Ionic charge manipulation using solution- and gas-phase chemistry to facilitate analysis of highly heterogeneous proteins by ESI-MS

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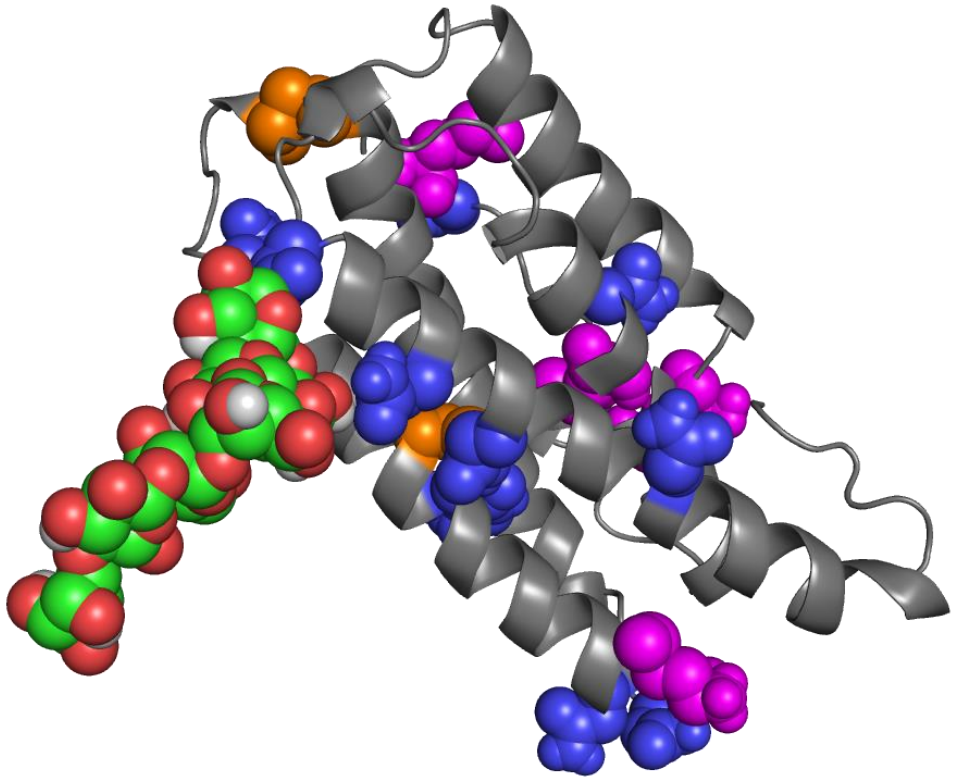
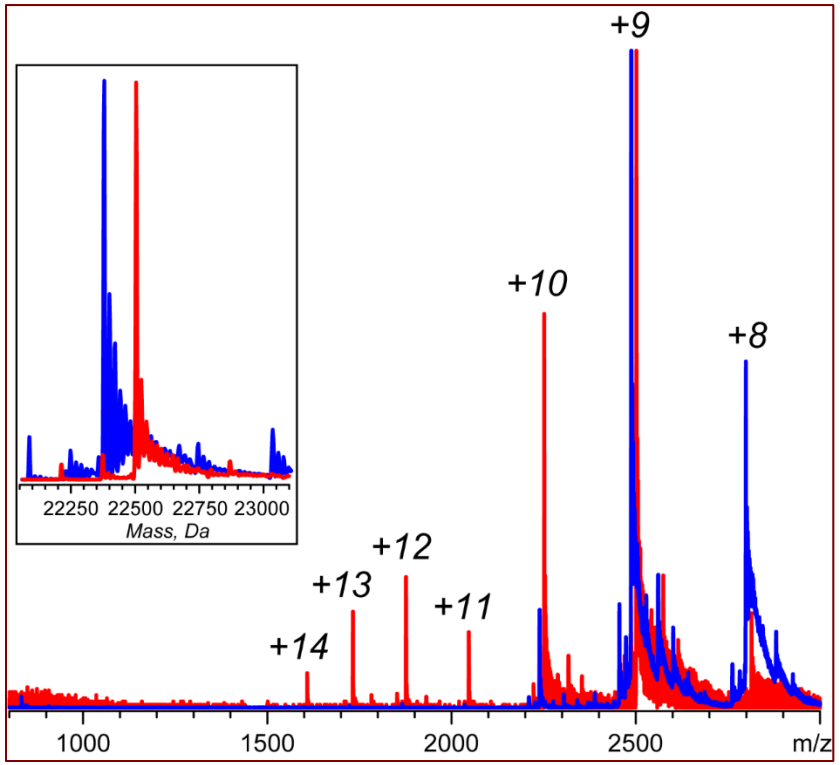
Structural heterogeneity of protein therapeutics



- enzymatic PTMs (glycosylation, etc.)
- non-enzymatic PTMs (oxidation, deamidation, disulfide scrambling, *etc*.)
- “designer” PTMs (PEGylation, small-molecule drug conjugation, *etc*.)
- Conformational heterogeneity (misfolding, mis-assembly, aggregation, *etc*.)

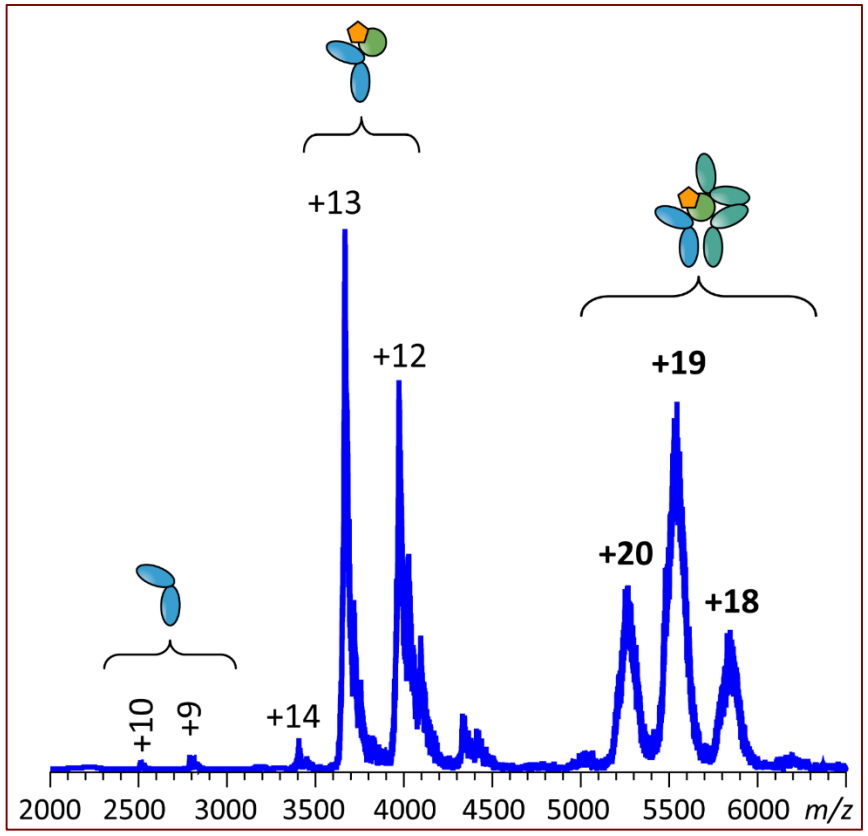


Intact mass measurements of biopharmaceutical products

Enable straightforward assessment of structural heterogeneity

Can be carried out in the on-line format (LC/MS)

