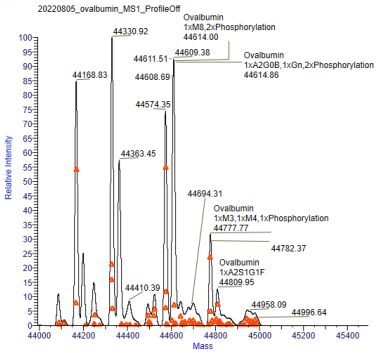
Analyze & annotate

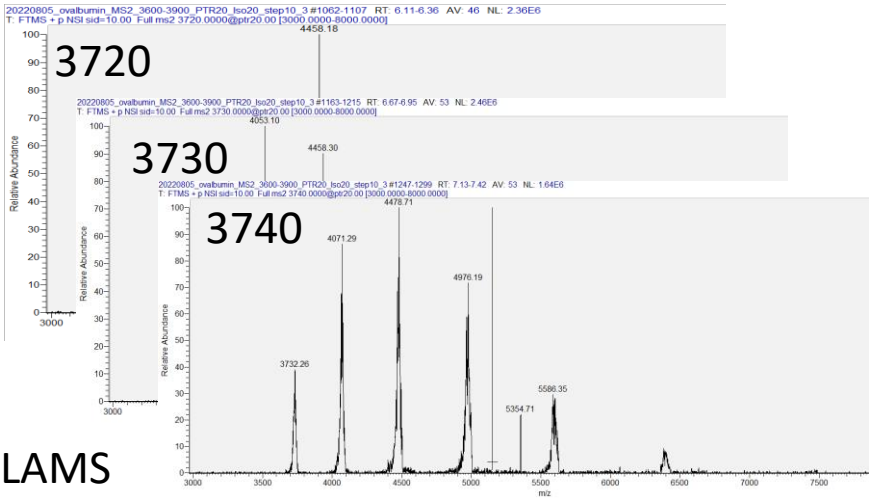
Ovalbumin contains a single N-linked glycan yet has multiple proteoforms

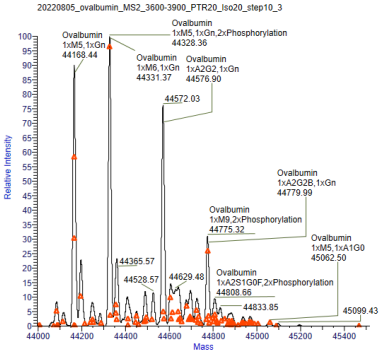
MS1





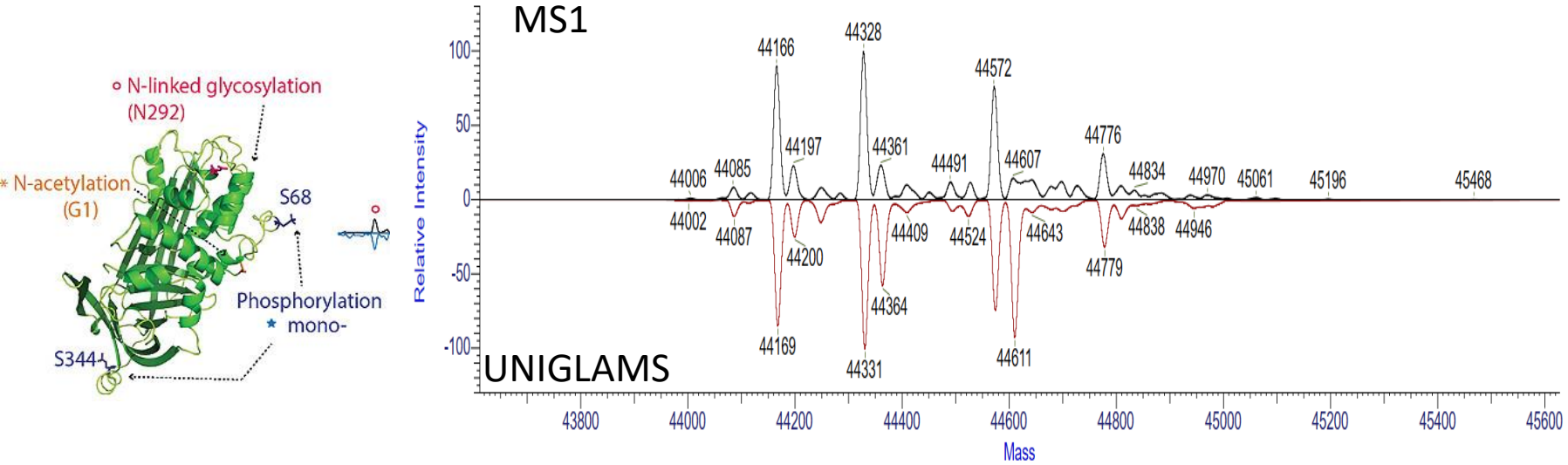




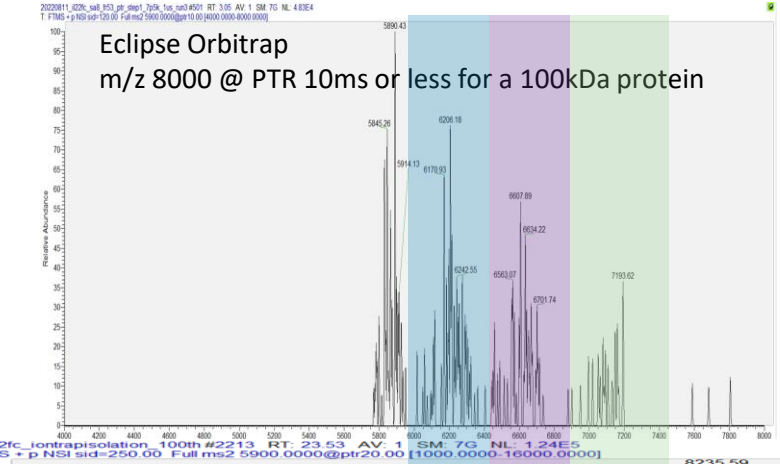


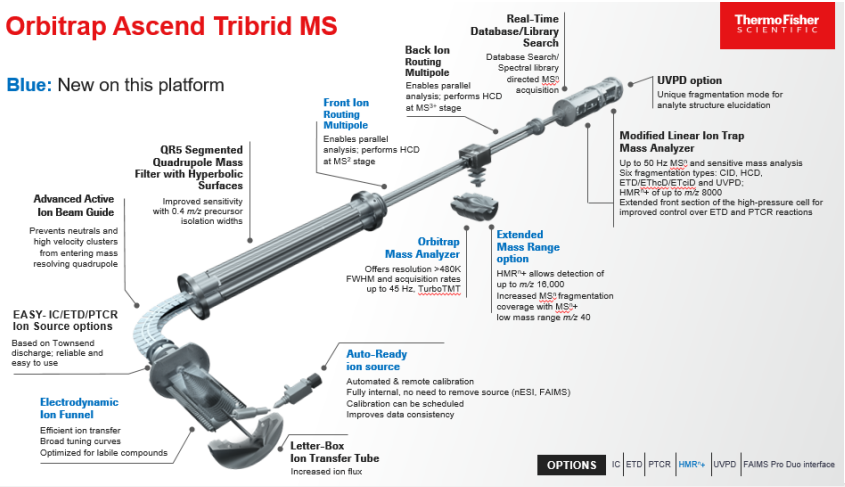
UNIGLAMS

Ovalbumin contains a single N-linked glycan yet has multiple proteoforms

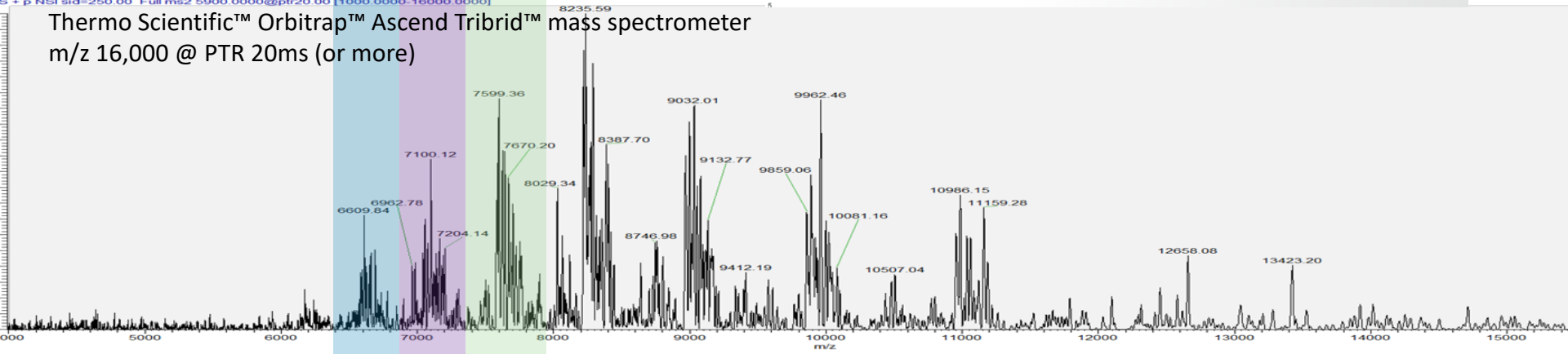


Utility of PTCR is limited by Eclipse m/z range

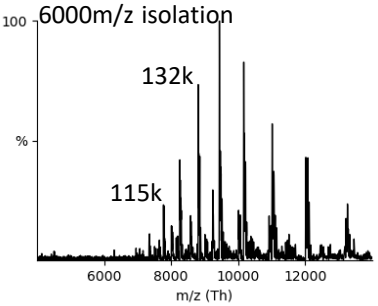


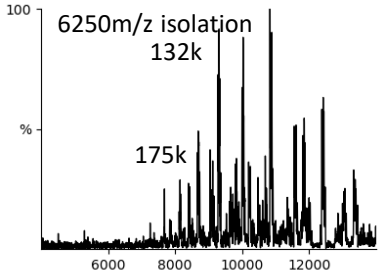


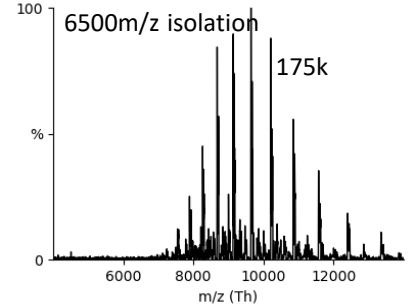
Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer
m/z 16,000 @ PTR 20ms (or more)



