Proteoform Peak Integration Facilitates DAR Estimation from Orbitrap Mass Spectra of Complex ADCs

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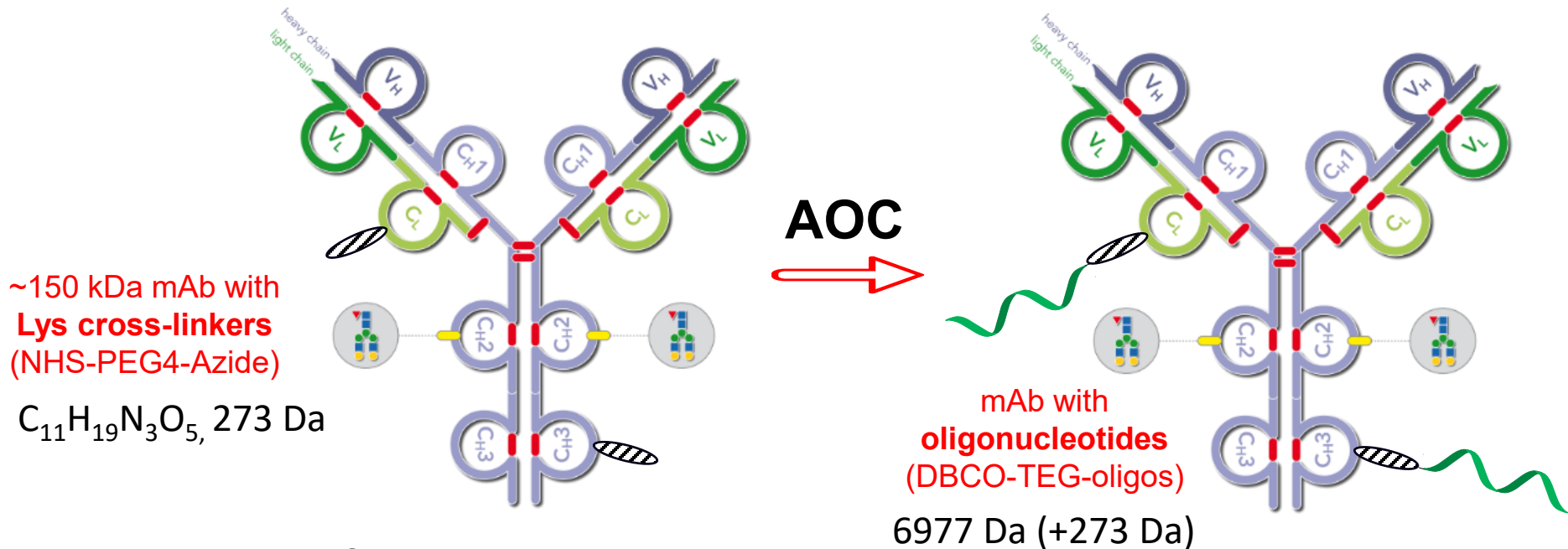[4] Protein and Antibody Engineering Unit, Orion Pharma, Turku, Finland

November 2, 2021; TOH pm – 03:50

Antibody-Oligonucleotide Conjugate (AOC)

Oligonucleotides – **selectively** arrest protein production by targeting specific genes

AOCs: Jain *et al., J. Clin. Med*. 2021   *Wagner et al., Bioconj. Chem.* 2019

Sample: a trastuzumab-oligonucleotide conjugate with ~7 kDa per payload (size & ionization)

~150 kDa mAb with
Lys cross-linkers
(NHS-PEG4-Azide)

$C_{11}H_{19}N_3O_5$, 273 Da

AOC

mAb with
oligonucleotides
(DBCO-TEG-oligos)

6977 Da (+273 Da)

Nagornov et al., Anal Chem, 2021, 12930

Objective: To Estimate the Drug-to-Antibody Ratio

Drug-to-antibody ratio (**DAR**) is the average number of drugs conjugated to an antibody

Native SEC
Q Exactive HF BioPharma
60k @ 200 *m/z*

unresolved proteoforms
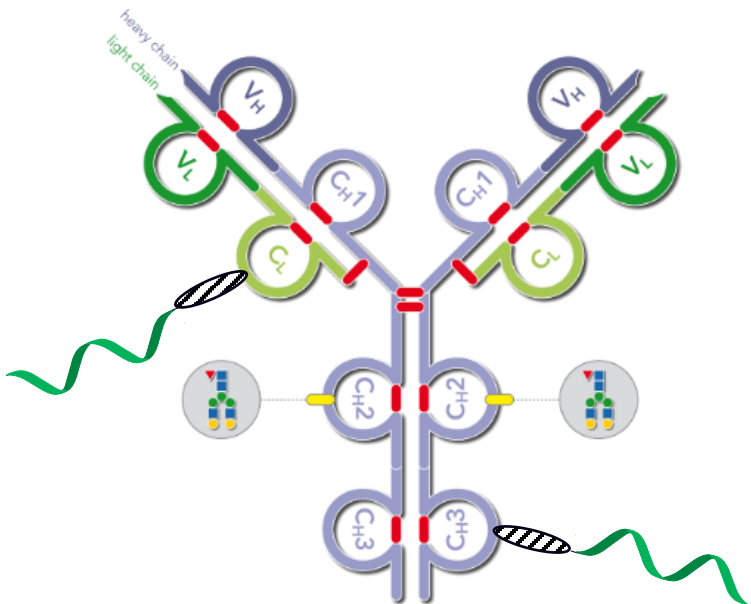
trastuzumab conjugated
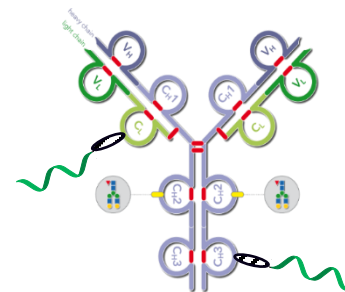with oligonucleotides

SEC: size-exclusion chromatography
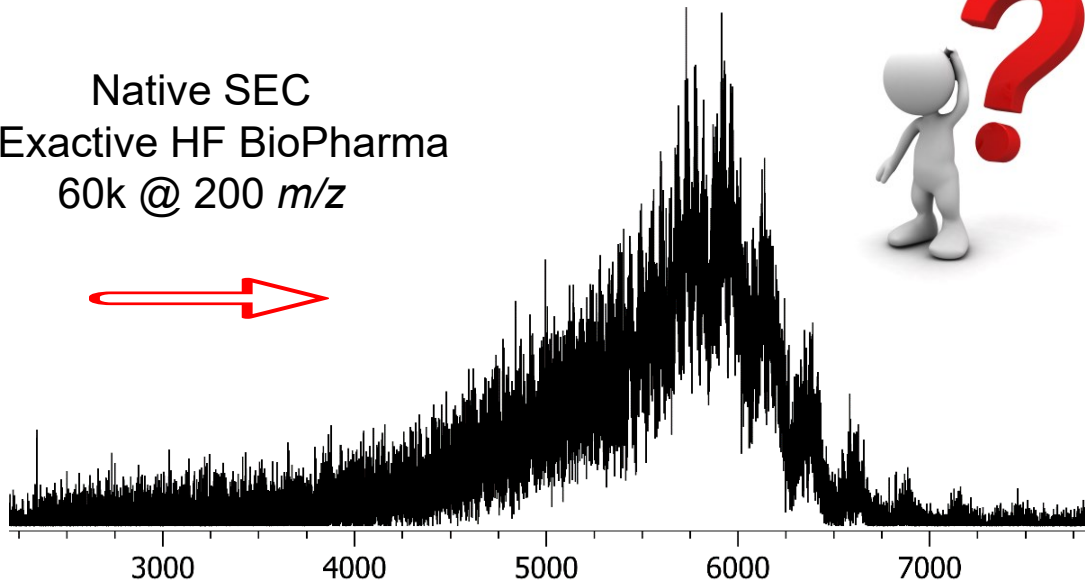
- **Sample preparation (deglycosylation)**

