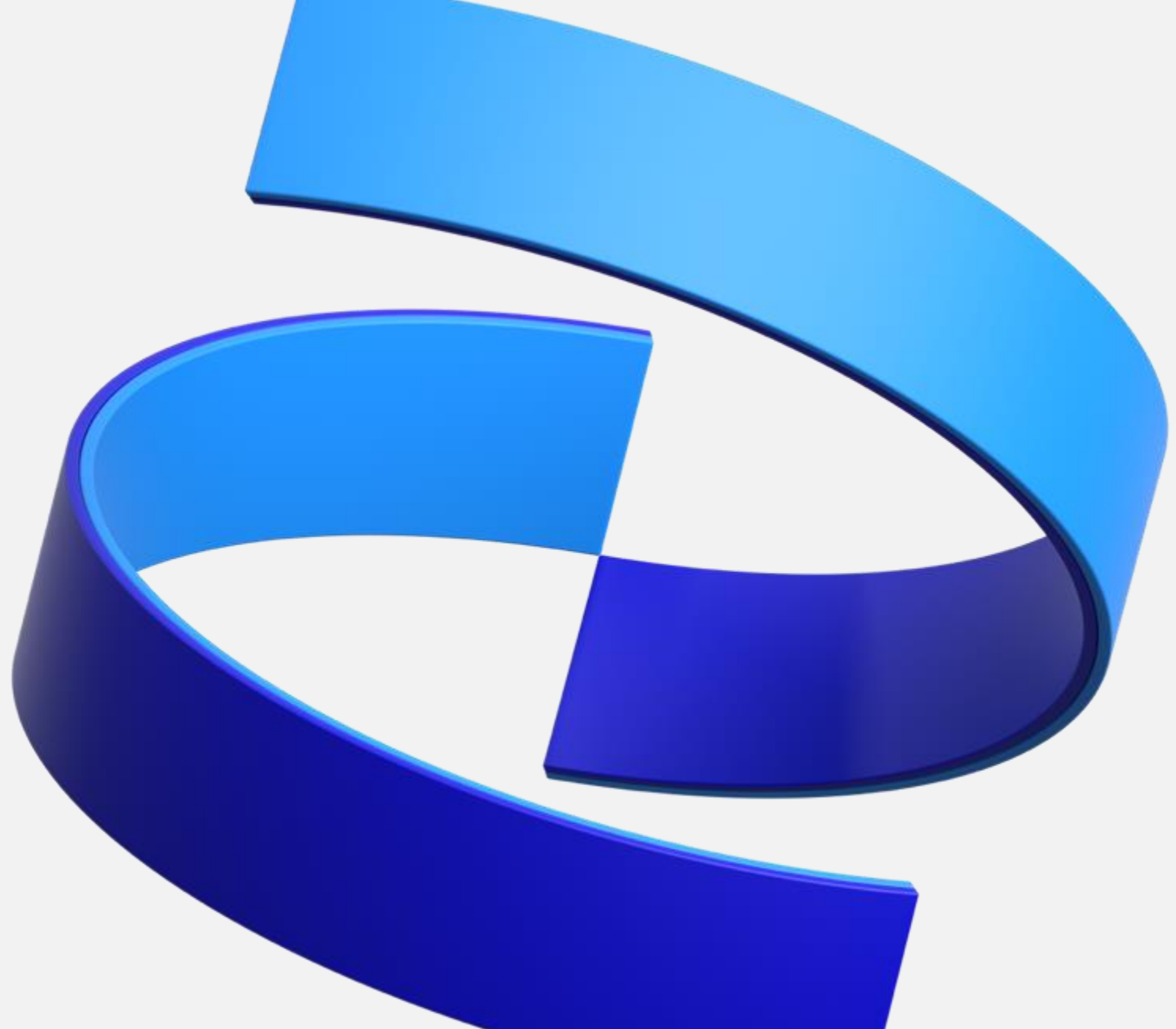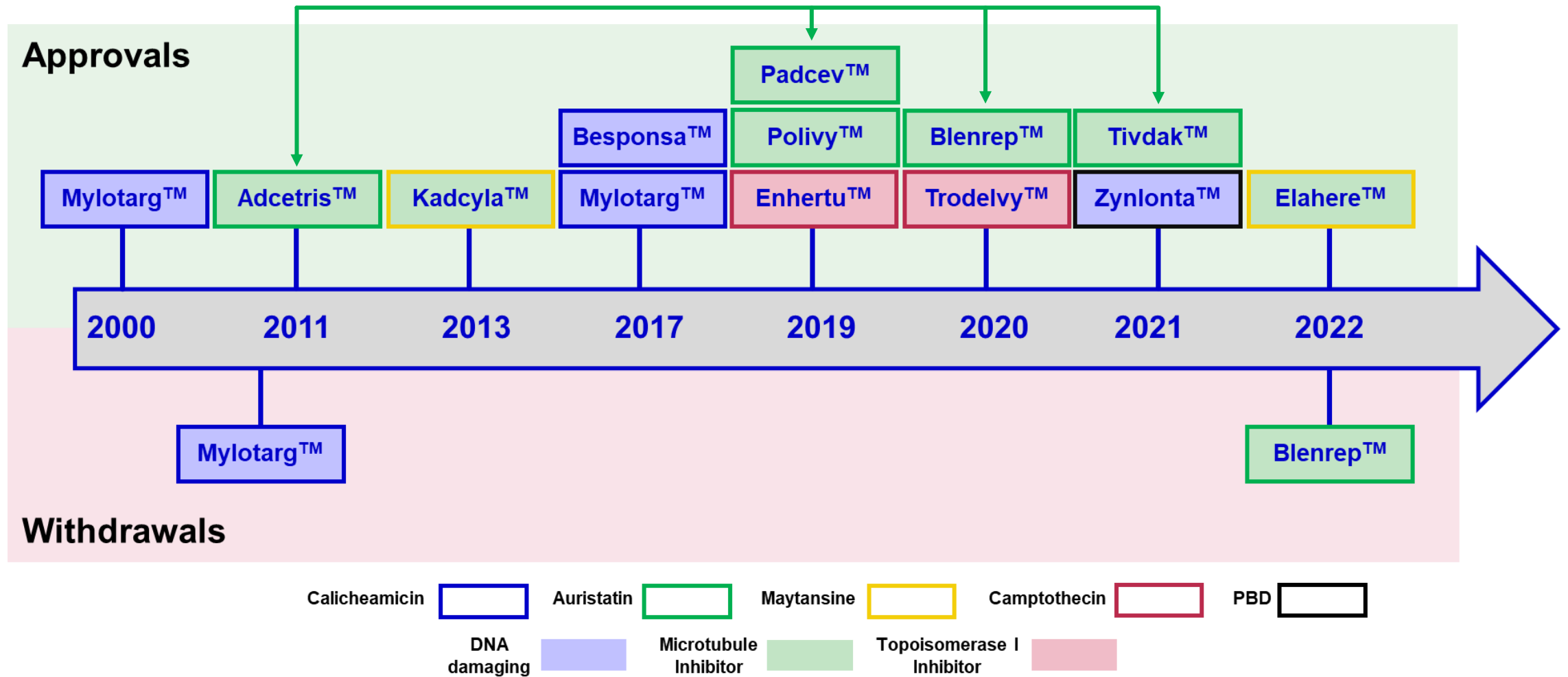# Integration of Mass Spectrometry into ADC Release and Stability Method Development and Process-to-Product Characterization