Charge reduced segmented isolation resolved proteoforms for identification

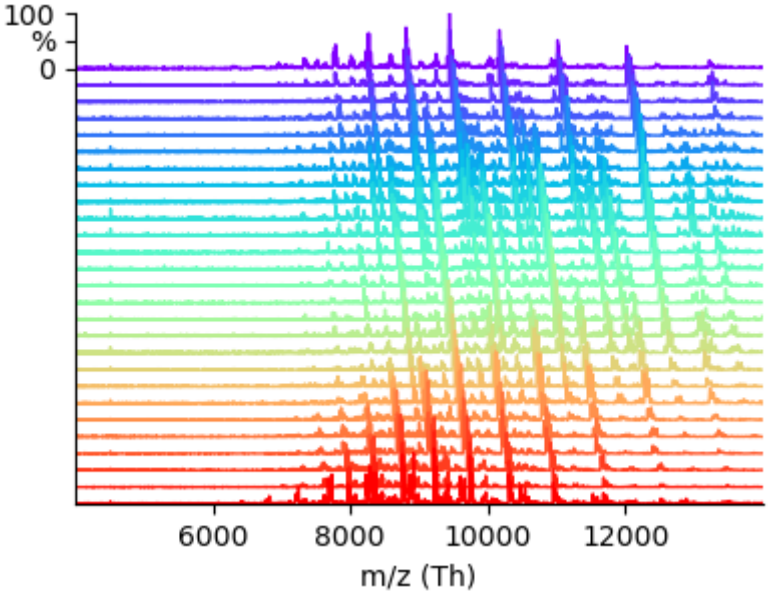


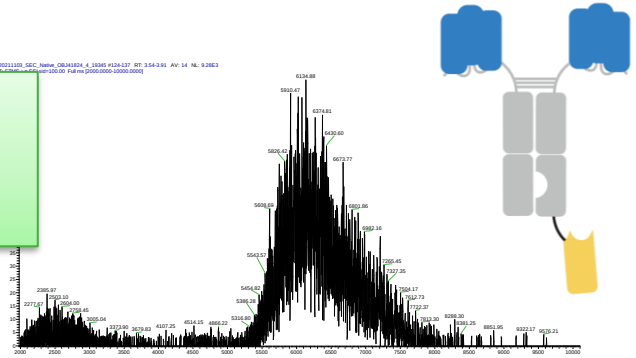


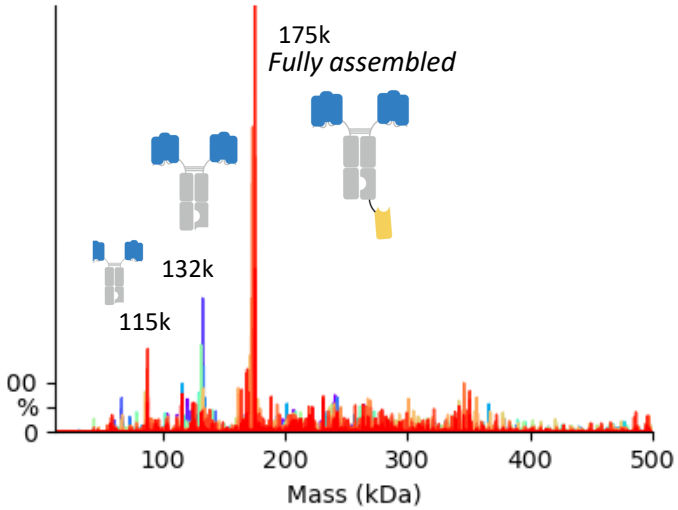


20k ligand scTrimer fused to muIgG2A

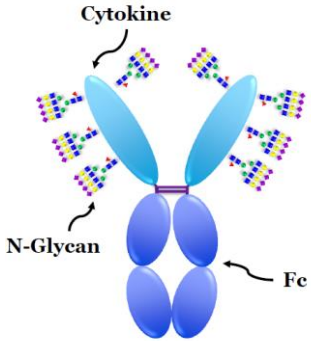
Each ligand subunit has 1 N-linked glycan (on NVT)
GSS linkers of varying lengths (why there are multiple samples)
Unresovable by native MS or SEC-MS, even after N&O degly
Expected MW = ~160 kDa





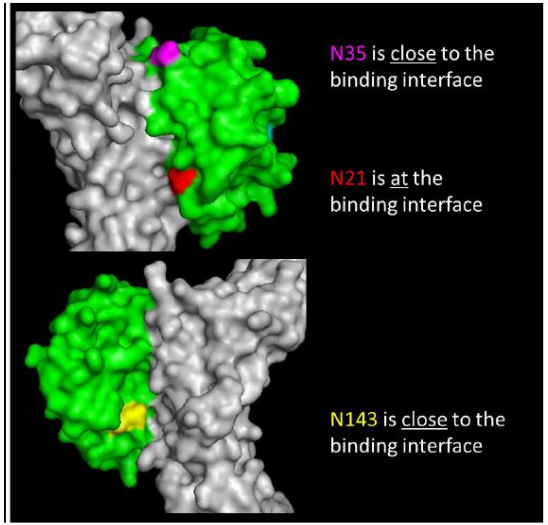


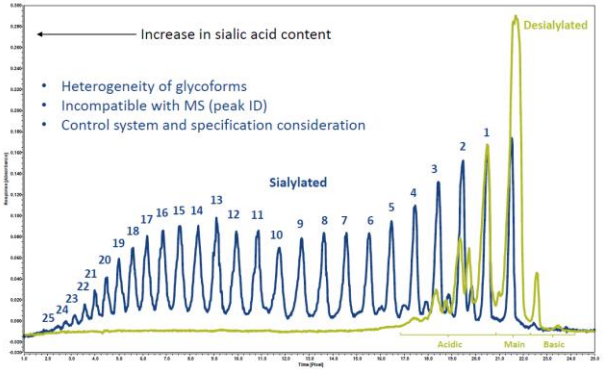
A Phase IIb heterogeneous glycosylated cytokine Fusion protein

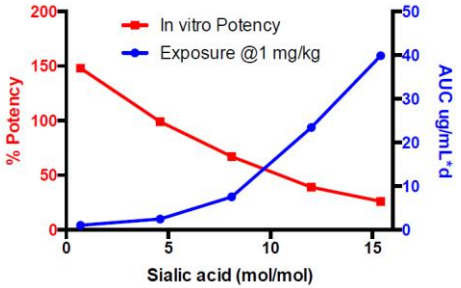


- CHO derived cytokine Fc-fusion protein
- Two IL-22 cytokines fused with the Fc portion of an IgG to prolong half-life
- Aglycosylated Fc to minimize effector function (N to G)
- Heavily glycosylated cytokines
  - ✓ 8 N-glycosylation sites
  - ✓ > 60 N-Glycans identified

4 N-glycosylation sites on each cytokine (N21, N35, N64, N143)



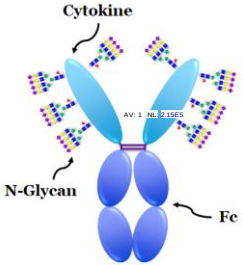




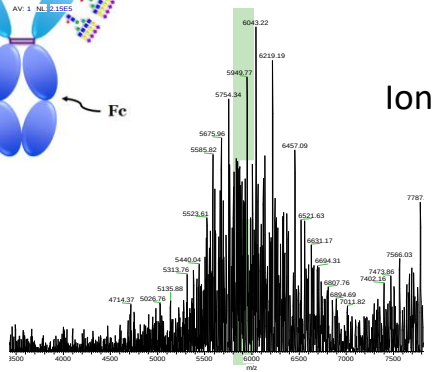
- With increase in sialic acid levels, exposure increases, *in vitro* potency decreases
- Drug exposure is the primary driver of *in vivo* PD response