- **Data analysis algorithms**
  Approaches to Heterogeneity in Native MS
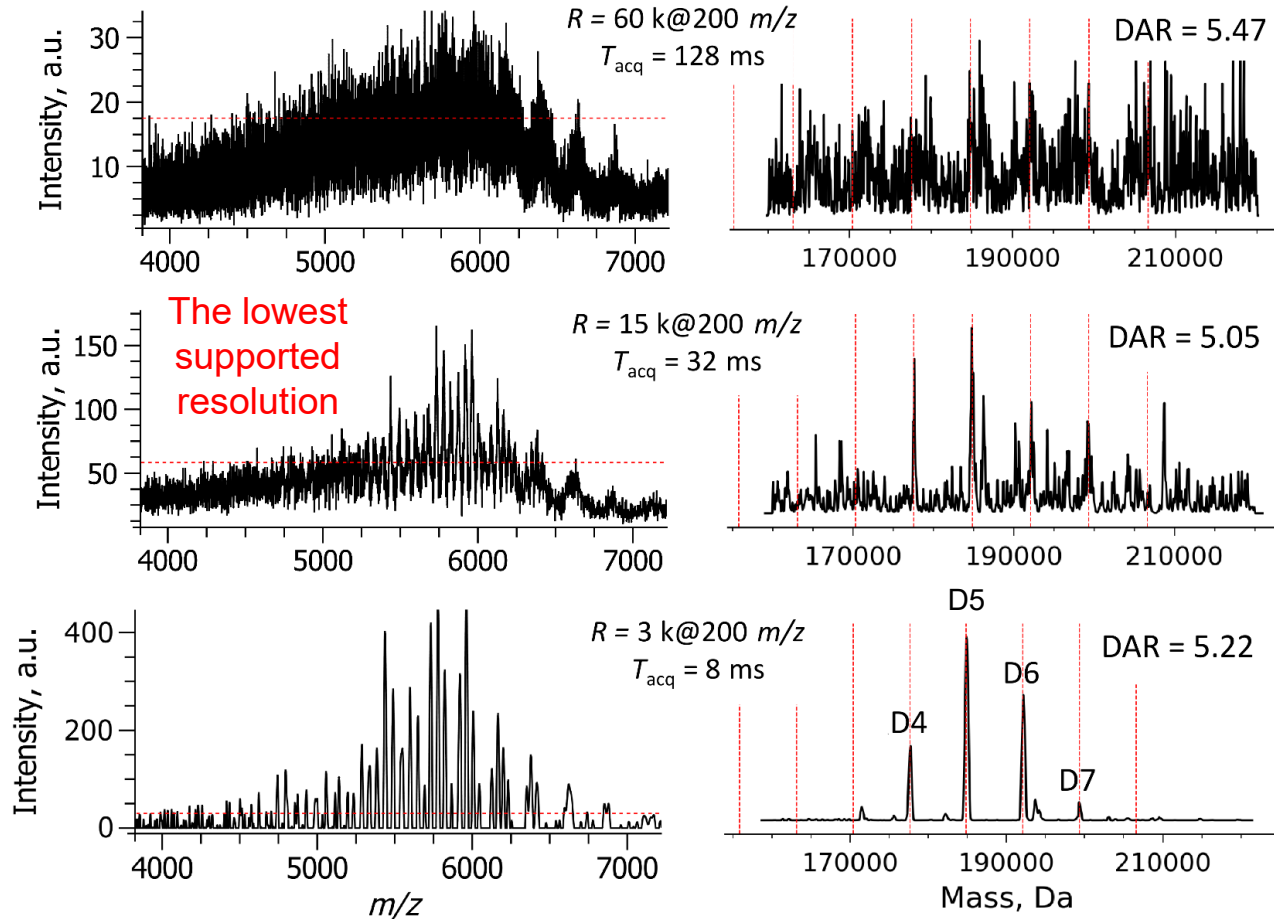 *Prell et al., Chem. Rev., 2021*

Addressing Mass Spectrum Complexity

resolution

R = 60 k@200 m/z
T_{acq} = 128 ms

DAR = 5.47

The lowest supported resolution

R = 15 k@200 m/z
T_{acq} = 32 ms

DAR = 5.05

R = 3 k@200 m/z
T_{acq} = 8 ms

DAR = 5.22

D5

D6

D4

D7

Sample:
Intact AOC

Experiment:
Native conditions
SEC-Orbitrap
(Q Exactive HF)

Anal Chem, 2021, 12930

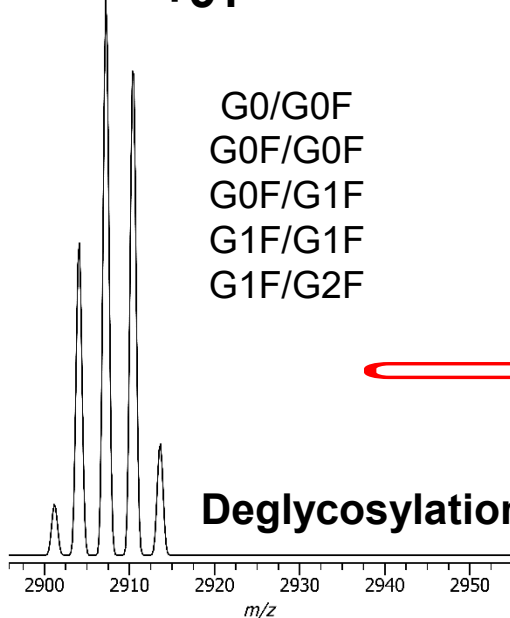# The AOC Sample Complexity

mAb glycoforms
(charge states)
+51
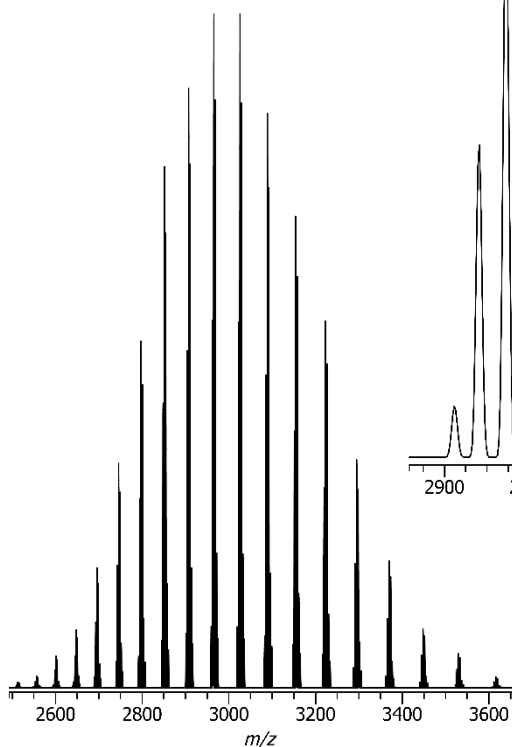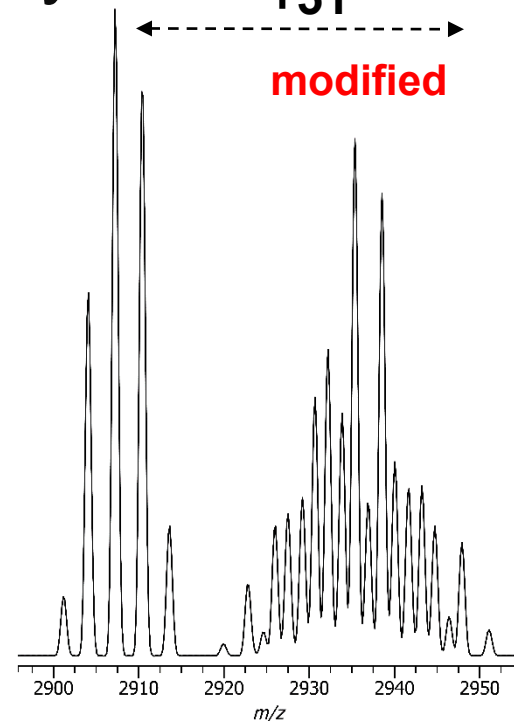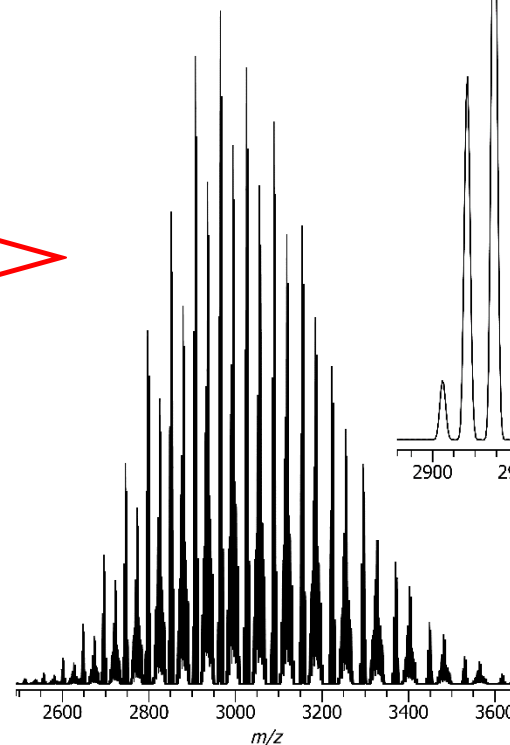
+51

G0/G0F
G0F/G0F
G0F/G1F
G1F/G1F
G1F/G2F

Deglycosylation?