Can be carried out in the “native MS” format



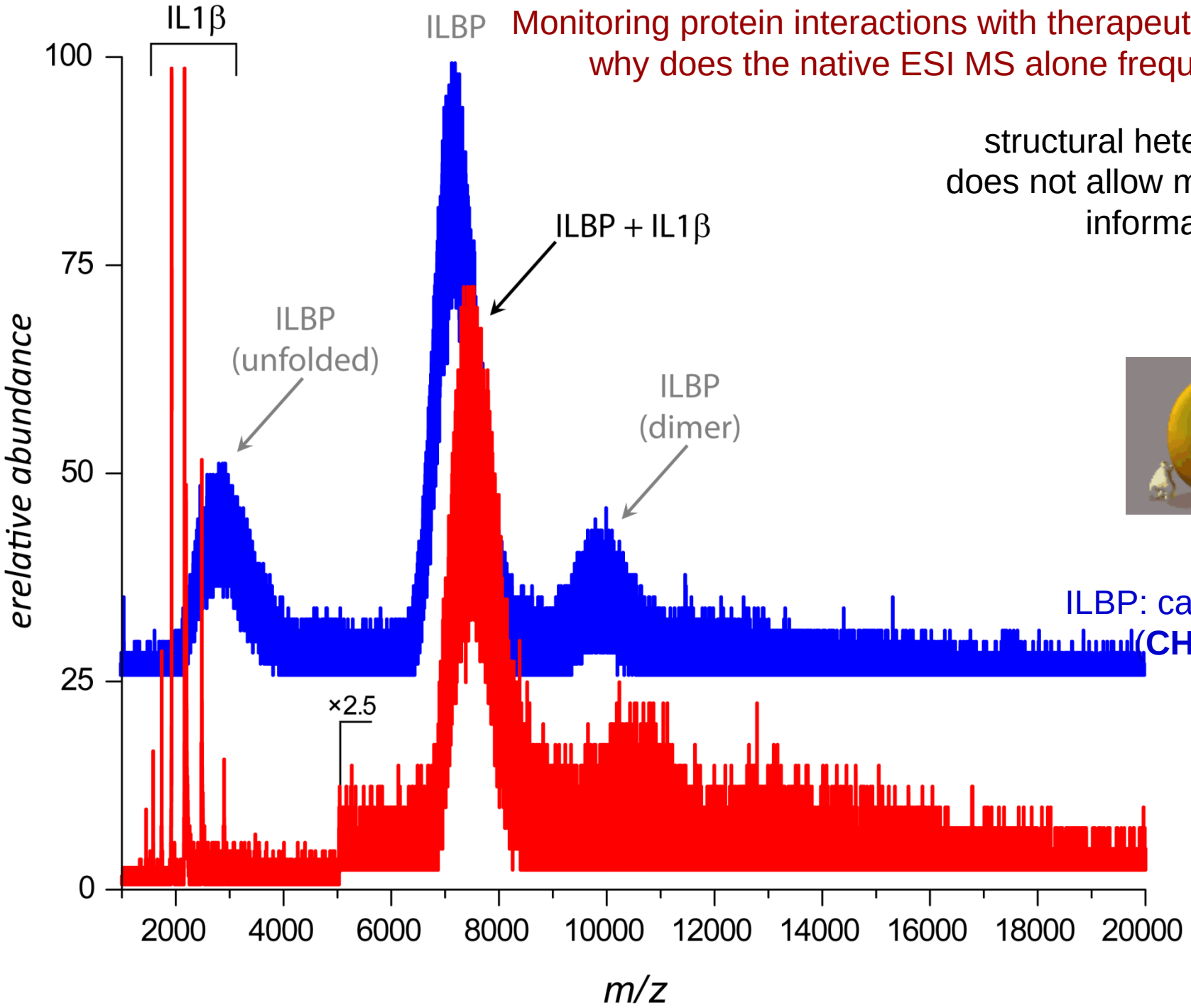
- Higher order structure integrity (conformational heterogeneity assessment)
- Interaction with physiological partners/therapeutic targets (function assessment)

Kaltashov, et al. Conformation and dynamics of biopharmaceuticals: transition of mass spectrometry-based tools from academe to industry. *J. Am. Soc. Mass. Spectrom.* **2010**, *21*, 323

Monitoring protein interactions with therapeutic targets: why does the native ESI MS alone frequently fail?