Michelle Irwin Beardsley & IL22Fc dev team

Stepping through glycoforms with UNIGLAMS



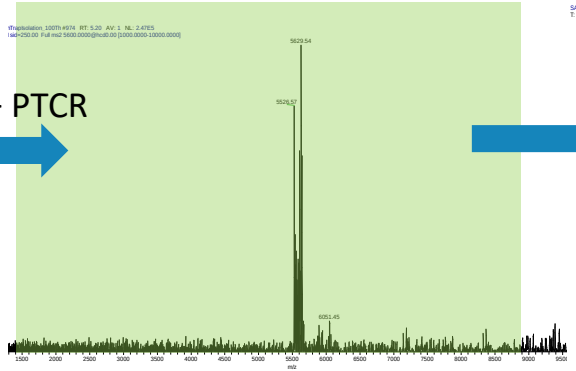
MS1



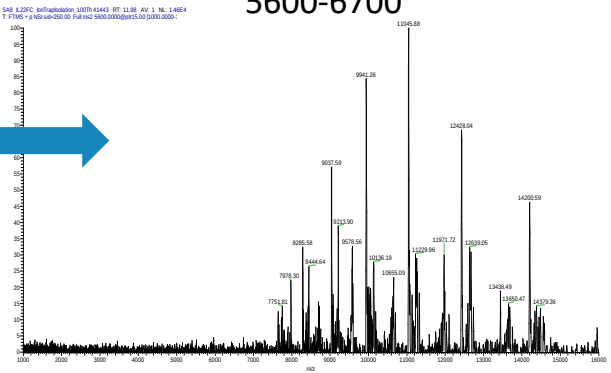
Ion isolation + PTCR

MS^2

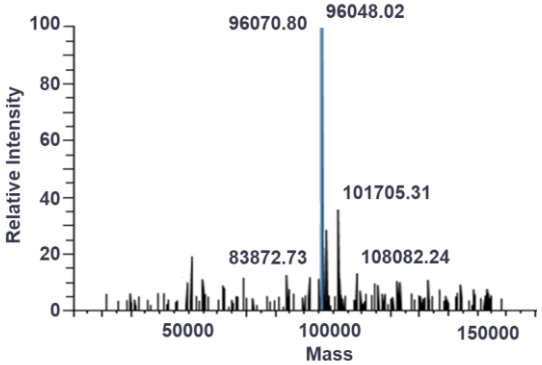
Isolation 100 Th window



PTR (@15ms) 50 Th segments 5600-6700

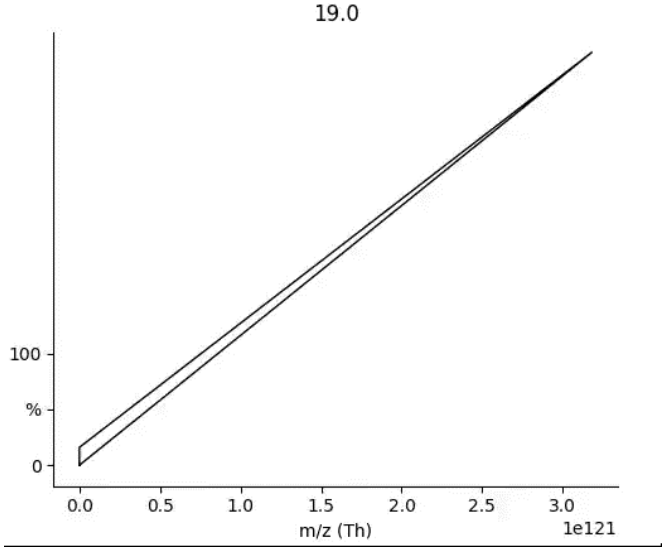


Deconvolution

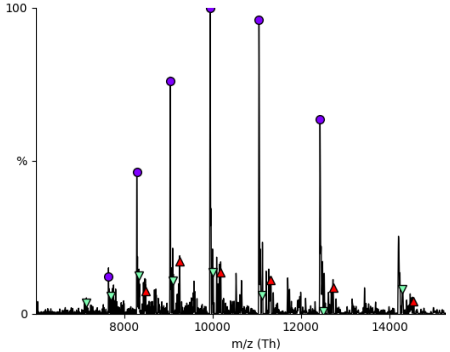


MPL

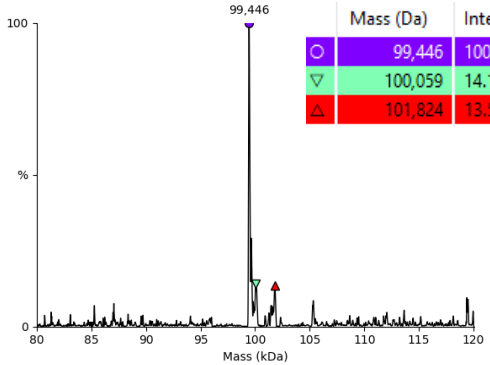
Stepping through Spectra on Orbitrap Ascend



m/z 5950 segment
1uScan (IIT 16ms)

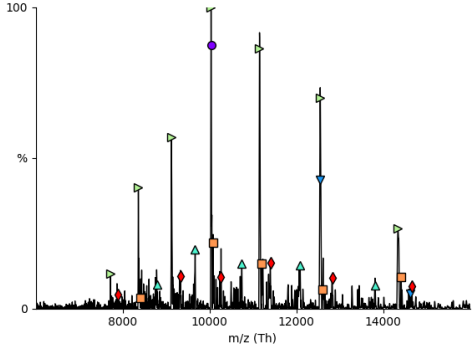


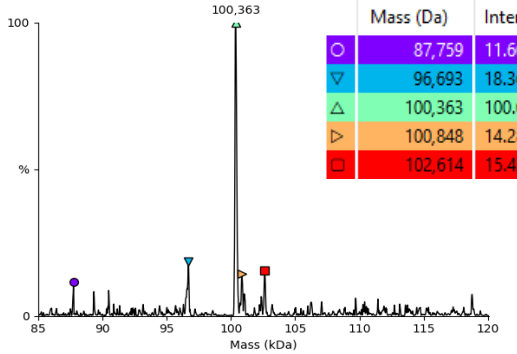
Deconvolved Single Scans



	Mass (Da)	Intensity	DScore
○	99,446	100.00	60.35
▽	100,059	14.11	29.33
△	101,824	13.52	38.19

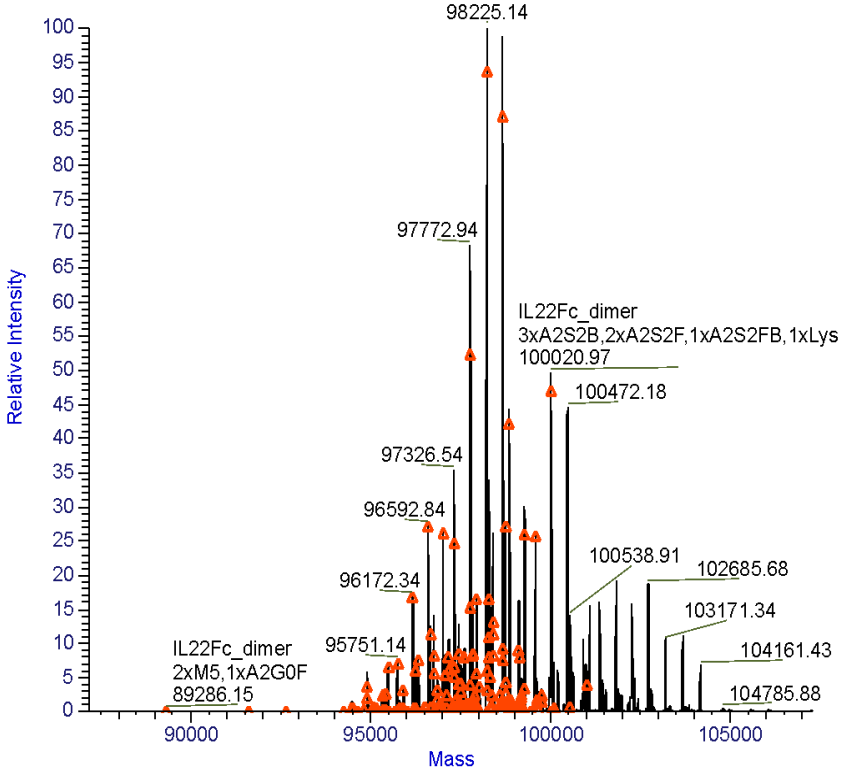
m/z 6000 segment
1uScan (IIT 16ms)





	Mass (Da)	Intensity	DScore
○	87,759	11.60	44.1
▽	96,693	18.36	54.7
△	100,363	100.00	63.36
▷	100,848	14.28	38.1
□	102,614	15.42	41.29