+ cross-linkers
(4-8 cross linkers)

+51

modified

FTMSSIMULATOR

Q Exactive HF
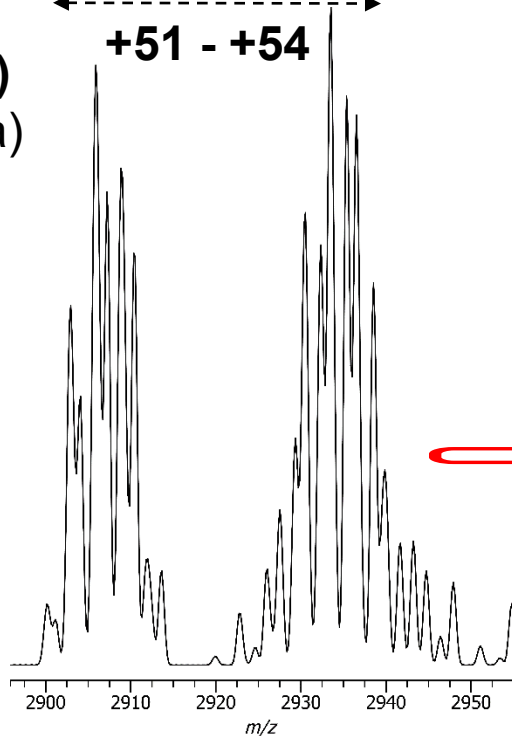15k @ 200 *m/z*

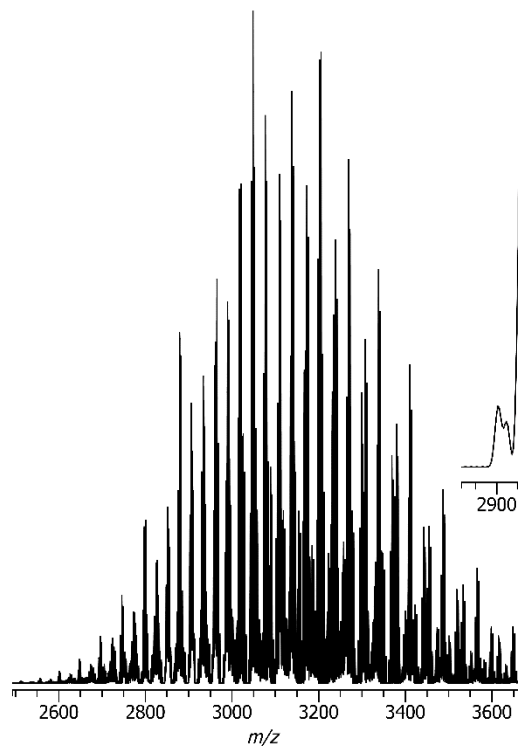**WP051**

JASMS 2020, 1927

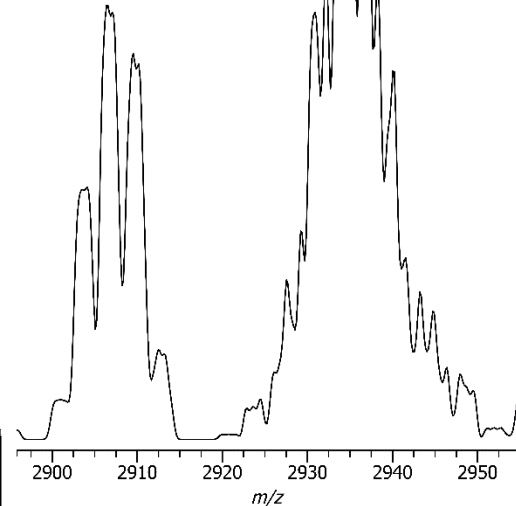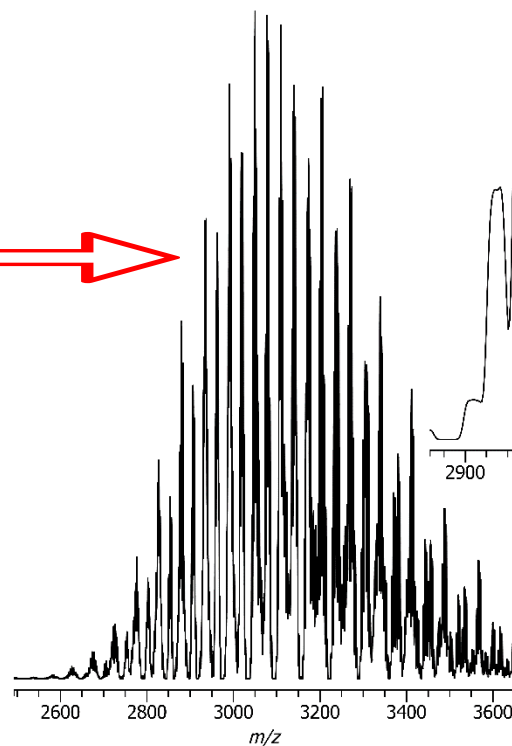# The AOC Sample Complexity

+ payload (drugs)
(4-8 drugs, 1.2 kDa)

+51 - +54

+ modifications
(16, 32, 44, … Da)

+51 - +54

FTMSSIMULATOR

Q Exactive HF
15k @ 200 *m/z*

WP051

JASMS 2020, 1927

Solution: Proteoform Peak Integration

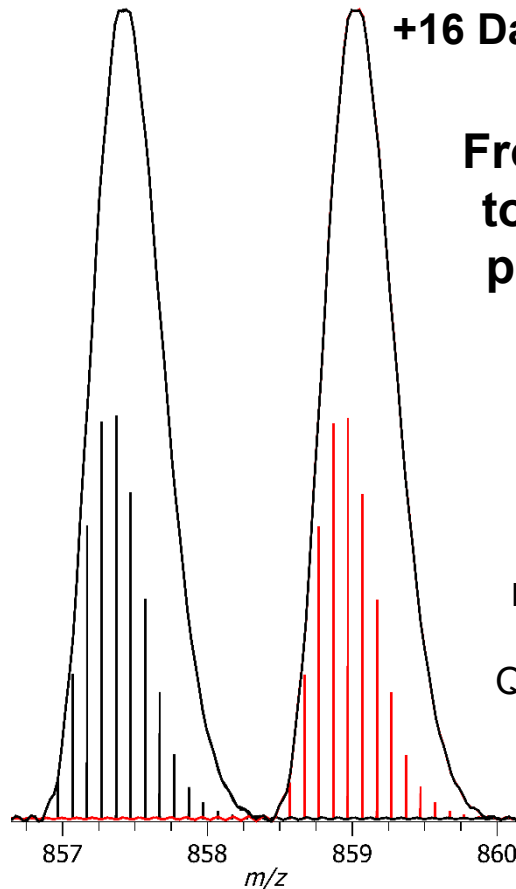**Isotopes** (fine structure)

Isotopologues (monoisotopic mass)

Proteoforms (average mass)

Integrated Proteoforms

R resolution

R = 1.5k

R = 0.5k

oxidized ubiquitin, +16 Da, [M+10H]$^{10+}$

S/N ↑

From resolved to **integrated** proteoforms

R ↓

FTMSSIMULATOR

Q Exactive HF 30k @ 200 *m/z*

857   858   859   860   *m/z*
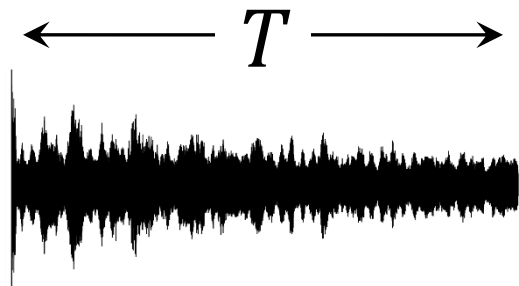
850   860   870   *m/z*
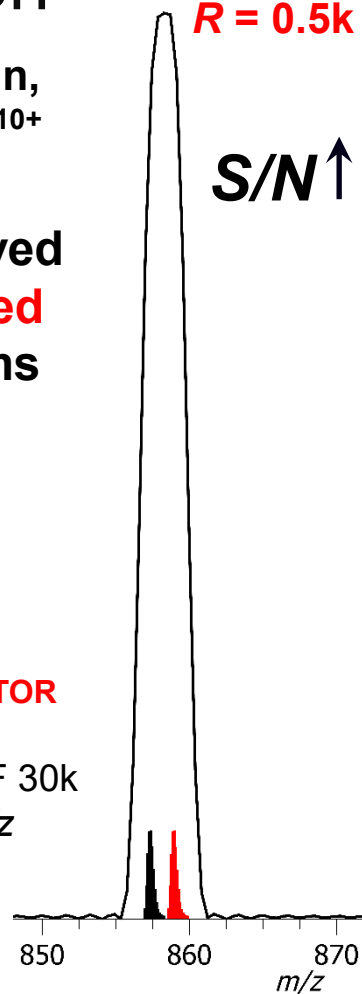
How to Reduce Resolution to Integrate Proteoforms?