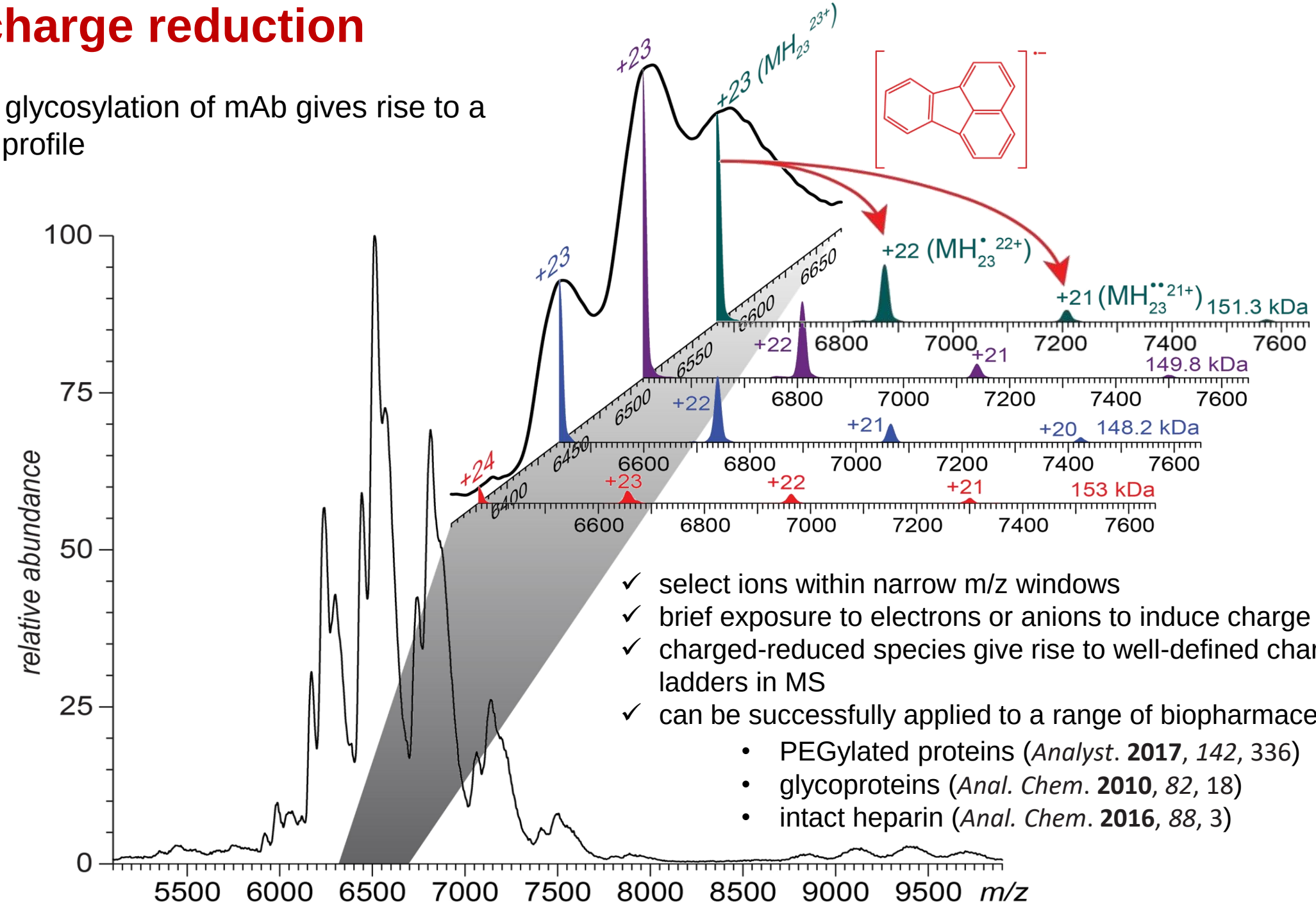
structural heterogeneity does not allow meaningful information to be obtained

ILBP: ca. 300 kDa
(**CHO > 20%**)



Limited charge reduction

- extensively glycosylation of mAb gives rise to a convoluted profile



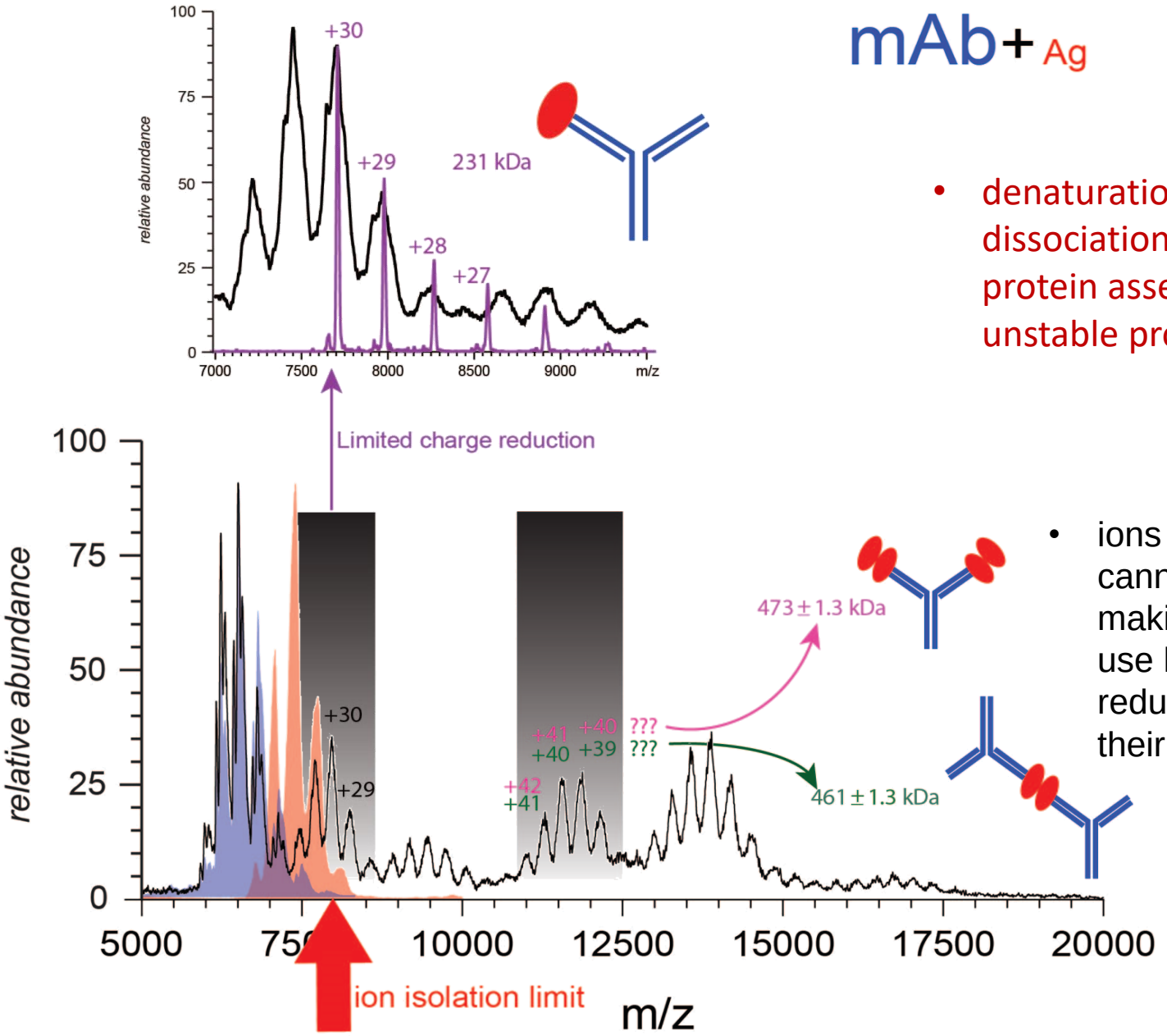
- ✓ select ions within narrow m/z windows
- ✓ brief exposure to electrons or anions to induce charge transfer
- ✓ charged-reduced species give rise to well-defined charge ladders in MS
- ✓ can be successfully applied to a range of biopharmaceuticals
  - PEGylated proteins (*Analyst*. **2017**, *142*, 336)
  - glycoproteins (*Anal. Chem*. **2010**, *82*, 18)
  - intact heparin (*Anal. Chem*. **2016**, *88*, 3)

Limitation

the inability of most mass spectrometers to isolate ions beyond certain threshold at high m/z values

- limited charge redaction of lower-m/z ions can be used to determine their masses (and assign the binding stoichiometry for the lower-mass complexes)

- the mAb/Ag mixture contains ionic signals corresponding to complexes with different binding stoichiometry
- composition & binding stoichiometry cannot be assigned unambiguously