>160 glycoforms identified by BPF from UNIGLAMS on cytokine fusion protein

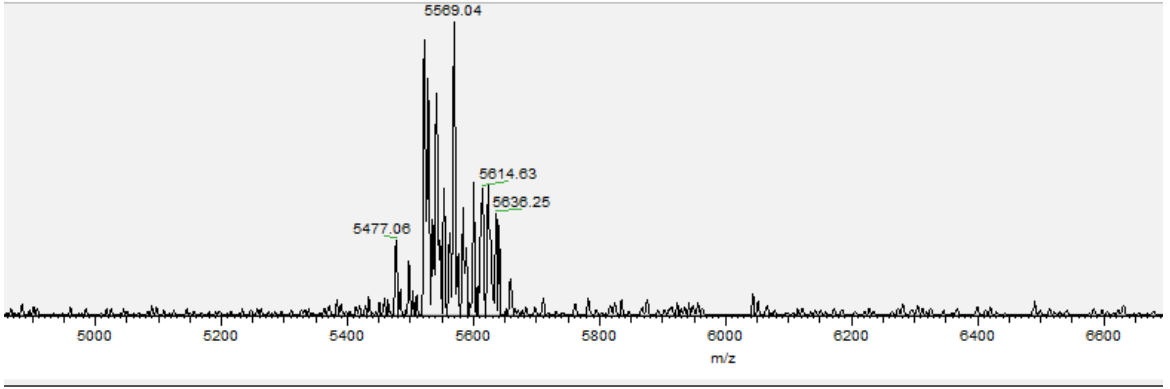


Modification	Average Mass	Theoretical Mass (Da)	Matched Mass Error (ppm)	Sum Intensity	Relative Abundance	Fractional Abundance	Score
1xA2G1F,1xA2G2F,1xA2S1G0FB,1xA2S1G1B,1xA2S2B,1xA2S2FB,2xLys	98225.10	98224.70	4.0	6.19E+07	100.00	7.64	51.85
1xA2G2F,1xA2S1G1F,1xA2S1G1B,1xA2S2B,1xA2S2F,1xA2S2FB	98673.08	98671.96	11.3	5.75E+07	92.92	7.10	46.14
1xM5,1xA2G2F,1xA2S1G1F,1xA2S2B,1xA2S2F,1xA2S2FB	97771.94	97771.12	8.3	3.45E+07	55.76	4.26	44.91
3xA2S2B,2xA2S2F,1xA2S2FB,1xLys	100020.97	100022.21	12.4	3.11E+07	50.24	3.84	43.57
1xA2S1G1F,3xA2S1G0FB,1xA2S1G1FB,1xA2S2FB,2xLys	98849.75	98849.30	4.6	2.79E+07	45.12	3.45	43.29
1xM6,1xA2S1G1B,1xA2S1G1FB,1xA2S2B,1xA2S2F,1xA2S2FB,2xLys	98740.78	98741.11	3.3	1.80E+07	29.11	2.22	36.55
1xM5,1xA2G1F,1xA2G2F,1xA2S1G1F,1xM9,1xA2S2B,2xLys	96592.80	96593.15	3.5	1.80E+07	29.05	2.22	36.72
2xA2G1F,1xA2G2F,1xA2S1G0F,1xA2S1G1F,1xA2S2B,2xLys	97016.36	97016.61	2.6	1.73E+07	27.90	2.13	37.89
1xA2S1G1F,1xA2S1G1B,1xA2S1G1FB,1xA2S2B,1xA2S2F,1xA2S2FB,1xLys	99294.49	99294.58	0.9	1.72E+07	27.84	2.13	36.96
3xA2S1G1FB,2xA2S2B,1xA2S2FB	99572.42	99572.79	3.7	1.71E+07	27.60	2.11	42.85
1xA2G0F,1xA2G1F,1xA2G2F,1xA2S1G1F,1xA2S2F,1xA2S2FB,1xLys	97325.50	97325.84	3.5	1.65E+07	26.57	2.03	43.37
	100472.18	0.00	0.0	1.61E+07	25.96	1.98	40.41
	100468.33	0.00	0.0	1.33E+07	21.47	1.64	45.15
	101822.03	0.00	0.0	1.25E+07	20.24	1.55	40.34
1xM5,1xA2G0F,1xA2G1F,1xA2G2F,1xA2S1G1F,1xA2S2B,2xLys	96172.29	96172.83	5.6	1.11E+07	17.91	1.37	36.31
1xA2G0F,1xA2G1F,1xA2S1G1F,1xA2S2,1xA2S2B,1xA2S2FB,2xLys	97947.79	97947.43	3.7	1.10E+07	17.70	1.35	39.10
1xA2S1G0F,1xA2S1G1F,1xM8,1xA2S2,1xA2S2B,1xA2S2F,2xLys	98294.62	98293.67	9.6	1.09E+07	17.62	1.35	28.80
1xM6,1xA2S1G0F,1xA2S1G1F,1xM9,1xA2S2F,1xA2S2FB,2xLys	97775.89	97775.20	7.1	1.01E+07	16.38	1.25	39.86
	101370.28	0.00	0.0	9.75E+06	15.75	1.20	41.29
1xA2S1G1F,1xM9,1xA1S1F,1xA2S2,1xA2S2F,1xA2S2FB,2xLys	98397.63	98398.76	11.5	8.71E+06	14.06	1.07	42.35
	101088.63	0.00	0.0	7.67E+06	12.39	0.95	30.17
1xM5,1xA2G2F,1xA2S1G0F,1xA2S1G1F,1xM8,1xA2S2F,2xLys	96665.13	96665.21	0.8	7.66E+06	12.37	0.95	33.53
1xA2S1G0F,1xA2S1G1F,1xA2S1G1,1xA2S2,1xA2S2F,1xA2S2FB	98395.55	98394.69	8.8	7.62E+06	12.30	0.94	39.28
1xA2G1F,1xA2G2F,1xA2S1G1F,1xA2S2B,1xA2S2F,1xA2S2FB,1xLys	98288.89	98289.68	8.1	7.25E+06	11.71	0.89	34.91