John Valliere-Douglass
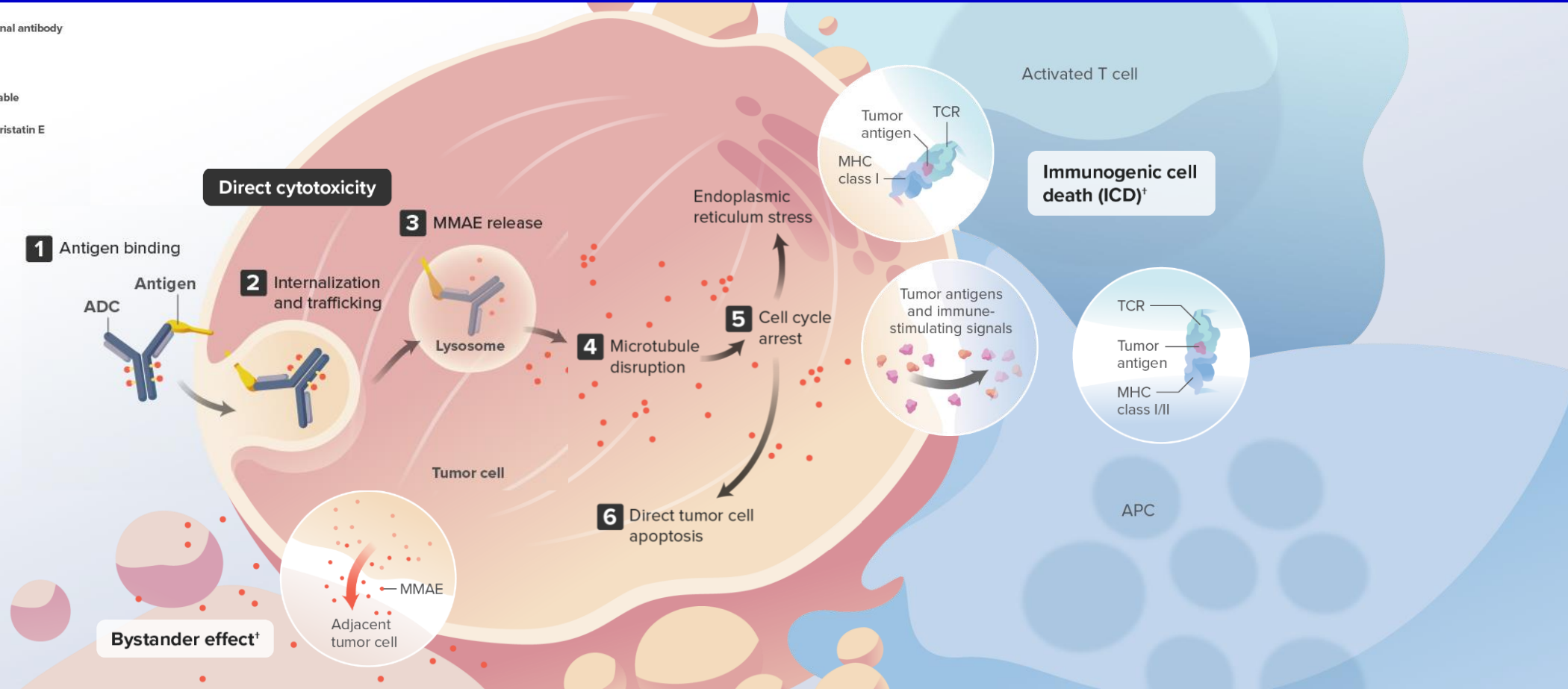
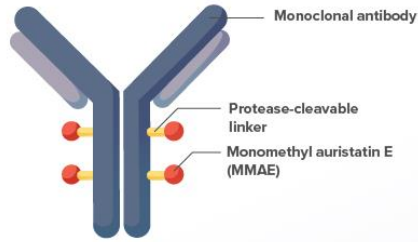Sept 2024