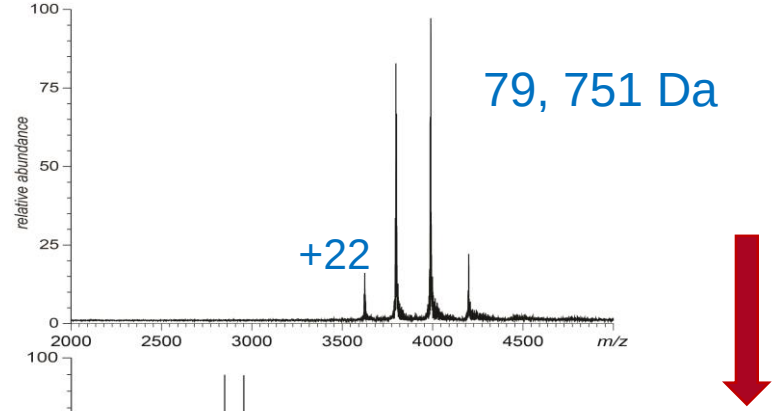


- denaturation results in dissociation of non-covalent protein assemblies or unstable proteins

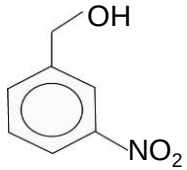
- ions at higher m/z cannot be isolated, making it impossible to use limited charge reduction to determine their masses

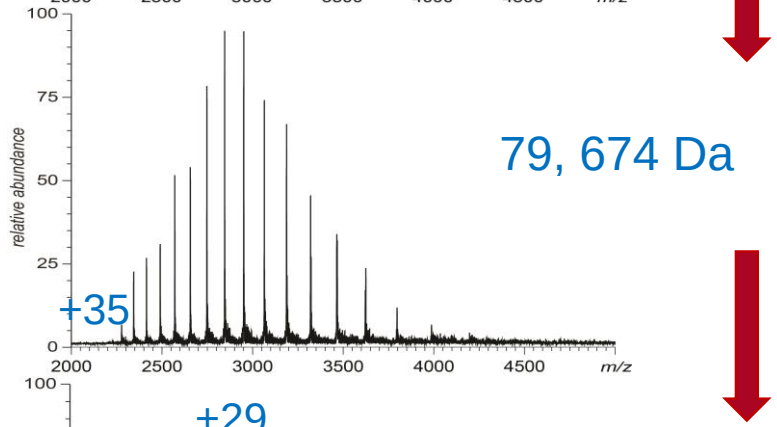
Strategy

- the utility of supercharging
- supercharging is achieved by adding m-nitrobenzyl alcohol (mNBA)
- higher charges enhance the charge reduction efficiency
- monitor the onset of denaturation

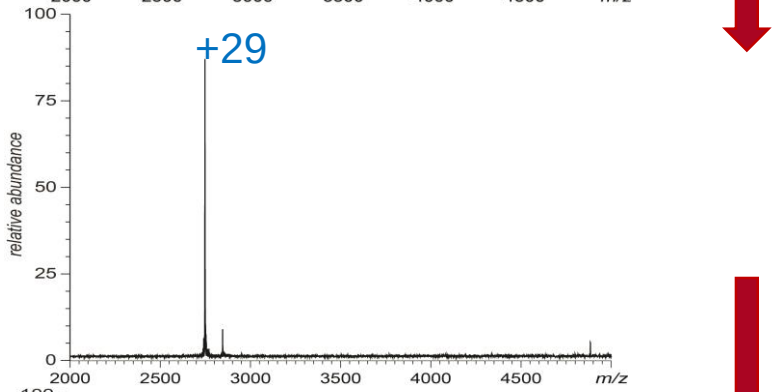


Add supercharging reagent

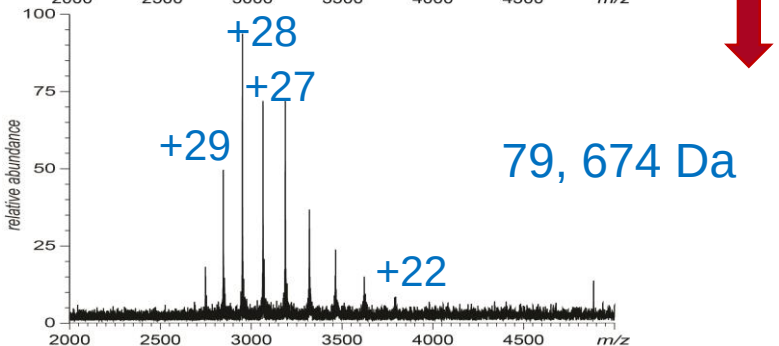


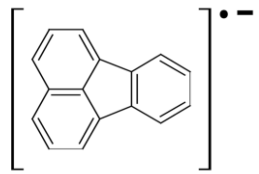


Isolate an ionic population within a narrow m/z window



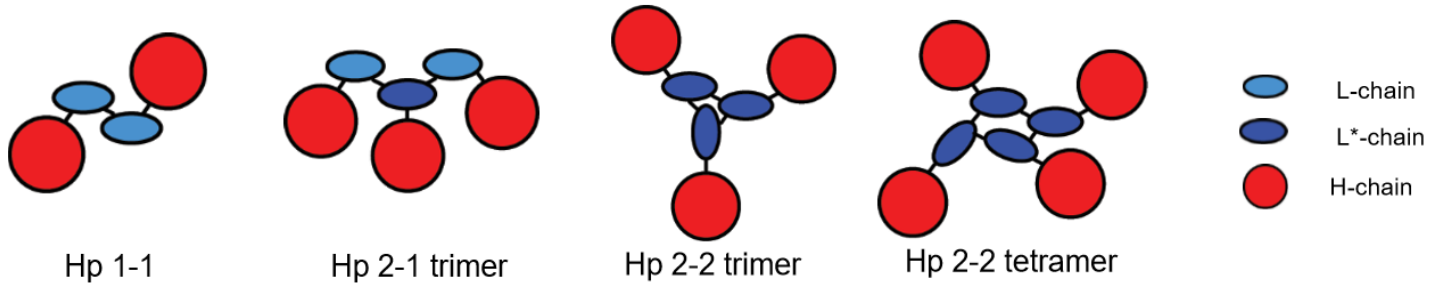
Induce limited charge reduction in the gas phase by standard reagent