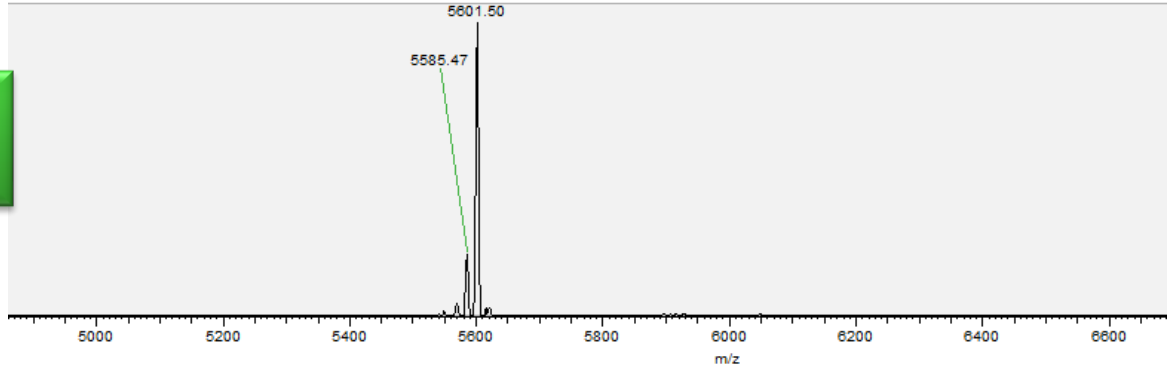
Challenges with Ion Isolation

m/z 5600

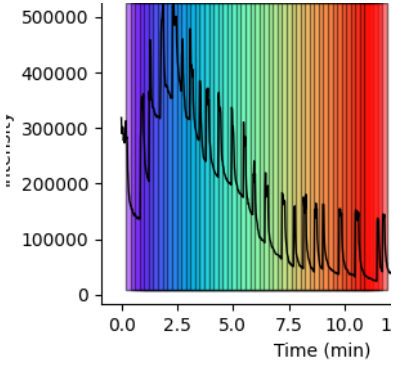
Ion Trap Isolation
100 Th

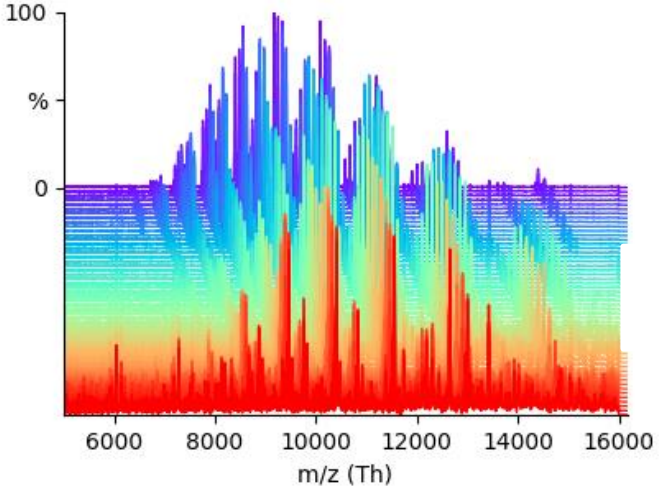


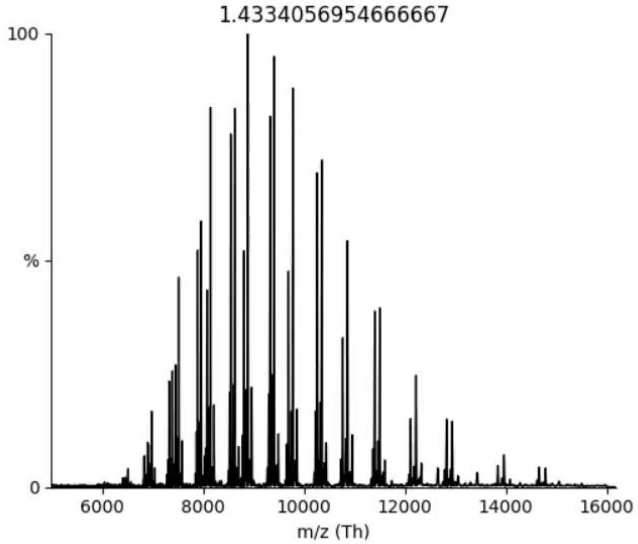
Quadrupole Isolation
~20 Th



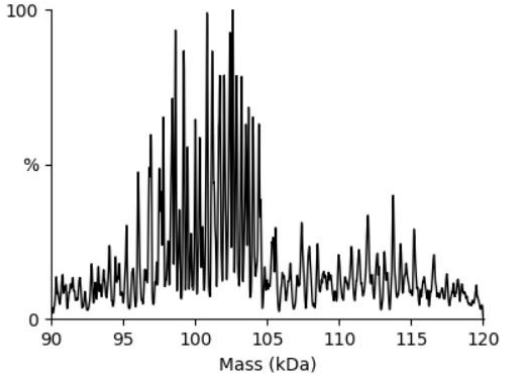
UNIGLAMS with Quadrupole isolation



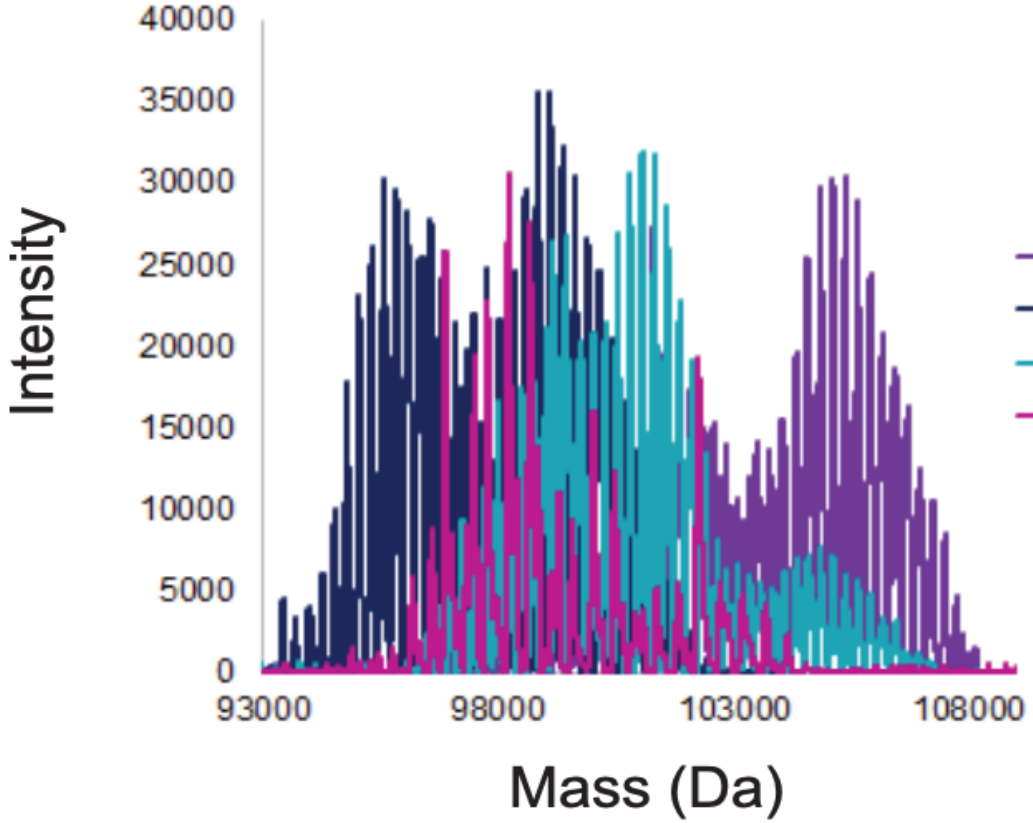


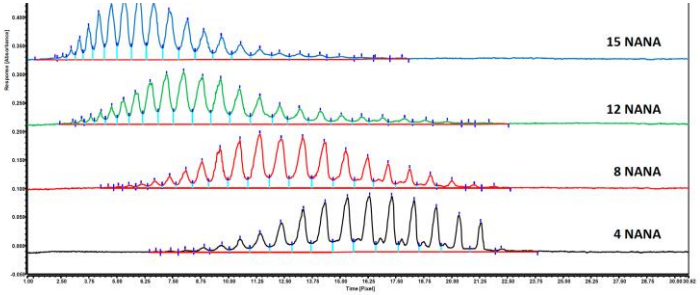


Peak Mass (Da)	Match	Error	Name
95211	95210.3476000...	0.65239999999...	2[HexNAc(5)Hex(3)Fuc(1)] 2[...
96019	96018.7549	0.24510000000...	2[HexNAc(5)Hex(4)Fuc(1)Ne...
96913	96912.9417000...	0.05829999998...	2[HexNAc(5)Hex(4)NeuAc(1)...
97496	97495.2786000...	0.72139999998...	1[HexNAc(5)Hex(3)Fuc(1)] 1[...
97771	97771.3742	-0.3742000000...	1[HexNAc(5)Hex(3)Fuc(1)] 2[...
98400	98400.0667000...	-0.0667000000...	1[HexNAc(5)Hex(3)Fuc(1)] 1[...
98617	98616.7398000...	0.26019999998...	1[G1F] 2[HexNAc(6)Hex(3)] 2...
98885	98884.8445	0.15549999999...	1[G1F] 2[HexNAc(6)Hex(3)Fu...
99197	99196.3891	0.61089999999...	3[G1F] 4[HexNAc(6)Hex(3)Fu...

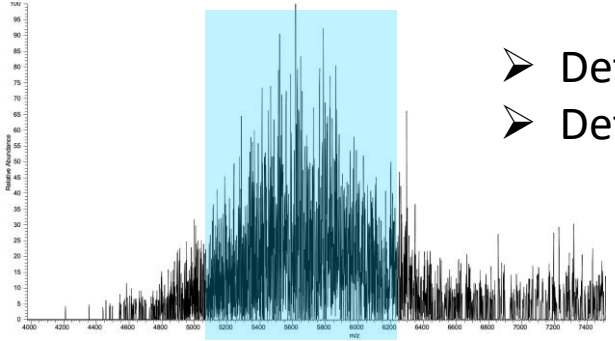


Separation of four Sialic acid fractions with UNIGLAMS

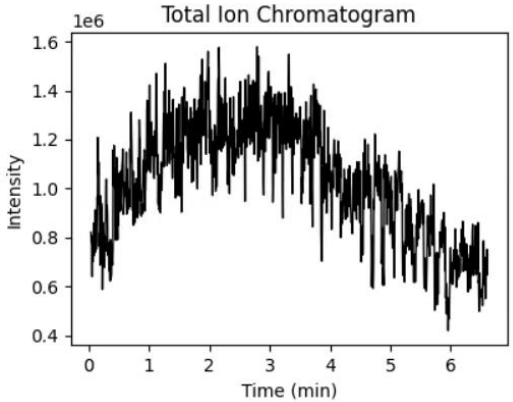


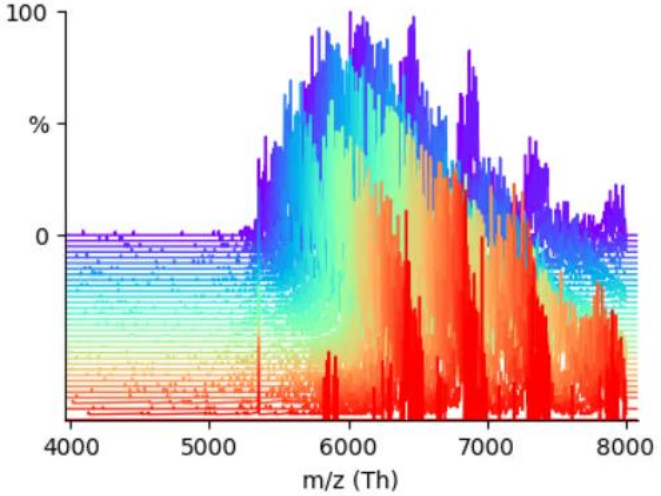


Automating UNIGLAMS



- Determine m/z range by MS1
- Determine optimal PTCR parameters

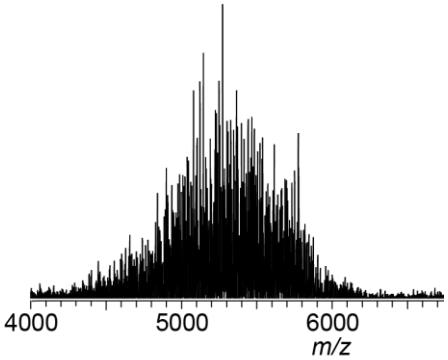


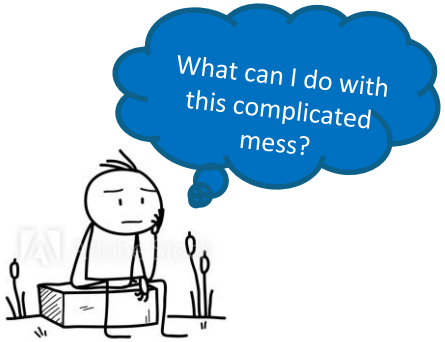


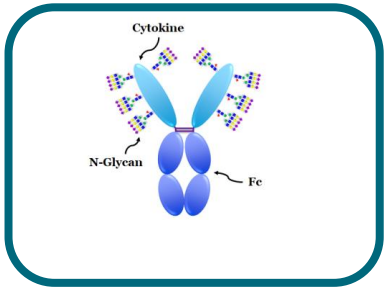
- Method Wizard

- Step by 1 Th, can do 1 or more uScans
- 6 min run time for 1uS @ res 15000

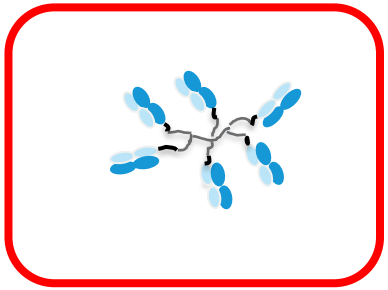
Intact Mass Measurement Approaches for Biotherapeutics