- directly via lower resolution mass spectra – limit of 15k @ *m/z* 200 (Q Exactive HF)

- via truncation of the **time-domain transients**, e.g., using an add-on DAQ system
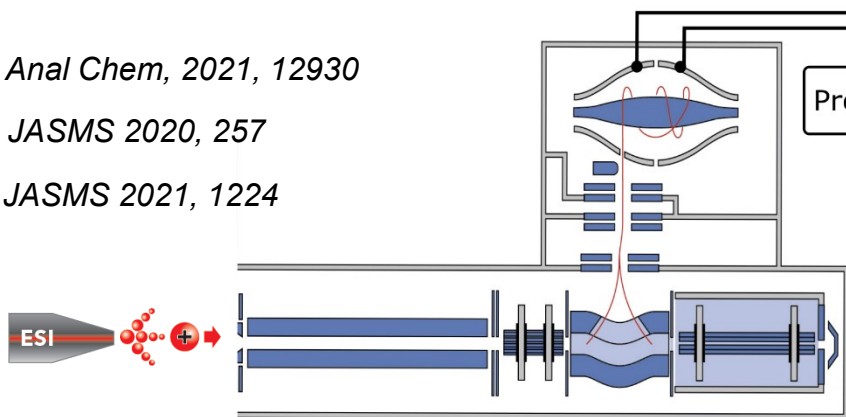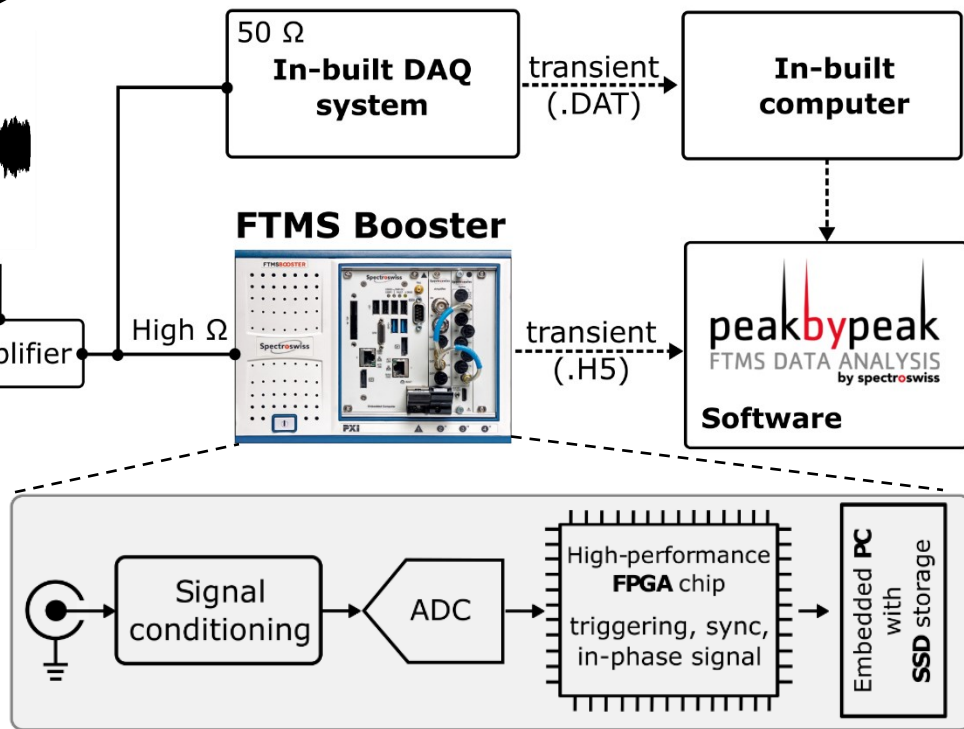
time-domain transient