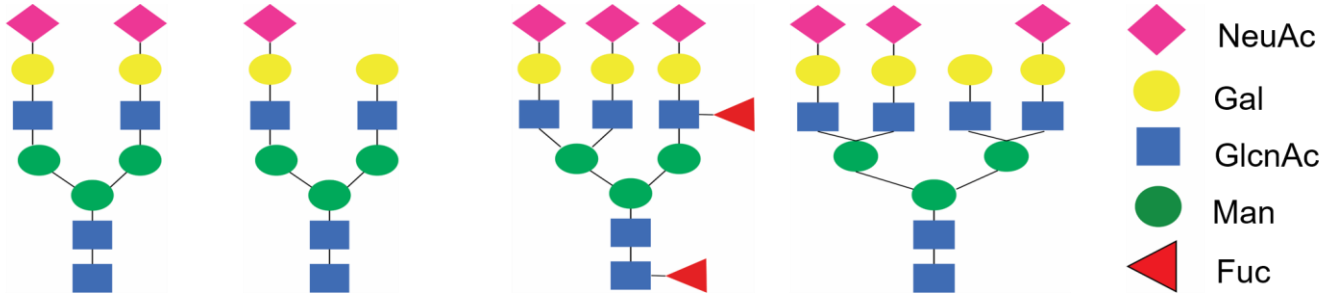
Haptoglobin (Hp)

- acute phase glycoprotein
- binds free hemoglobin (Hb) in circulation
- several isoforms
- high degree of heterogeneity (CHO ≥ 20%)



Haptoglobin human P00738

MSALGAVIAL LLWGQLFAVD SGNDVTDIAD DGCPKPPEIA HGYVEHSVRY[32]
QCKNYYKLRT EGDGVYTLND KKQWINKAVG DKLPECEADD GCPKPPEIAH[82]
GYVEHSVRYQ CKNYYKLRTE GDGVYTLNNE KQWINKAVGD KLPECEAVCG[132]
KPKNPANPVQ RILGGHLDAK GSFPWQAKMV SHHNLTTGAT LINEQWLLTT[39]
AKNLFLNHSE NATAKDIAPT LTLYVGKKQL VEIEKVVLHP NYSQVDIGLI[89]
KLKQKVSVNE RVMPICLPSK DYAEVGRVGY VSGWGRNANF KFTDHLKYVM[139]
LPVADQDQCI RHYEGSTVPE KKTPKSPVGV QPILNEHTFC AGMSKYQEDT[189]
CYGDAGSAFA VHDLEEDTWY ATGILSFDKS CAVAEYGVYV KVTSIQDWVQ[239]
KTIAEN[245]



Di-asialylated Bi-antennary N-glycan 2.2 kDa

Mono-sialylated Bi-antennary N-glycan 1.9 kDa

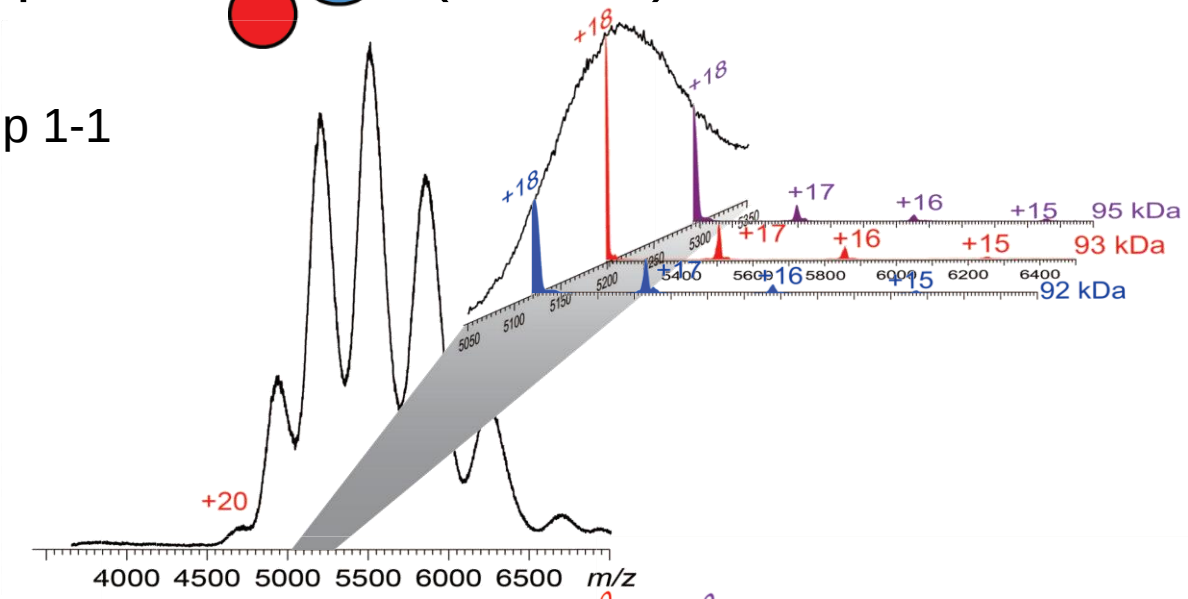
Tri-sialylated Tri-antennary N-glycan 2.9 kDa

Tri-asialylated Tera-antennary N-glycan 2.8 kDa

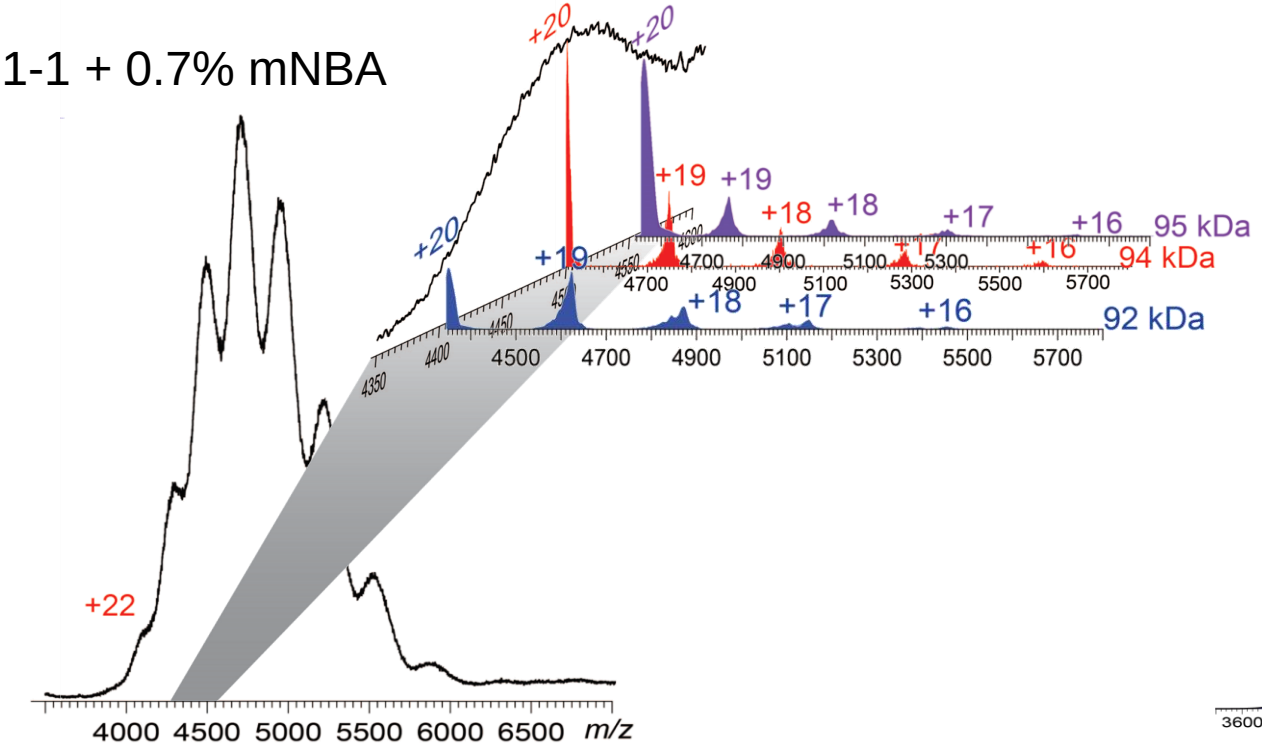
L*-chain (15.9 kDa) containing L-chain (9.1 kDa) sequence and an *extra segment*; H-chain (27.2 kDa) including ***four glycosylation sites.***