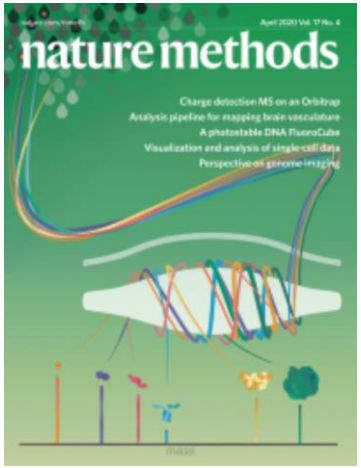


Charge reduction (PTCR)



Direct Mass Technology (DMT)

Direct Mass Technology (DMT)



a.k.a. CD-MS, single particle MS, individual ion MS

Journal of proteome research

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Letter

Individual Ion Mass Spectrometry Enhances the Sensitivity and Sequence Coverage of Top-Down Mass Spectrometry

Jared O. Kafader, Kenneth R. Durbin, Rafael D. Melani, Benjamin J. Des Soye, Luis F. Schachner, Michael W. Senko, Philip D. Compton, and Neil L. Kelleher*

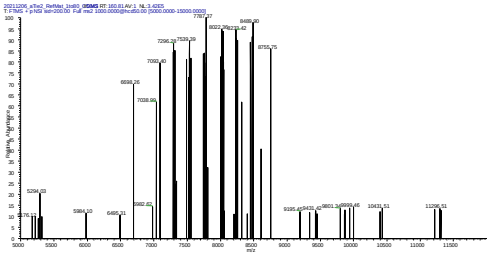
nature methods

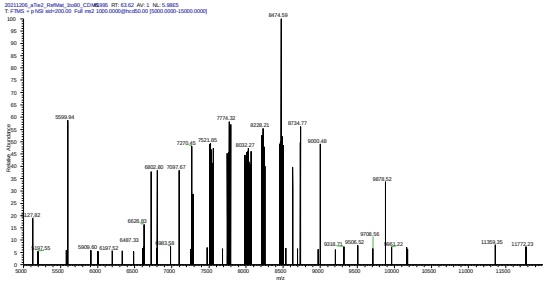
BRIEF COMMUNICATION

https://doi.org/10.1038/s41592-020-0770-7

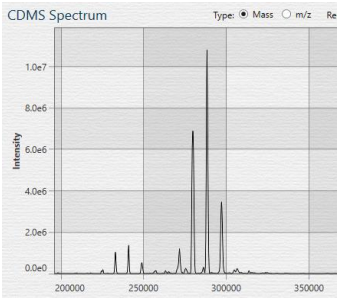
Resolving heterogeneous macromolecular assemblies by Orbitrap-based single-particle charge detection mass spectrometry

Tobias P. Wörner[1,2], Joost Snijder[1,2], Antonette Bennett[3], Mavis Agbandje-McKenna[3], Alexander A. Makarov[1,4] and Albert J. R. Heck[1,2]

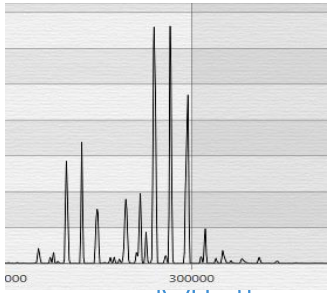




GMP Lot

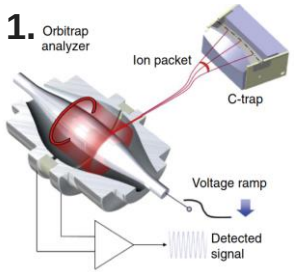


TOX Lot



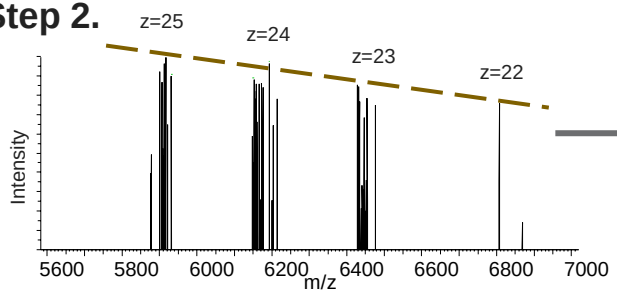
DMT workflow

Step 1.



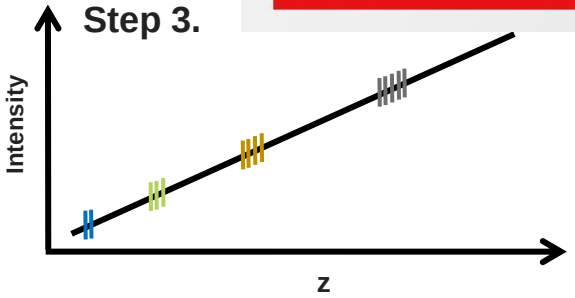
Enable Direct Mass technology mode

Step 2.



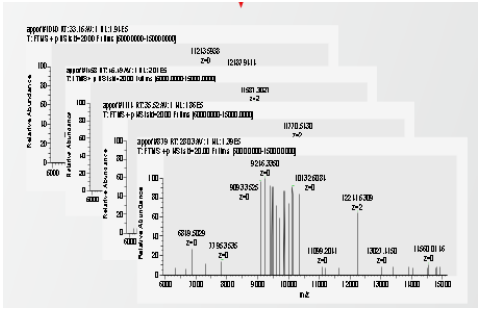
Single ion injection at each m/z

Step 3.



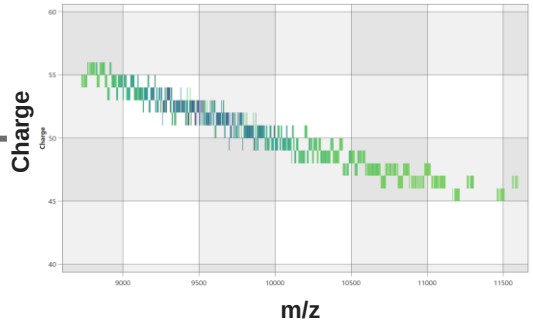
Establish calibration curve

Step 4.



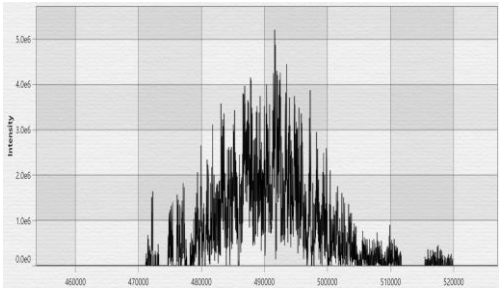
Collect scans of individual ions

Step 5.



Obtain charge state and m/z

Step 6.