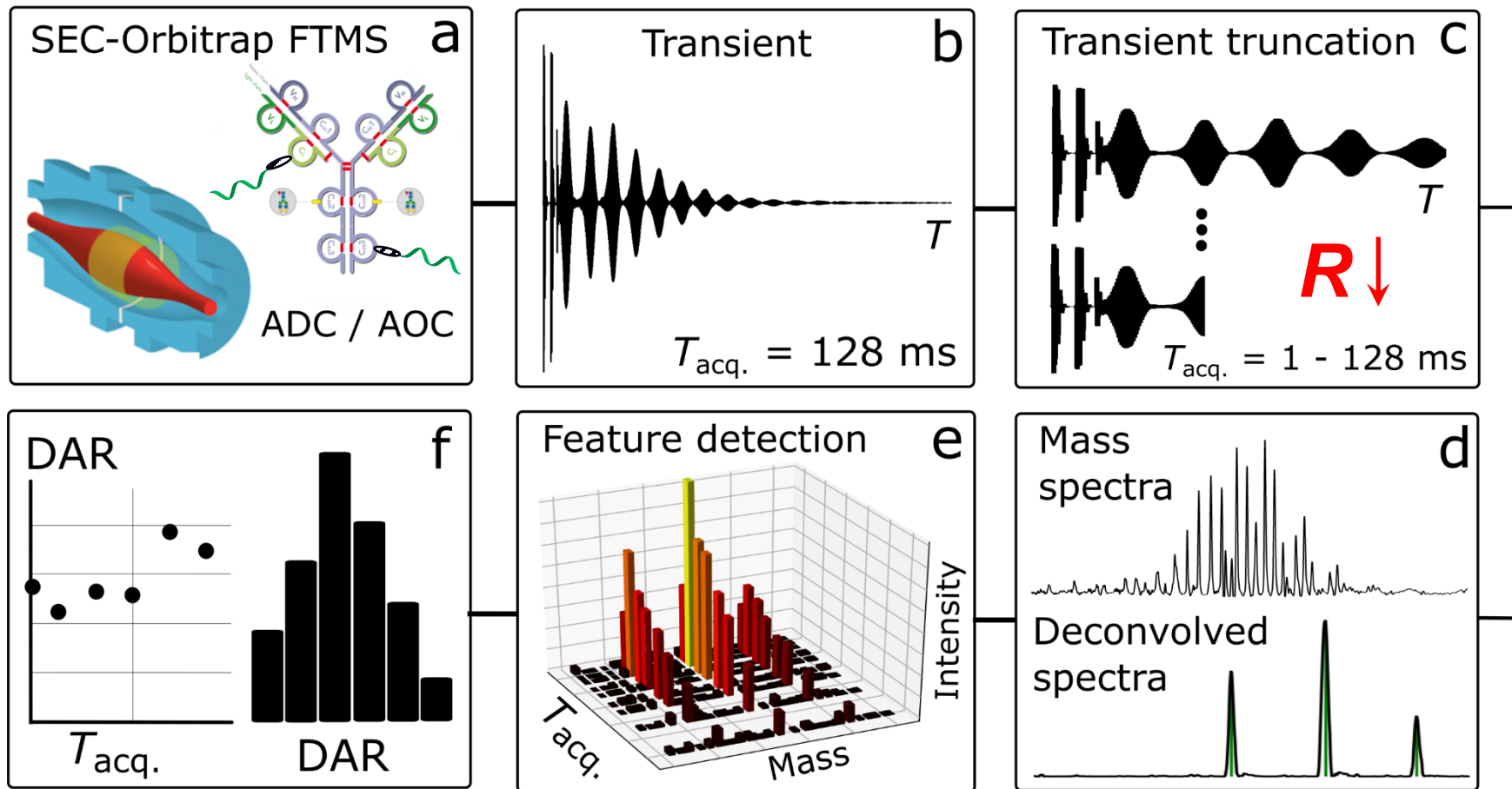$$R \sim T$$

Anal Chem, 2021, 12930
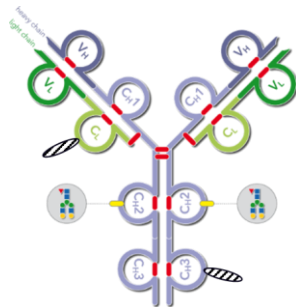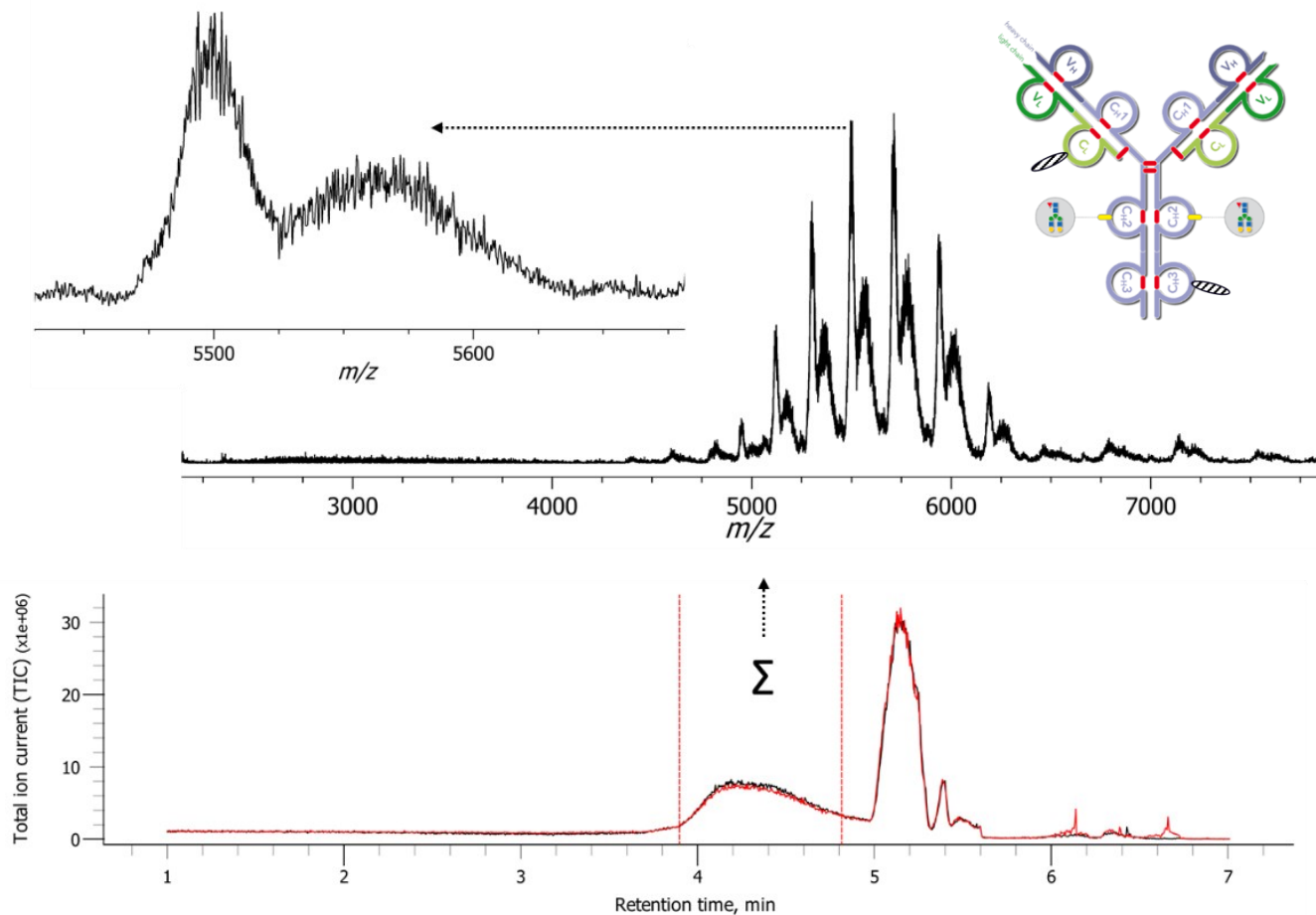
JASMS 2020, 257

JASMS 2021, 1224

T

50 Ω
In-built DAQ system

transient (.DAT)

In-built computer

FTMS Booster

Preamplifier

High Ω

transient (.H5)

peak by peak
FTMS DATA ANALYSIS
by spectroswiss

Software

High-performance **FPGA** chip

triggering, sync, in-phase signal

Signal conditioning

ADC

Embedded **PC** with **SSD** storage

ESI

QE schematics: www.planetorbitrap.com

Proteoform Peak Integration Workflow

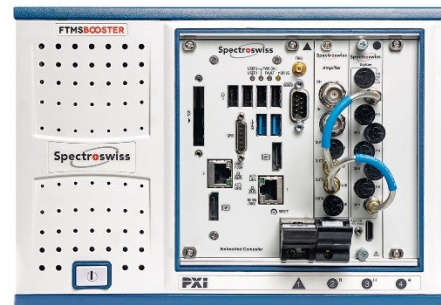*Nagornov et al., Anal Chem, 2021, 12930*

Analysis of an Antibody-Crosslinker Conjugate

Native mode analysis of trastuzumab modified with cross-linkers

Sample separation with SEC (20 ug per infusion)

FTMS: a Q Exactive HF BioPharma. Settings: AGC 5e6, resolution 60k at *m/z* 200 (128 ms)

Analysis of an Antibody-Crosslinker Conjugate

simulated

mAb glycoforms + cross-linkers

Q Exactive HF 15k @ 200 *m/z*

FTMS**SIMULATOR**

Native mode analysis of trastuzumab modified with cross-linkers

Sample separation with SEC (20 ug per infusion)

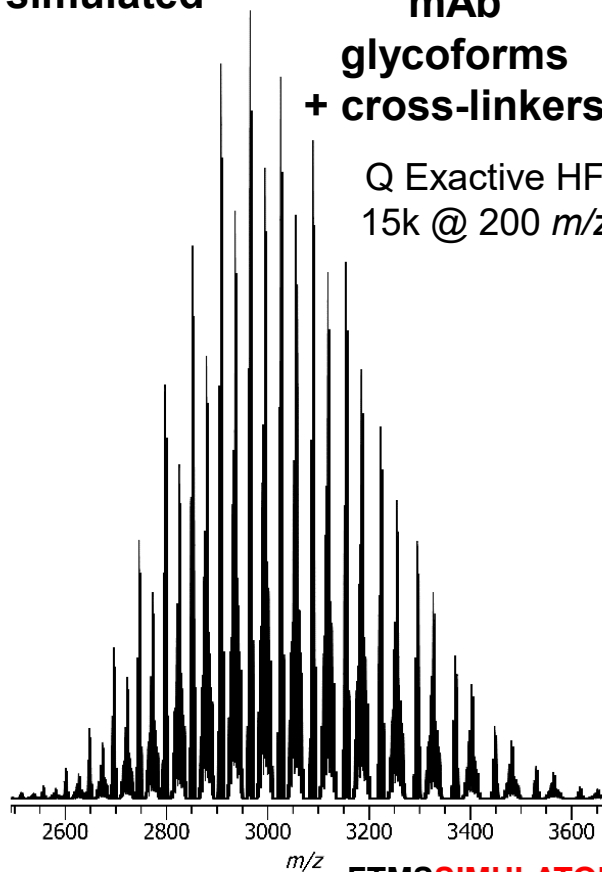FTMS: a Q Exactive HF BioPharma. Settings: AGC 5e6, resolution 60k at *m/z* 200 (128 ms)