Currently approved ADCs

Exploration of the antibody–drug conjugate clinical landscape Heather Maecker, Vidya Jonnalagadda, Sunil Bhakta, Vasu Jammalamadaka & Jagath R. Junutula, *MABS* 2023, VOL. 15, NO. 1, 2229101

Vedotin ADC mechanism of action involves direct drug-linker mediated cytotoxic killing and immune system recruitment

APC: antigen-presenting cell; MHC: major histocompatibility complex; MMAE: monomethyl auristatin E; TCR: T-cell receptor
†Additional mechanisms of action and their potential to complement the direct cytotoxicity of some MMAE-based antibody-drug conjugates are currently under investigation

Cysteine-linked vedotin ADCs are typically conjugated to a drug-to-antibody ratio (DAR) of ~4

TCEP Reducing Agent (2 M eq)

mc-vc-PAB-MMAE drug-linker

DAR 0

DAR 2

DAR 4

DAR 6

DAR 8
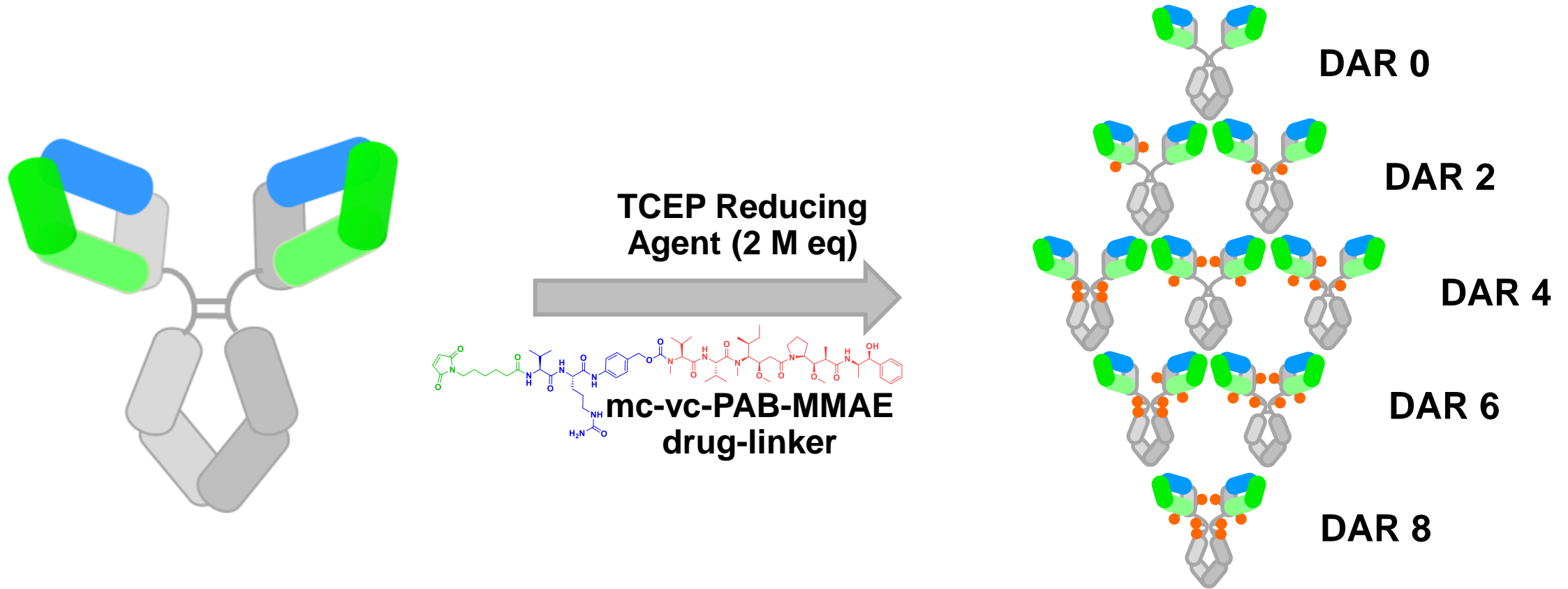
Key distinguishing feature of interchain cysteine-linked ADCs
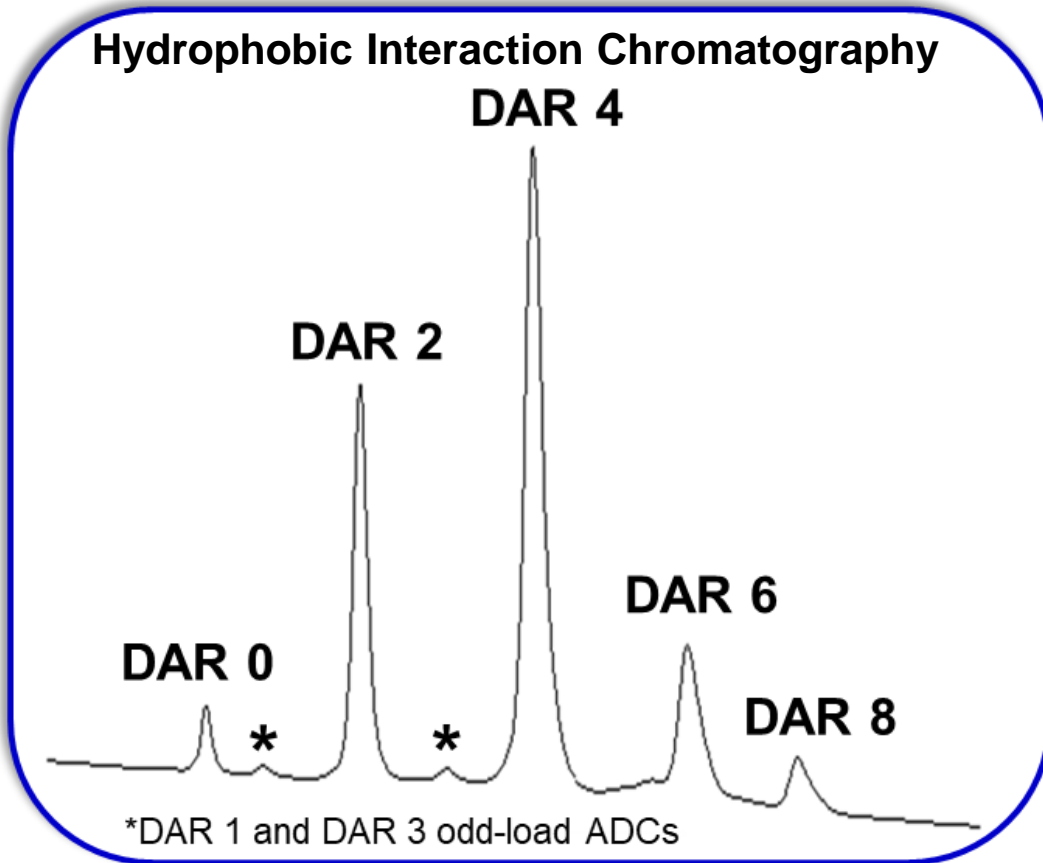
- The ADC is a composite of non-covalent and covalent linked assemblies of drug-linked HCs and LCs