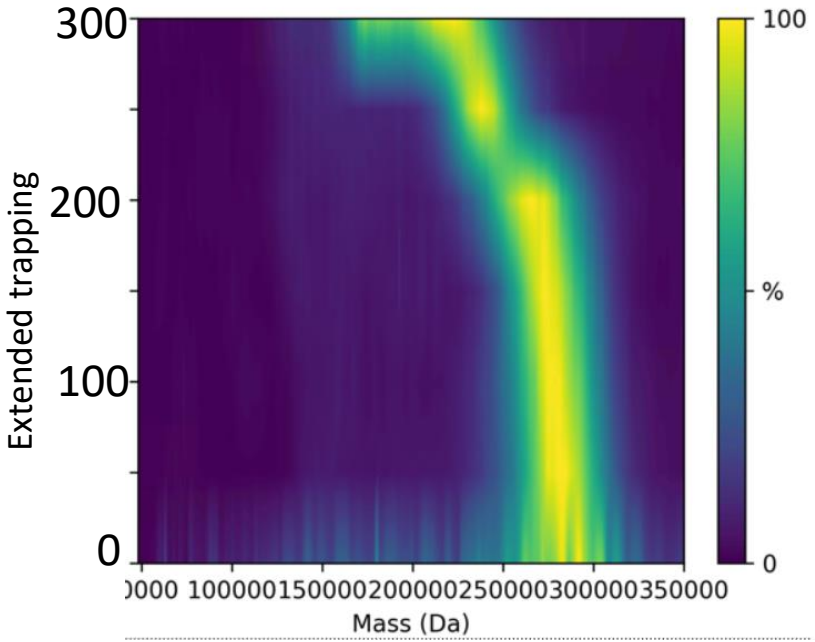


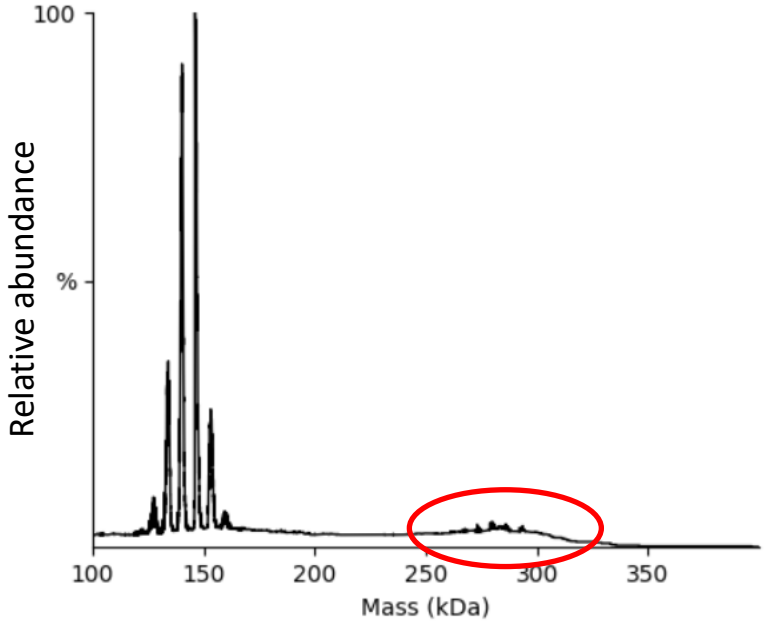
m/z × z → m for each ion

MW of heterogenous molecule

Nature Methods, Vol 17, 395–398, 2020

Some Applications of DMT



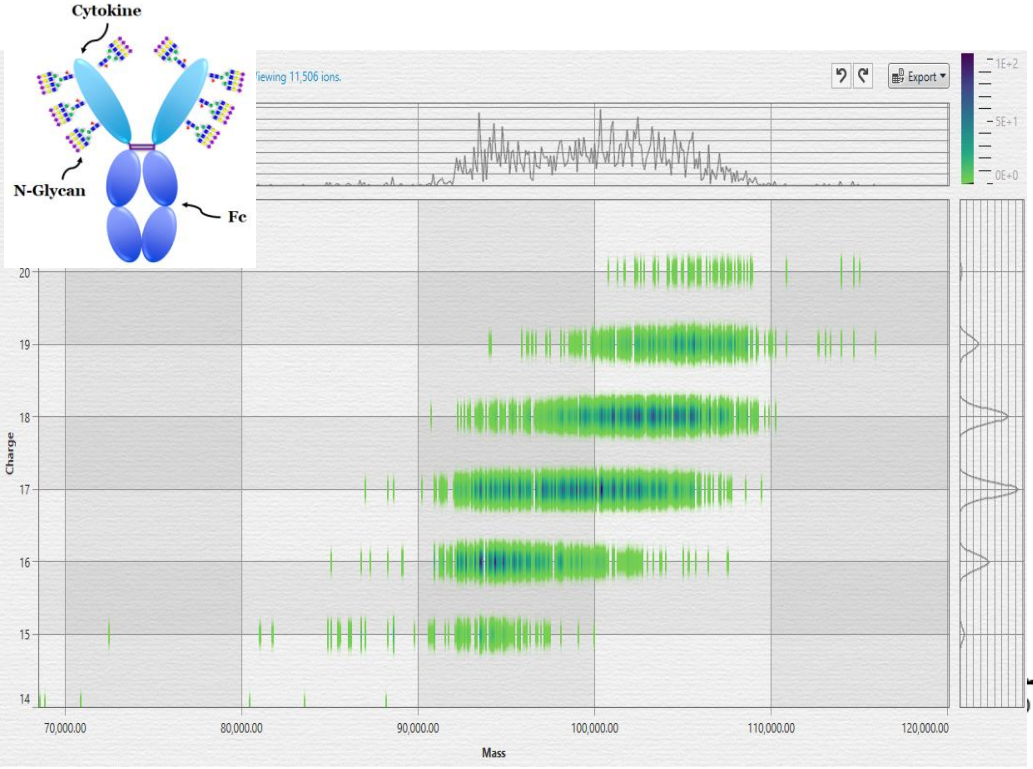


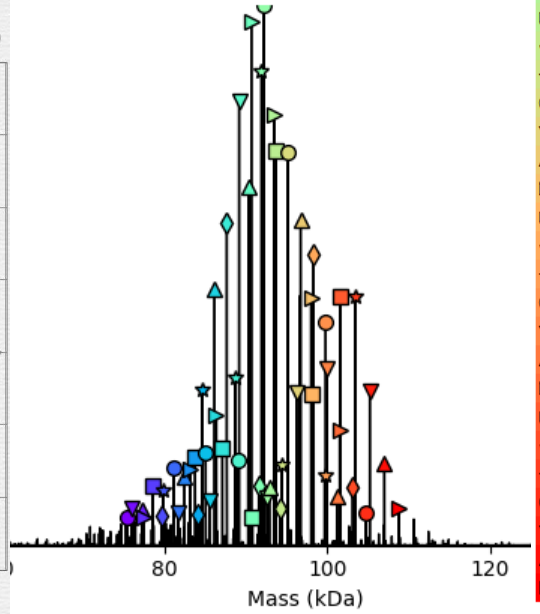
- Membrane protein in nanodisc
- 5 min collection per CE

- Low concentration BsAb (100ug/ml)

with Michael Marty

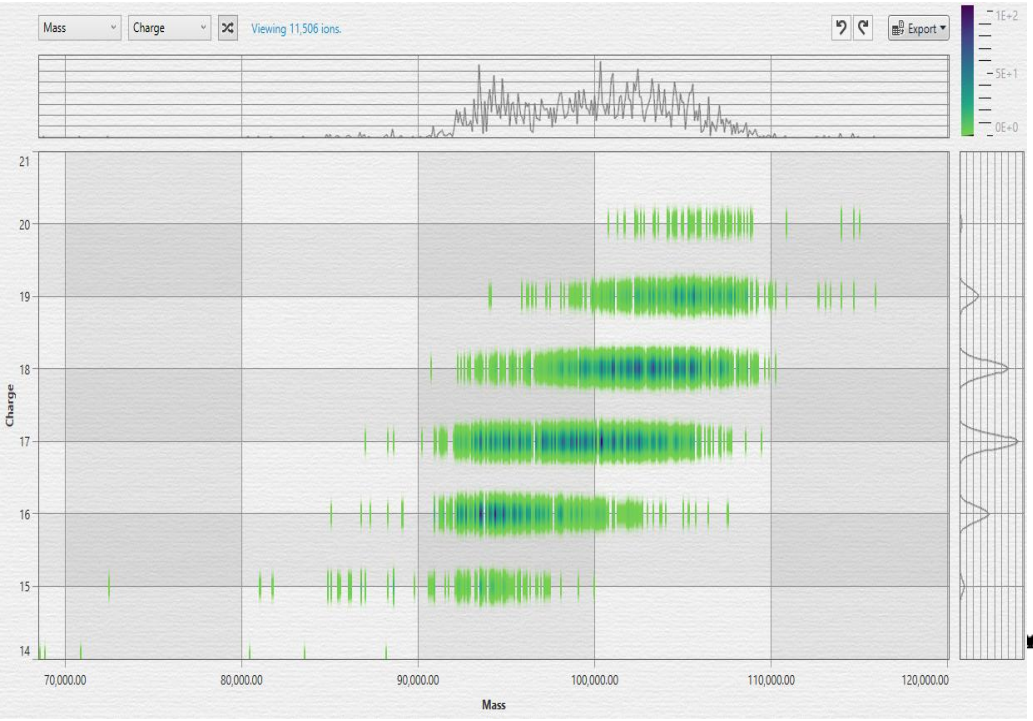
DMT on the low SA Fraction: a cautionary tale of oversampling?

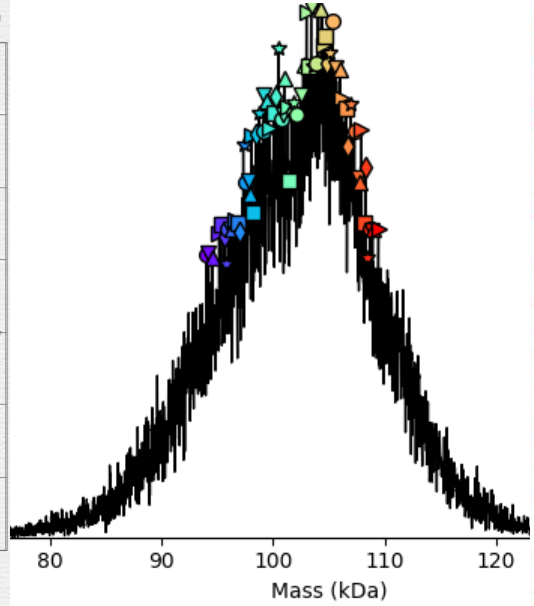




○	92,190	1[IL22Fc] 1[G0F-GlcNAc] 1[G0F] 2[G0] 1...
▽	92,630	1[IL22Fc] 5[G0F]
△	92,900	1[IL22Fc] 1[G0F] 1[HexNAc(5)Hex(4)Fu...
▷	93,540	1[IL22Fc] 2[G0F-GlcNAc] 1[G0F] 2[G0] 1...
□	93,690	1[IL22Fc] 2[G0F] 1[HexNAc(5)Hex(4)Fu...
◊	94,200	1[IL22Fc] 1[G0F] 1[HexNAc(5)Hex(4)Fu...
☆	94,430	1[IL22Fc] 2[G0F] 1[HexNAc(6)Hex(3)Fu...
○	95,140	1[IL22Fc] 2[G0F-GlcNAc] 1[G0F] 2[Hex...
▽	96,230	1[IL22Fc] 1[G0F] 1[M5-G1F] 1[HexNAc(...
△	96,740	1[IL22Fc] 2[G0F-GlcNAc] 1[G0F] 1[Hex...
▷	98,030	1[IL22Fc] 1[G0F] 3[G0] 1[HexNAc(5)Hex...
□	98,190	1[IL22Fc] 1[G0F] 1[HexNAc(5)Hex(4)Fu...
◊	98,340	1[IL22Fc] 3[G0F-GlcNAc] 1[G0F] 3[G0] 1...
☆	99,690	1[IL22Fc] 2[G0F-GlcNAc] 1[G0F] 3[G0] 1...
○	99,830	1[IL22Fc] 5[G0F] 3[M5-G1F] 1[G1F]
▽	99,940	1[IL22Fc] 2[G0F-GlcNAc] 1[G0F] 3[G0] 1...
△	101,1...	1[IL22Fc] 3[G0F] 2[HexNAc(6)Hex(3)Fu...
▷	101,5...	1[IL22Fc] 5[G0F] 2[M5-G1F] 1[HexNAc(...
□	101,6...	1[IL22Fc] 5[G0F] 1[M5-G1F] 3[G0] 1[G1...
◊	103,1...	1[IL22Fc] 2[G0F] 2[HexNAc(5)Hex(4)Fu...
☆	103,4...	1[IL22Fc] 1[G0F] 2[M5-G1F] 2[HexNAc(...
○	104,7...	1[IL22Fc] 2[G0F] 3[M5-G1F] 1[G0] 2[He...
▽	105,2...	1[IL22Fc] 4[G0F-GlcNAc] 3[G0F] 3[G0] 1...
△	107,0...	1[IL22Fc] 1[G0F-GlcNAc] 6[G0F] 2[Hex...
▷	108,8...	1[IL22Fc] 2[G0F-GlcNAc] 1[G0F] 3[G0] 2...