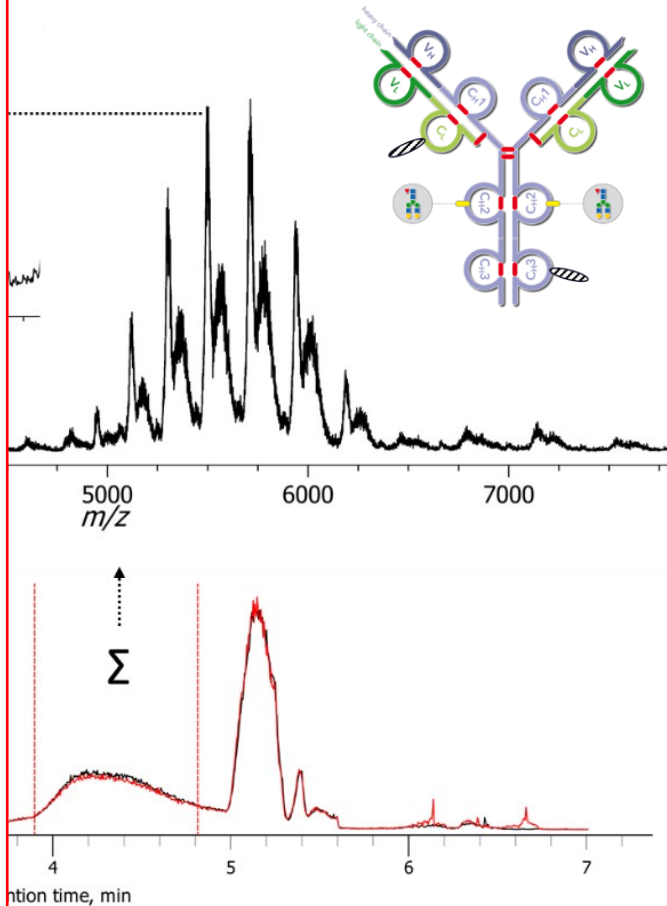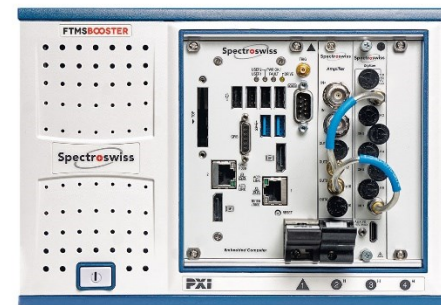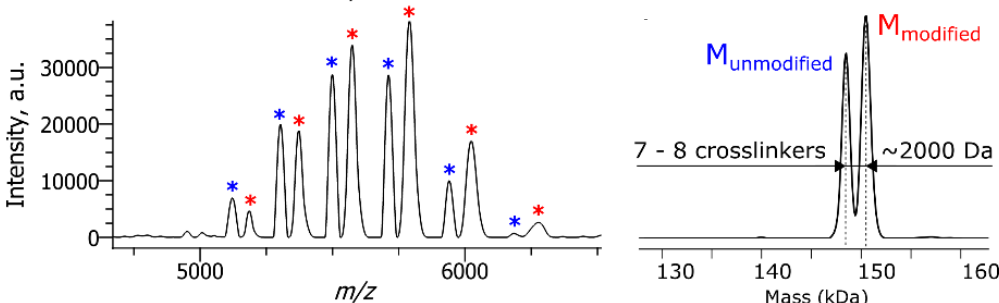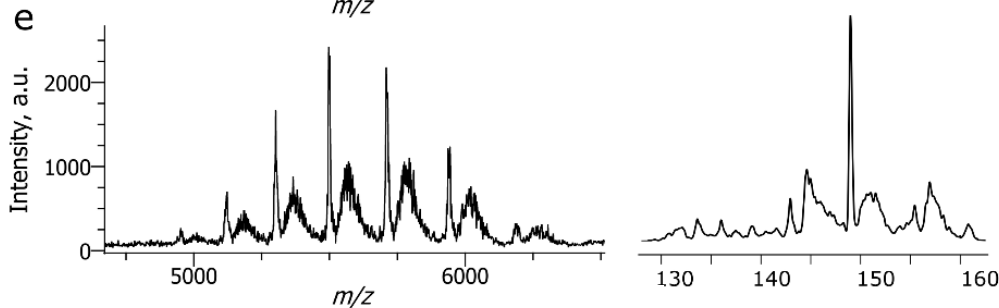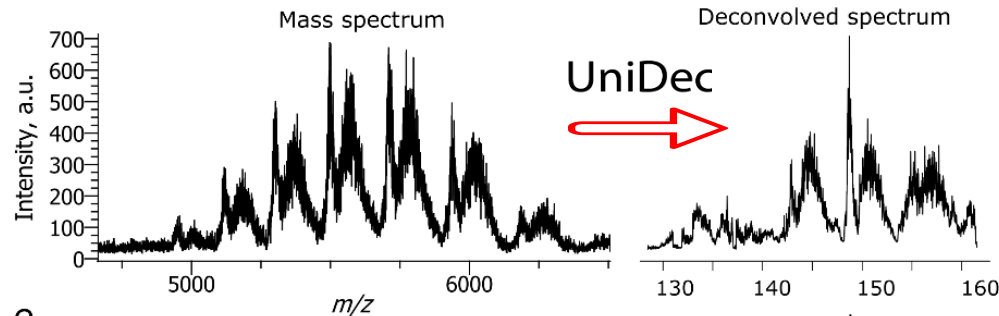# Analysis of an Antibody-Crosslinker Conjugate
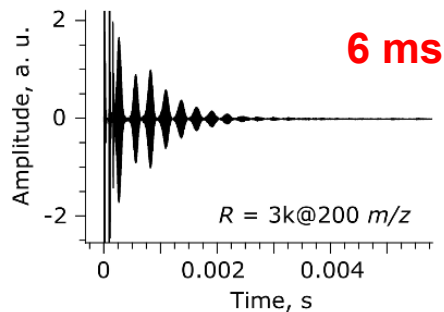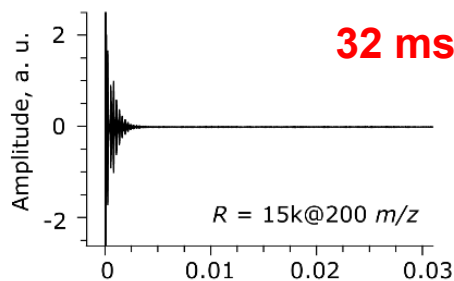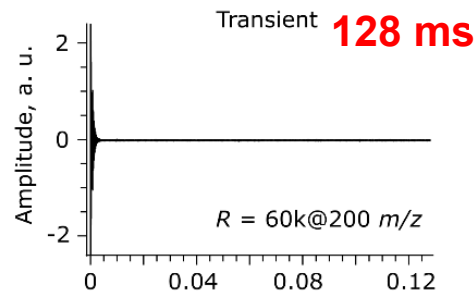
mAb sample:
trastuzumab
(Herceptin)

cross-linkers:
NHS-PEG4-Azide
$C_{11}H_{19}N_3O_5$
MW = 273.29 Da

Experiment:
Native conditions
SEC-Orbitrap
(Q Exactive HF)

Transients:
FTMS Booster
Peak-by-Peak

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Analysis of an Intact AOC Sample: Full Elution Peak

128 ms

$R = 60$ k@200 m/z
$T_{acq} = 128$ ms

DAR = 5.47

32 ms

$R = 15$ k@200 m/z
$T_{acq} = 32$ ms

DAR = 5.05

8 ms

$R = 3$ k@200 m/z
$T_{acq} = 8$ ms

DAR = 5.22

D5
D6
D4
D7

resolution

m/z

Mass, Da
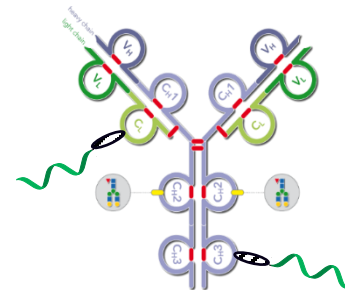
Sample:
Intact AOC

Experiment:
Native conditions
SEC-Orbitrap
(Q Exactive HF)