Interchain cysteine linked ADCs are rugged and stable non-covalent assemblies of drug-linked heavy and light chains
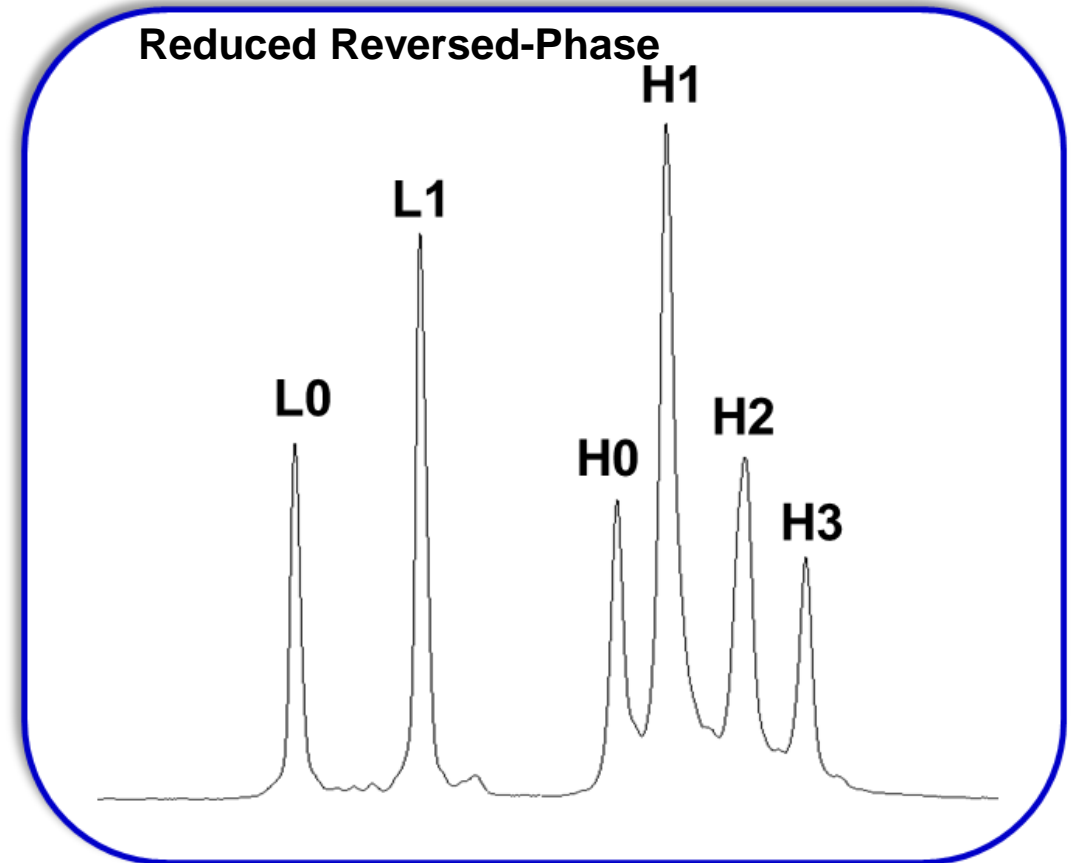