Hp 1-1: (92 kDa)

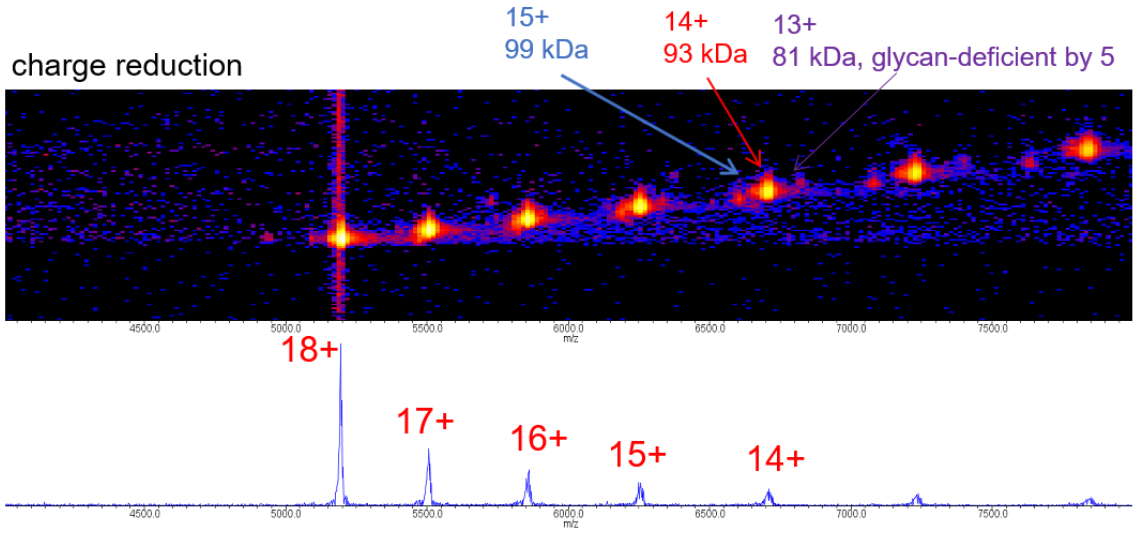
Hp 1-1



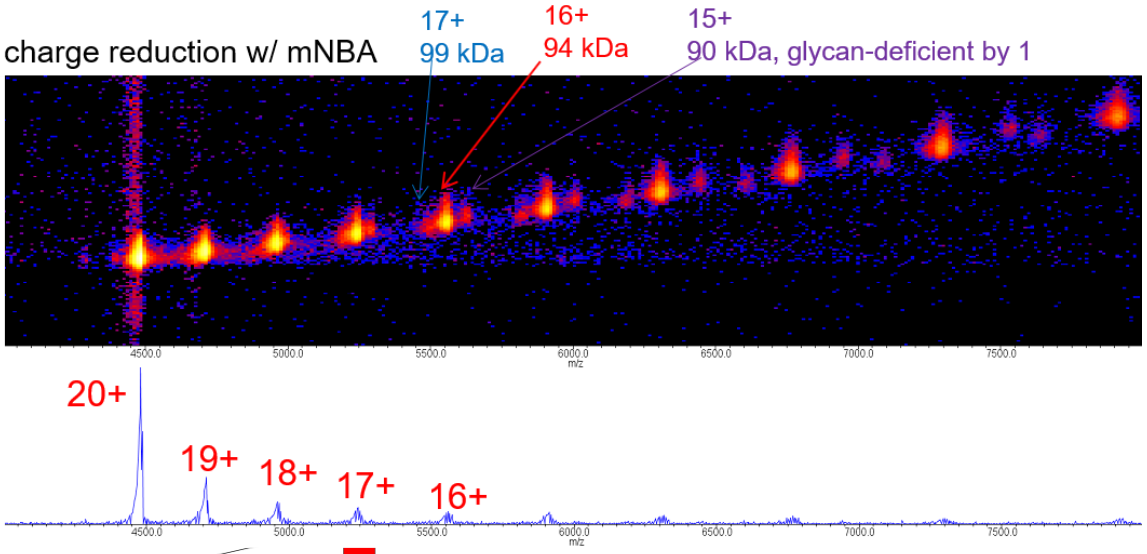
Hp 1-1 + 0.7% mNBA

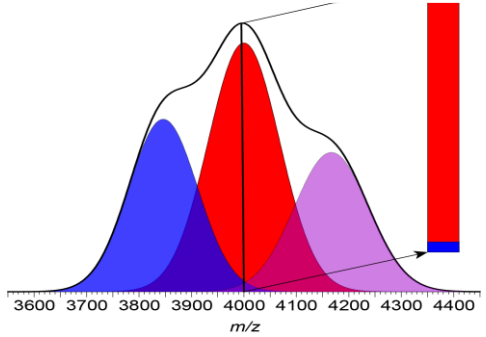


charge reduction



charge reduction w/ mNBA

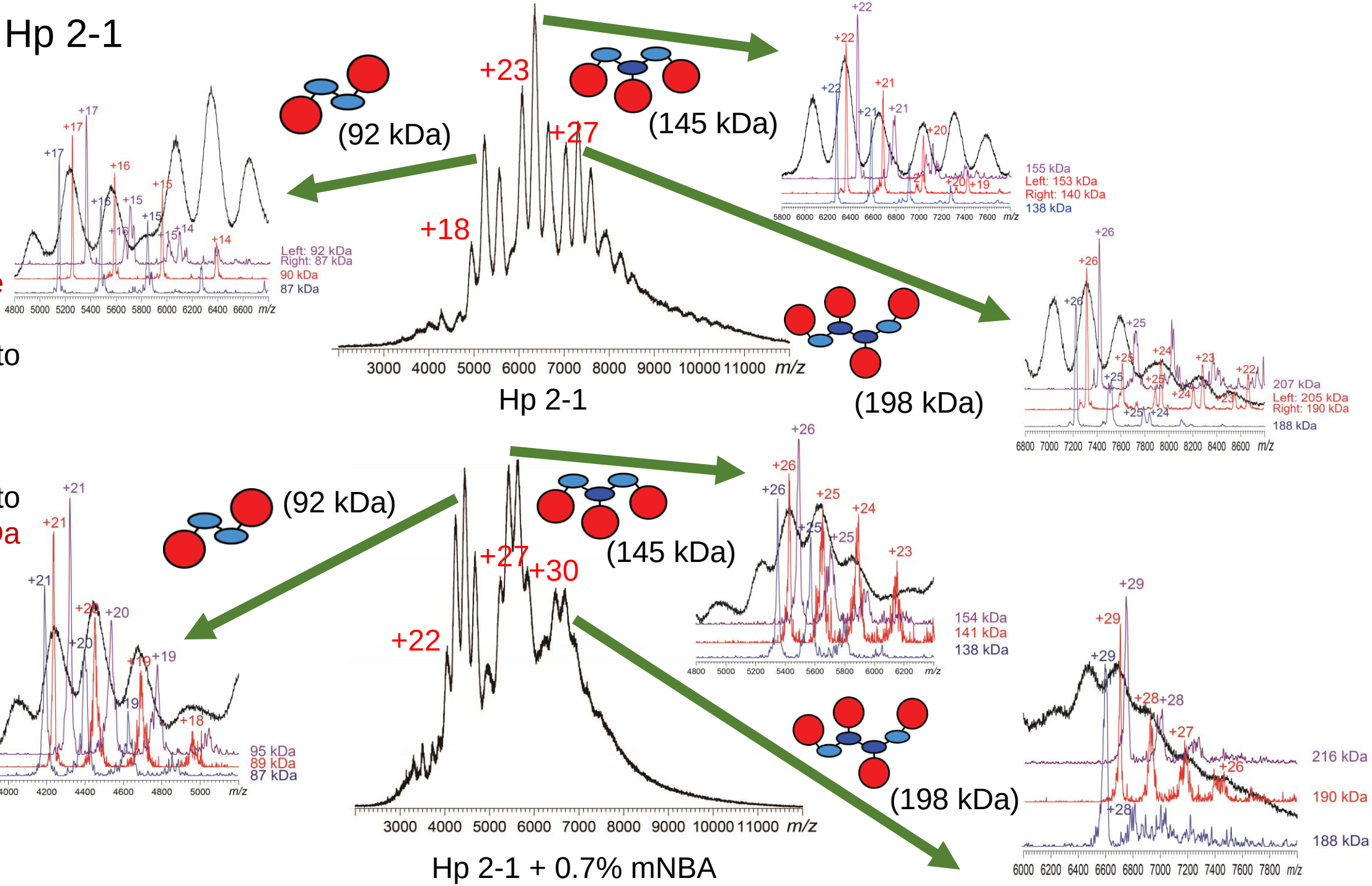




Gaussian distributions reveals the overlapping of adjacent peaks during mass selection process