Transients:
FTMS Booster
Peak-by-Peak

Analysis of an Intact AOC Sample: **Sliding Window**

Application of a sliding window approach to LC-MS analysis of AOCs
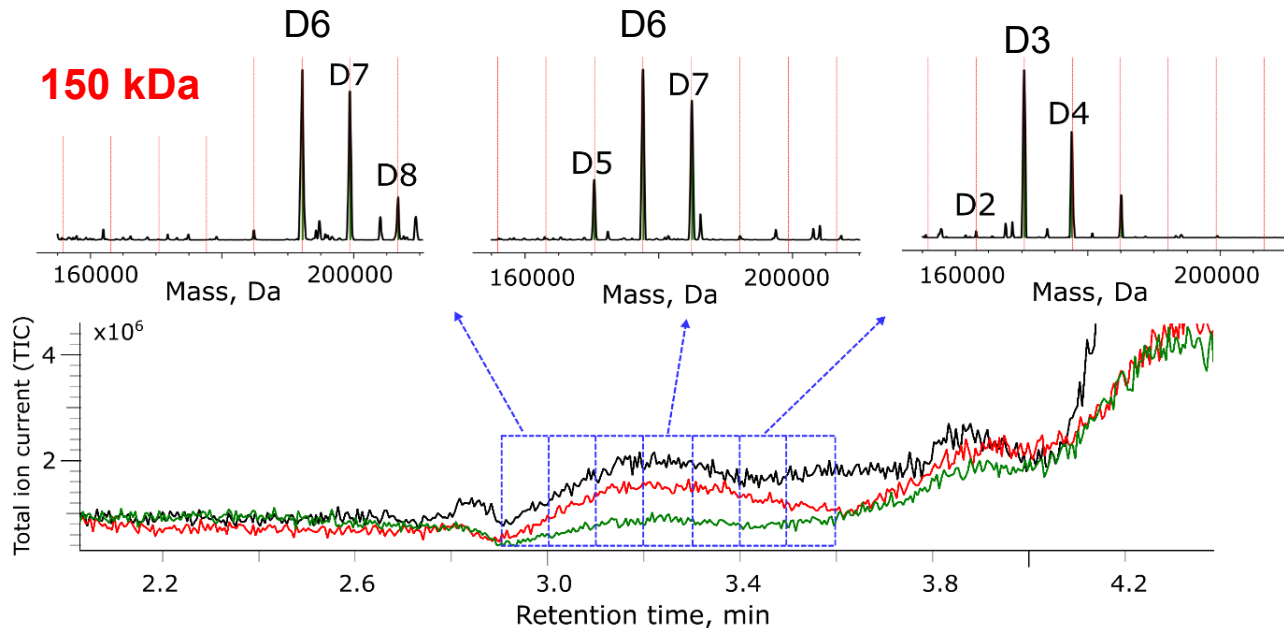

$$DAR = \frac{\sum_{n=0}^{m} (I_n \times n)}{\sum_{n=0}^{m} I_n}$$

Integrated approach

DARs: Integrated approach (**5.22**) *vs.* Sliding window approach (**4.55**)

Sliding window approach: ADC – Thermo PO72283; mAbs – *Eur J Pharm Bopharm* 2021 158: 83
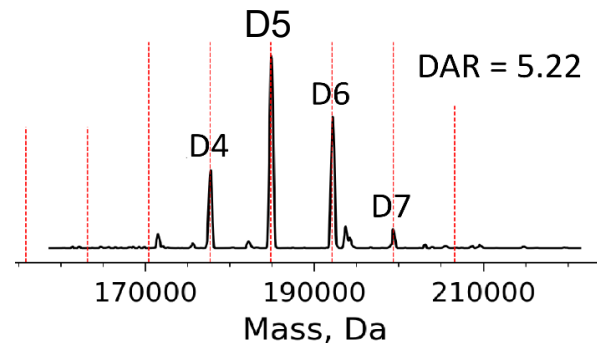
What if the AOC sample complexity is reduced?

Analysis of an Intact AOC Sample: **Sliding Window**

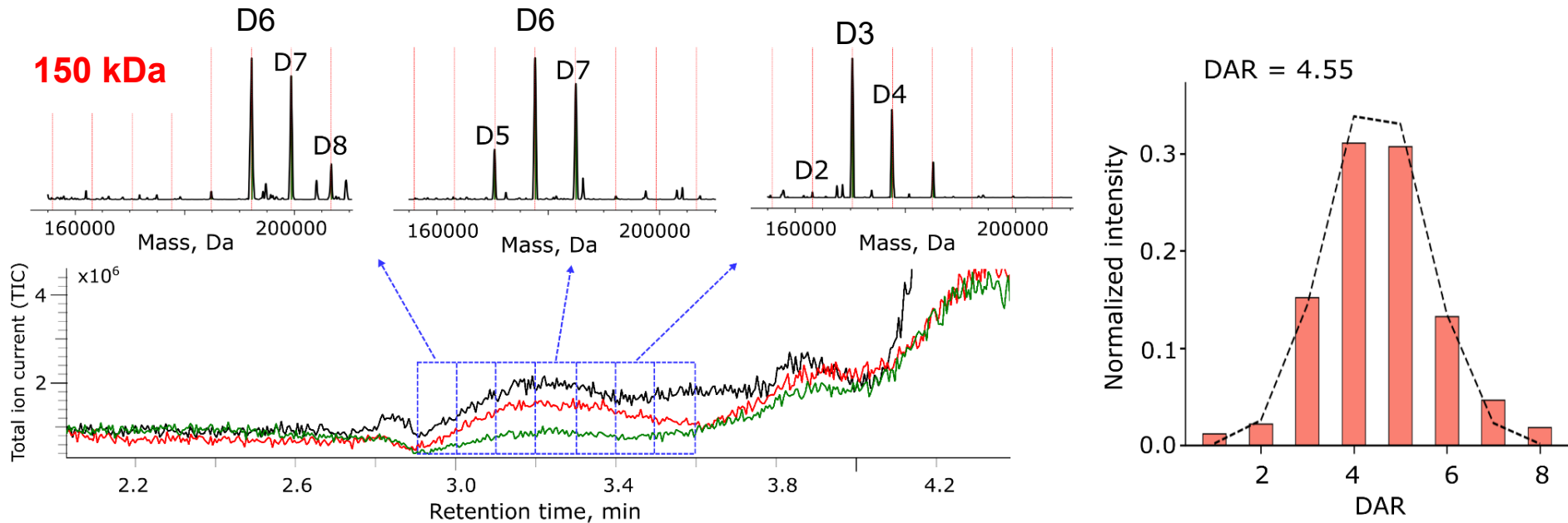**Application of a sliding window approach to LC-MS analysis of AOCs**
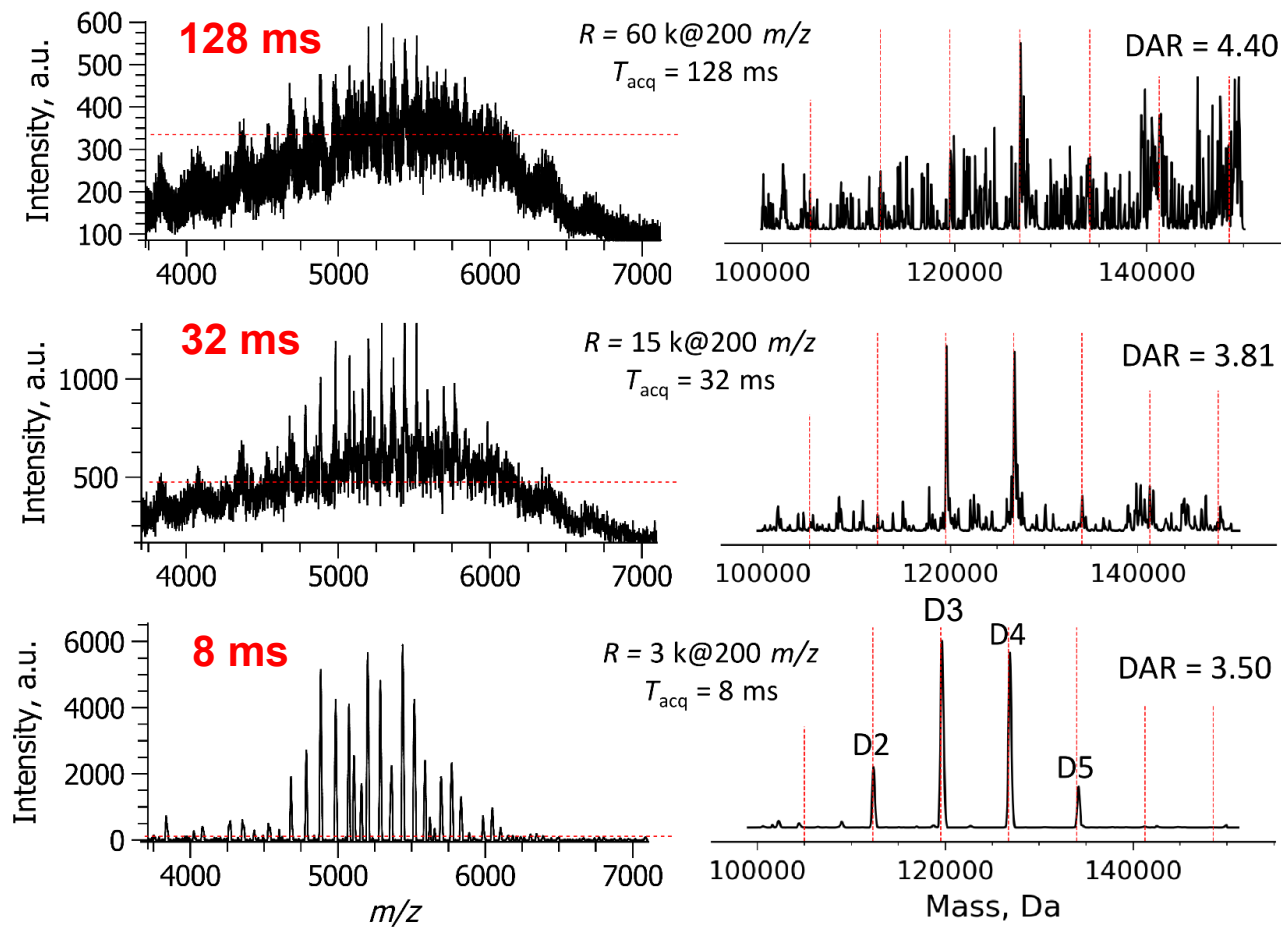