$$\Delta D = D_{ADC} - D_{mAb}$$
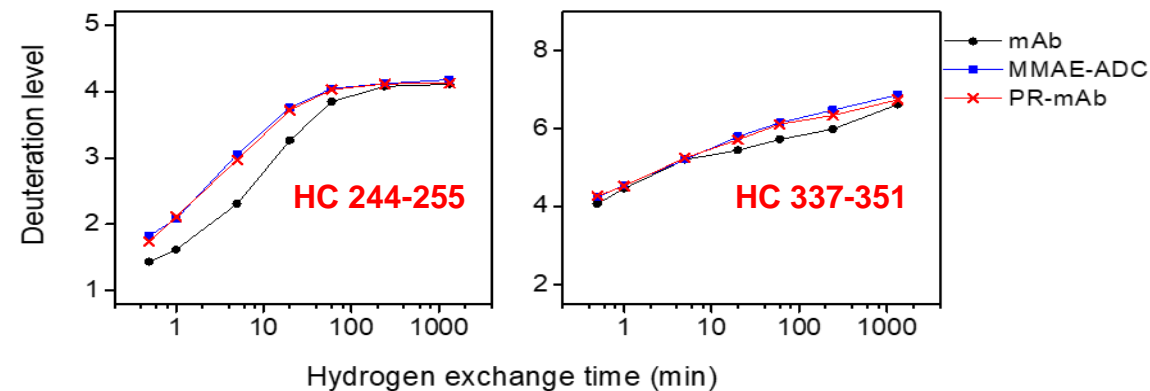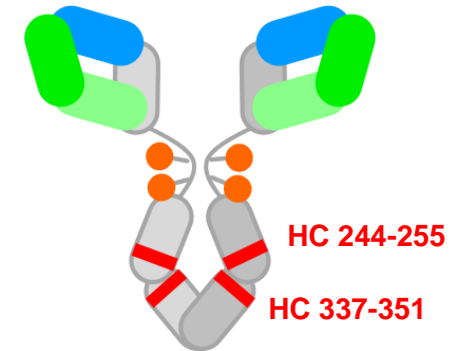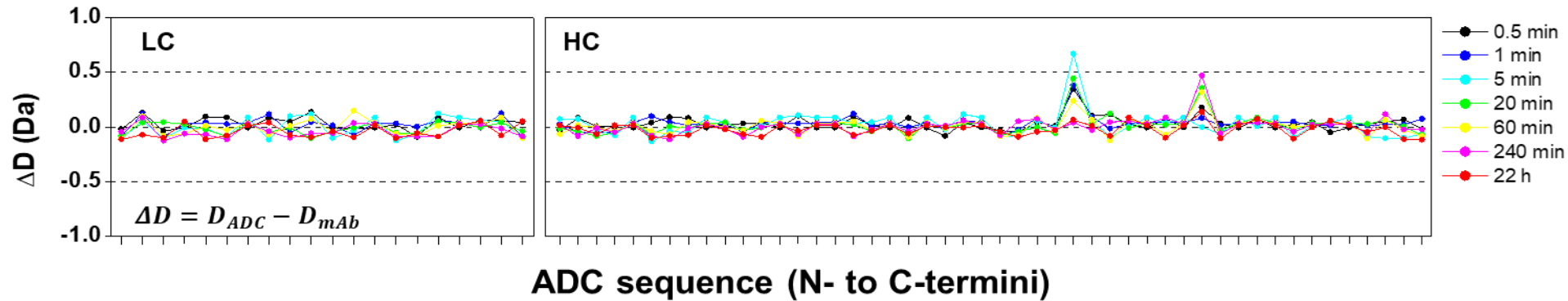
Conformation and Dynamics of Interchain Cysteine-Linked Antibody-Drug Conjugates as Revealed by Hydrogen/Deuterium Exchange Mass Spectrometry, Lucy Pan, Oscar Salas-Solano, John Valliere-Douglass; *Anal. Chem.* 2014 Mar 4;86(5):2657-64.