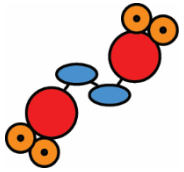
Mixed type Hp 2-1

Limited charge reduction of the most abundant ions gives rise to three well defined charge ladders corresponding to 90 kDa, 140 kDa and 190 kDa, respectively



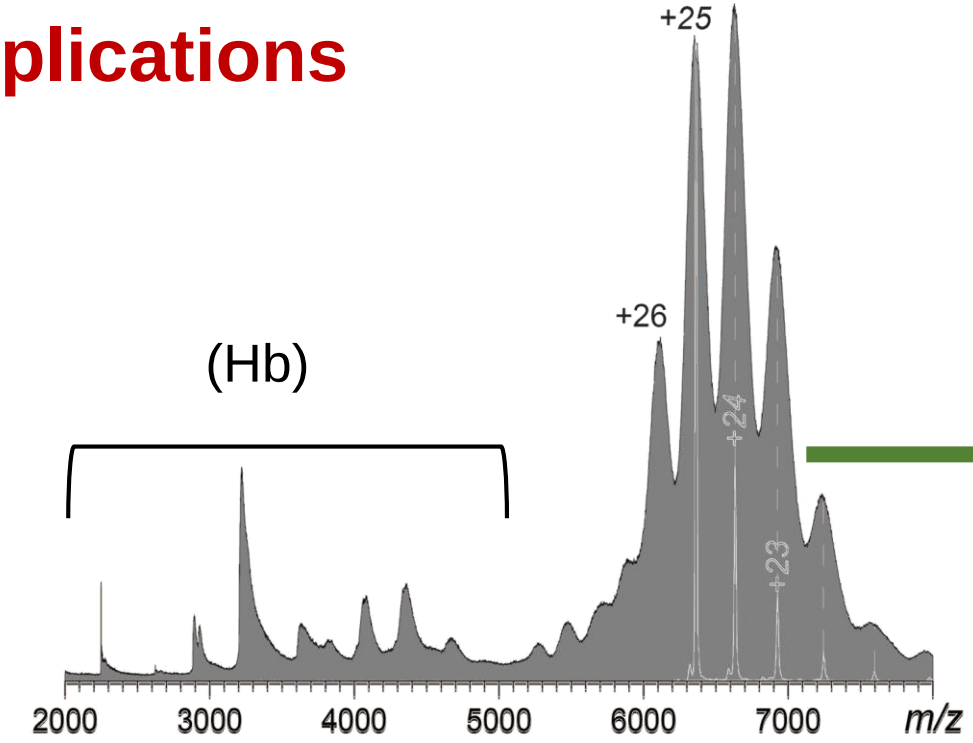
Expanding the scope of applications

Hp 1-1 & Hb complex

Hp 1-1 & Hb  (157 kDa)