DMT on the low SA Fraction: a more conservative analysis



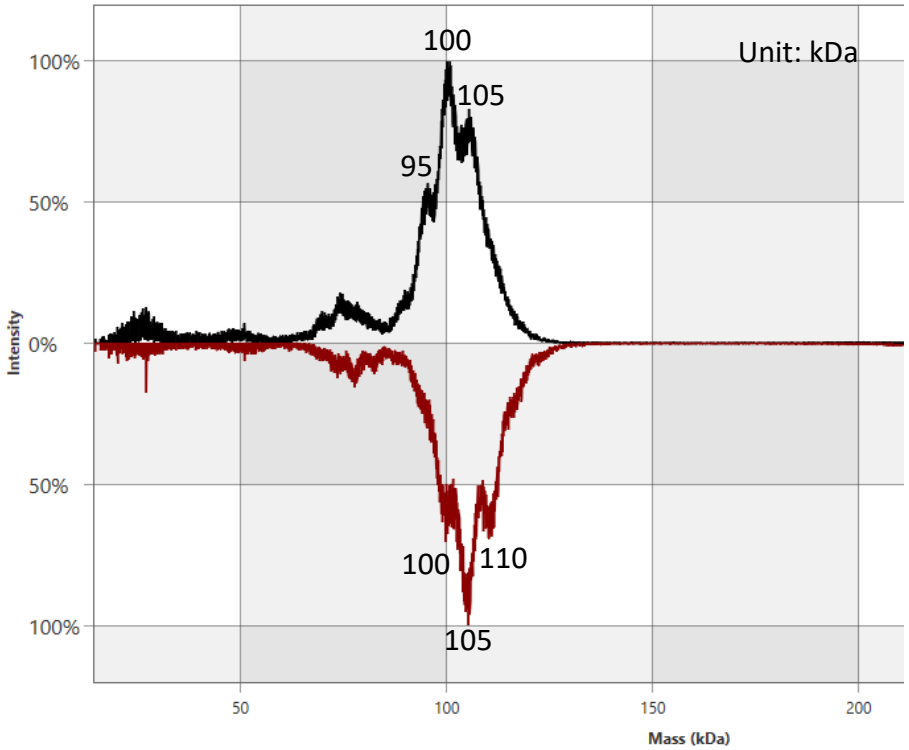


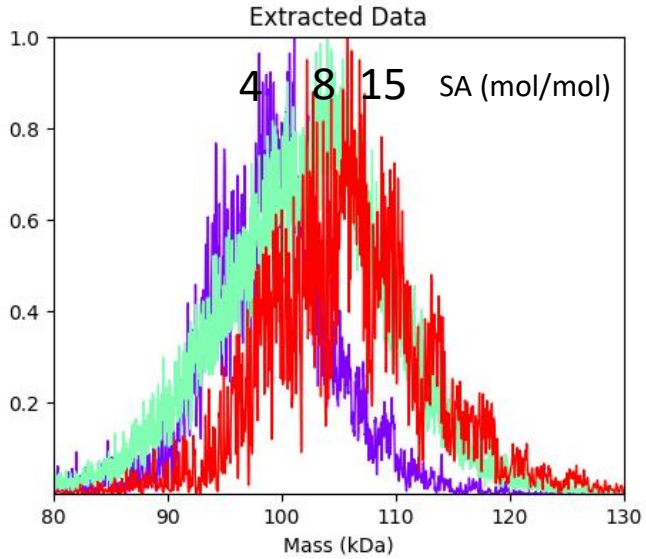
◇	97,035	1[IL22Fc] 1[G0F] 1[M5-G1F] ...
☆	97,330	1[IL22Fc] 1[G0F-GlcNAc] 1[...
○	97,505	1[IL22Fc] 2[G0F-GlcNAc] 1[...
▽	97,760	1[IL22Fc] 2[G0F] 1[M5-G1F] ...
△	97,875	1[IL22Fc] 2[G0F-GlcNAc] 1[...
▷	98,040	1[IL22Fc] 1[G0F] 1[HexNAc(...
□	98,240	1[IL22Fc] 4[G0F] 3[M5-G1F] ...
◇	98,475	1[IL22Fc] 2[G0F-GlcNAc] 1[...
☆	98,755	1[IL22Fc] 2[G0F] 2[HexNAc(...
○	99,075	1[IL22Fc] 1[G0F] 1[HexNAc(...
▽	99,195	1[IL22Fc] 4[G0F] 1[M5-G1F] ...
△	99,440	1[IL22Fc] 5[G0F] 2[M5-G1F] ...
▷	99,720	1[IL22Fc] 1[G0F] 2[G0] 1[He...
□	100,045	1[IL22Fc] 4[G0F-GlcNAc] 1[...
◇	100,275	1[IL22Fc] 1[G0F-GlcNAc] 5[...
☆	100,480	1[IL22Fc] 1[G0F-GlcNAc] 1[...
○	100,740	1[IL22Fc] 1[G0F] 1[HexNAc(...
▽	100,870	1[IL22Fc] 2[G0F] 1[M5-G1F] ...
△	100,995	1[IL22Fc] 4[G0F-GlcNAc] 5[...
▷	101,265	1[IL22Fc] 1[G0F-GlcNAc] 5[...
□	101,385	1[IL22Fc] 1[G0F-GlcNAc] 3[...
◇	101,700	1[IL22Fc] 1[G0F] 1[HexNAc(...
☆	101,865	1[IL22Fc] 2[G0F] 3[M5-G1F] ...
○	102,150	1[IL22Fc] 4[G0F-GlcNAc] 1[...
▽	102,495	1[IL22Fc] 1[G0F] 3[G0] 2[He...
△	102,800	1[IL22Fc] 1[G0F] 1[HexNAc(...
▷	102,995	1[IL22Fc] 1[G0F] 4[G0] 2[G2...
□	103,230	1[IL22Fc] 2[G0F-GlcNAc] 1[...
◇	103,410	1[IL22Fc] 1[G0F] 4[M5-G1F] ...
☆	103,630	1[IL22Fc] 1[G0F-GlcNAc] 5[...
○	103,805	1[IL22Fc] 1[G0F] 4[M5-G1F] ...
▽	104,020	1[IL22Fc] 2[G0F] 2[M5-G1F] ...
△	104,310	1[IL22Fc] 3[G0F] 1[M5-G1F] ...
▷	104,525	1[IL22Fc] 1[G0F] 1[M5-G1F] ...
□	104,640	1[IL22Fc] 2[G0F] 3[M5-G1F] ...
◇	104,850	1[IL22Fc] 1[G0F] 6[G0] 2[He...
☆	105,125	1[IL22Fc] 4[G0F-GlcNAc] 1[...
○	105,335	1[IL22Fc] 1[G0F] 6[G0] 1[He...

No resolution to the resolution issue

SA8

SA15



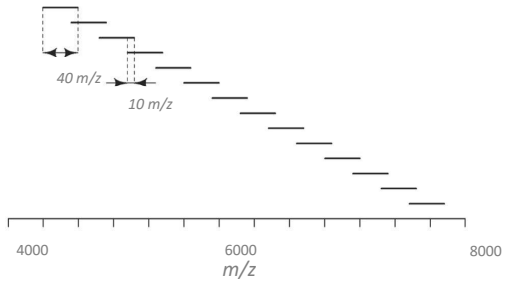


- MW of SA15 shifts higher compared to MW of SA8

Summary

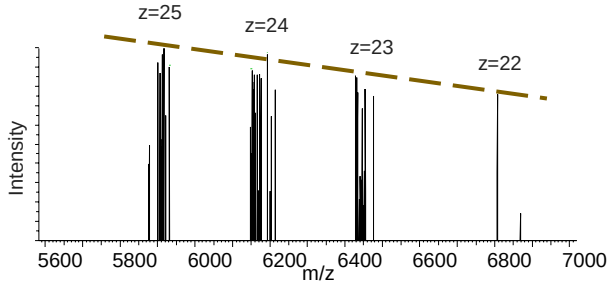
Two approaches to improve spatial resolution of heterogeneous biomolecules

UNIGLAMS



- Small window isolation followed by charge reduction.
- Multiple scan windows may be merged together after deconvolution to reveal proteoforms present
- Ideal for glycoproteins and other biomoelcules with overlapping charge states

DMT



- Single particle detection at high resolution
- No deconvolution needed (so complexity not an issue)
- Useful for large molecules or complexes, membrane proteins and oligomeric structures

Thank you!

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