DARs: Integrated approach (**5.22**) *vs.* Sliding window approach (**4.55**)

Sliding window approach: ADC – Thermo PO72283; mAbs – *Eur J Pharm Bopharm* 2021 158: 83

What if the AOC sample complexity is reduced?

Analysis of an Antibody **Subunit**-Drug Conjugate

128 ms
$R = 60\ k@200\ m/z$
$T_{acq} = 128\ ms$
DAR = 4.40

32 ms
$R = 15\ k@200\ m/z$
$T_{acq} = 32\ ms$
DAR = 3.81

8 ms
$R = 3\ k@200\ m/z$
$T_{acq} = 8\ ms$
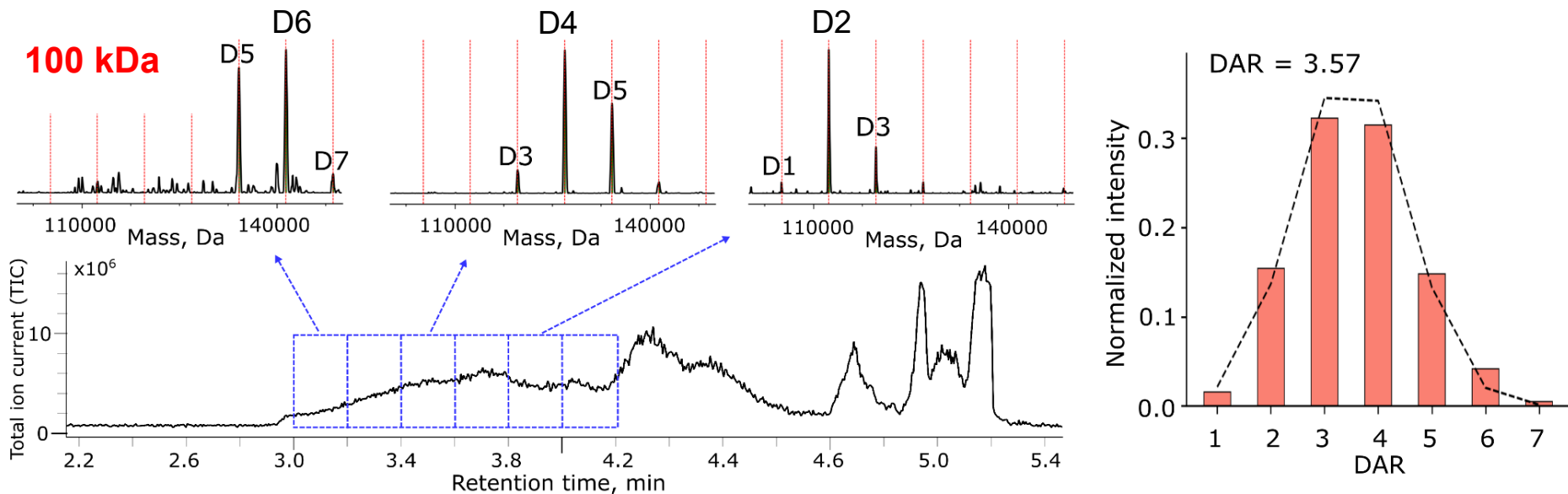DAR = 3.50

D2 D3 D4 D5

AOC sample:
IdeS-derived
F(ab)$_2$ subunit of
trastuzumab
(~100 kDa) with
oligonucleotides
of ~7 kDa each

Experiment:
Native conditions
SEC-Orbitrap
(Q Exactive HF)

Transients:
FTMS Booster
Peak-by-Peak

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Analysis of an Antibody Subunit-Drug Conjugate

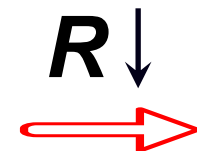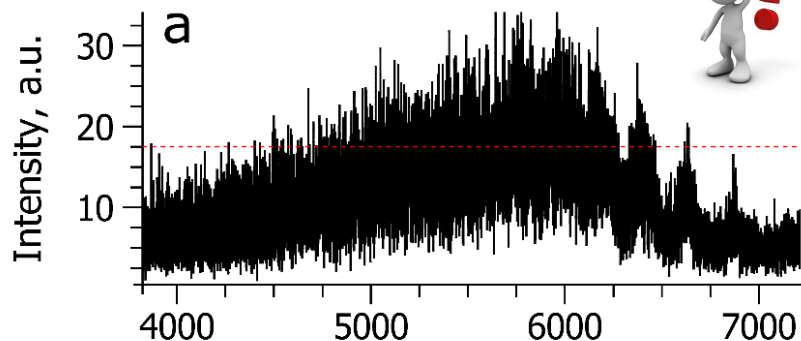**Application of a sliding window approach to LC-MS analysis of ADCs**

DAR estimate: Integrated approach (**3.50**) *vs.* Sliding window approach (**3.57**)

The revealed DARs are in line with the anticipated and simulated values
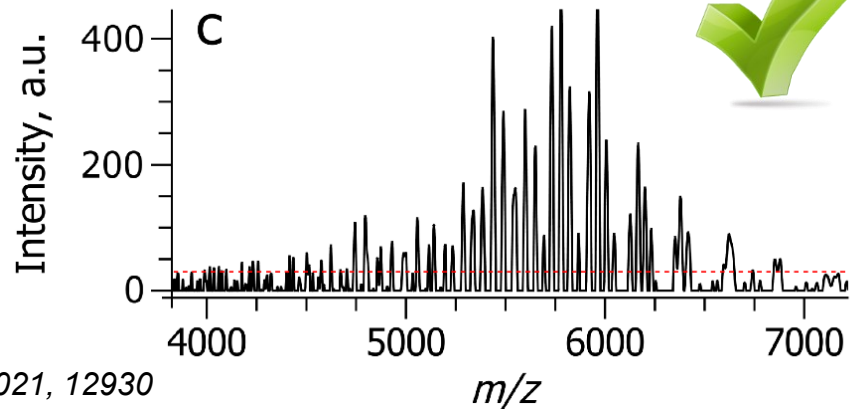
Conclusions

"resolved" proteoforms

integrated proteoforms

R↓

Anal Chem, 2021, 12930

- Proteoform peaks can be integrated by **significant** resolution reduction in Orbitrap FTMS
- **Proteoform integration shows utility for DAR analysis on complex ADC/AOC samples**
- **Sliding window** approach applied to ADC/AOC LC-MS data increases analysis accuracy
- Resolution reduction by user-controlled time-domain transient truncation is readily available
- Practical implementation: method automatization and optimization; comparison with TOF MS
- Other use cases in the analysis of heterogenous samples, different ADC/AOC complexity?

Resolution – Use with Caution! Sometimes Less is More.

Thank you!

Spectroswiss

TOP SPEC

WP051

ThP120

FTMS
Workshop
(Wednesday)

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