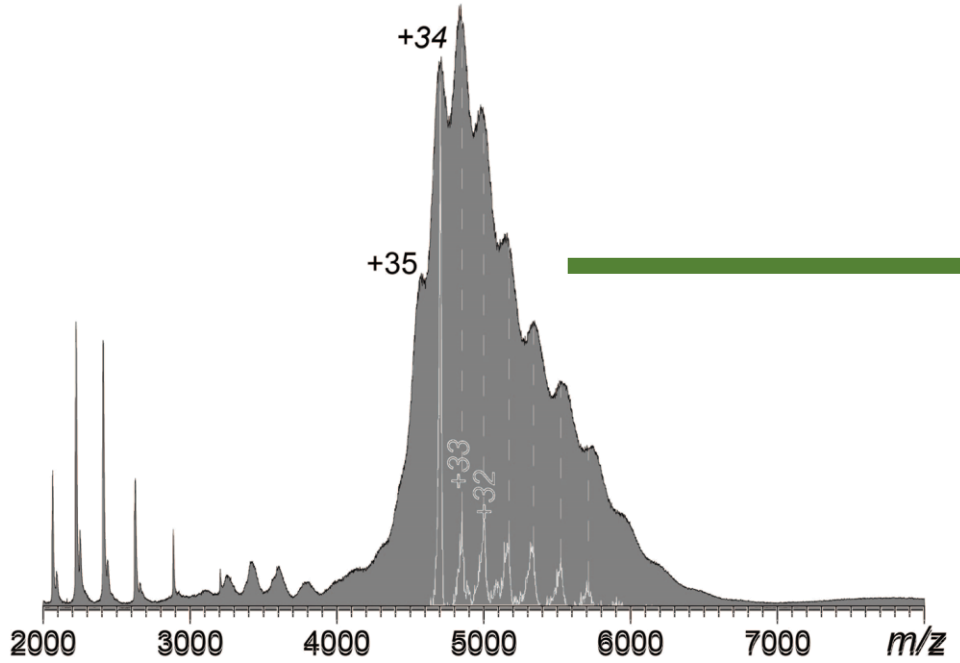
Hb dimer (α*β*)

- the mixture of Hp and Hb presents an overwhelmingly challenging case
- Hp/Hb proves that this method works well with non-covalent assemblies (native MS)



159 kDa

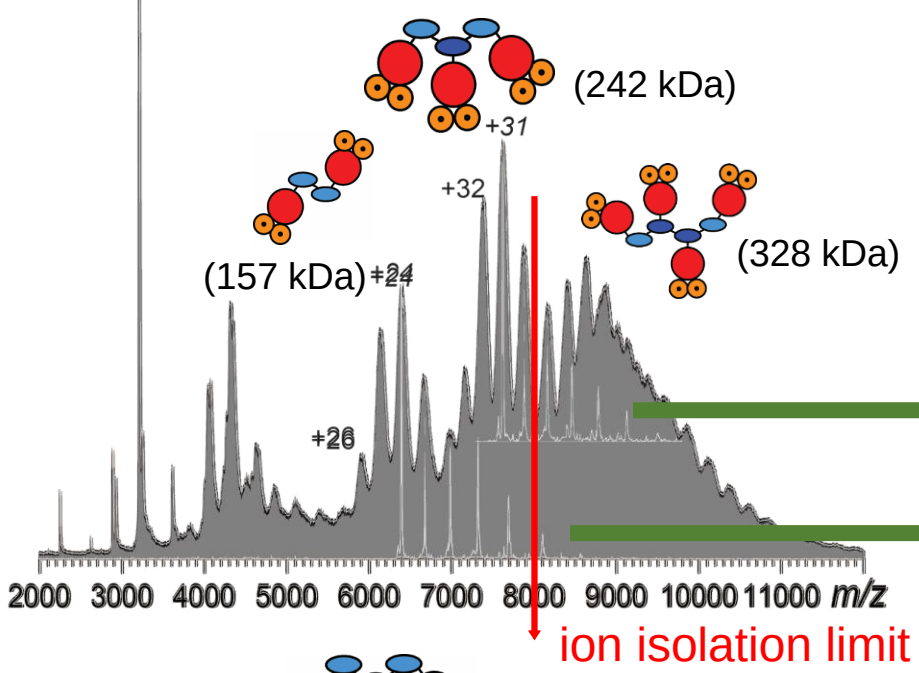
Hp 1-1 & Hb + 0.7% mNBA



160 kDa

Hp 2-1 & Hb complex

Hp 2-1 & Hb

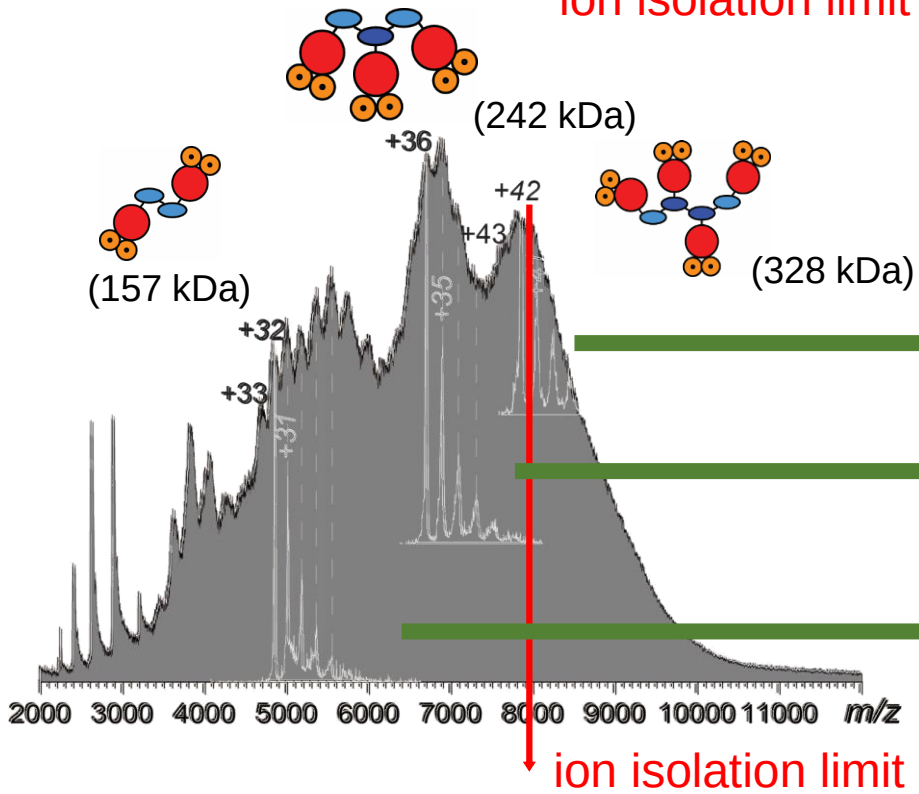


236 kDa

153 kDa

unable to generate charge ladder for this species because its ionic signal is above the mass-selection threshold

Hp 2-1 & Hb + 0.7% mNBA



336 kDa

241 kDa

154 kDa

type is well resolved now after adding mNBA to shift m/z value below the threshold

Conclusions

- a new analytical tool that manipulate ionic charge states using solution and gas phase chemistry opens up an exciting opportunity to make accurate mass determination of highly heterogeneous proteins

- each type of extensively glycosylated haptoglobin can be discerned from the convoluted MS spectrum

- this technique, for the first time, demonstrates the interpretable MS information for the Hp/Hb binding

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Ms. Miaowei Xu

Mass Spectrometry Core Facility

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- A.T. Iavarone & E.R. Williams. Mechanism of charging and supercharging molecules in electrospray ionization. *J. Am. Chem. Soc*. **2003**, *125*, 2319.