Rely on reversed-phase LC and HIC to assess the drug-to-antibody ratio (DAR) for cysteine-linked ADCs

Hydrophobic Interaction Chromatography

DAR 4

DAR 2

DAR 6

DAR 0

DAR 8

*

*

*DAR 1 and DAR 3 odd-load ADCs

QC release assay

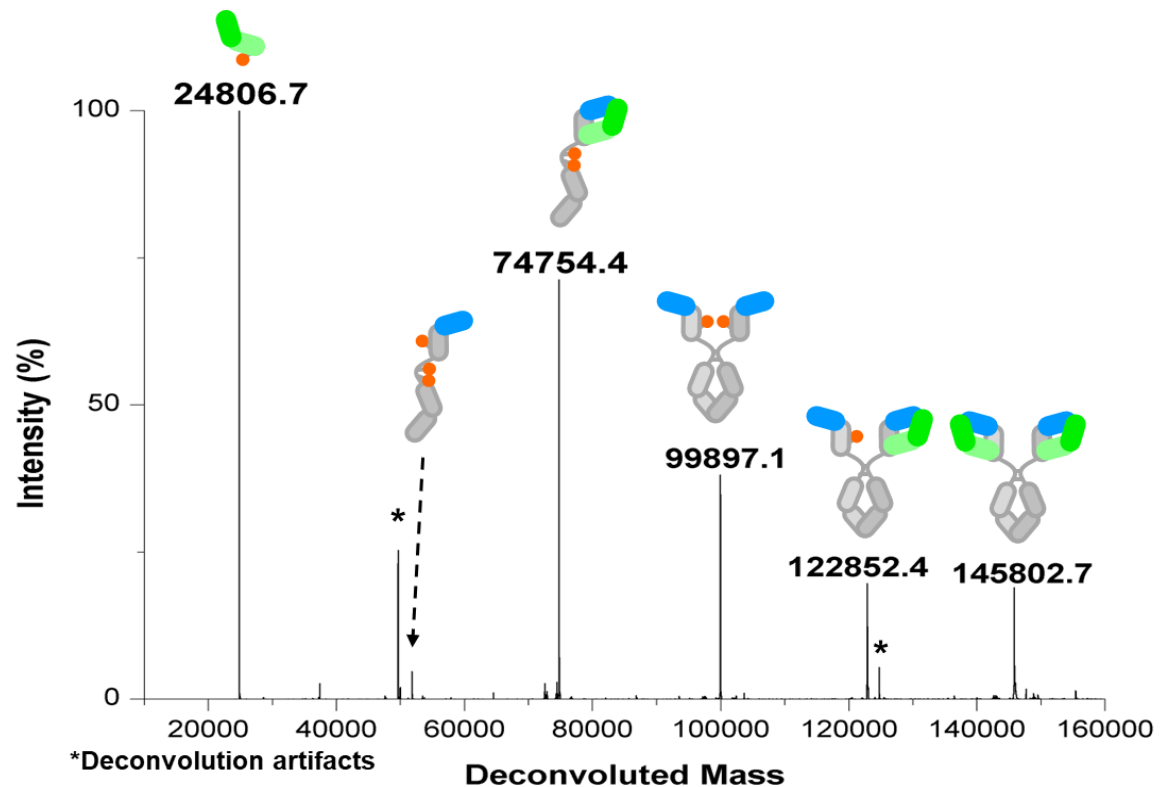**Reduced Reversed-Phase**

H1

L1

L0

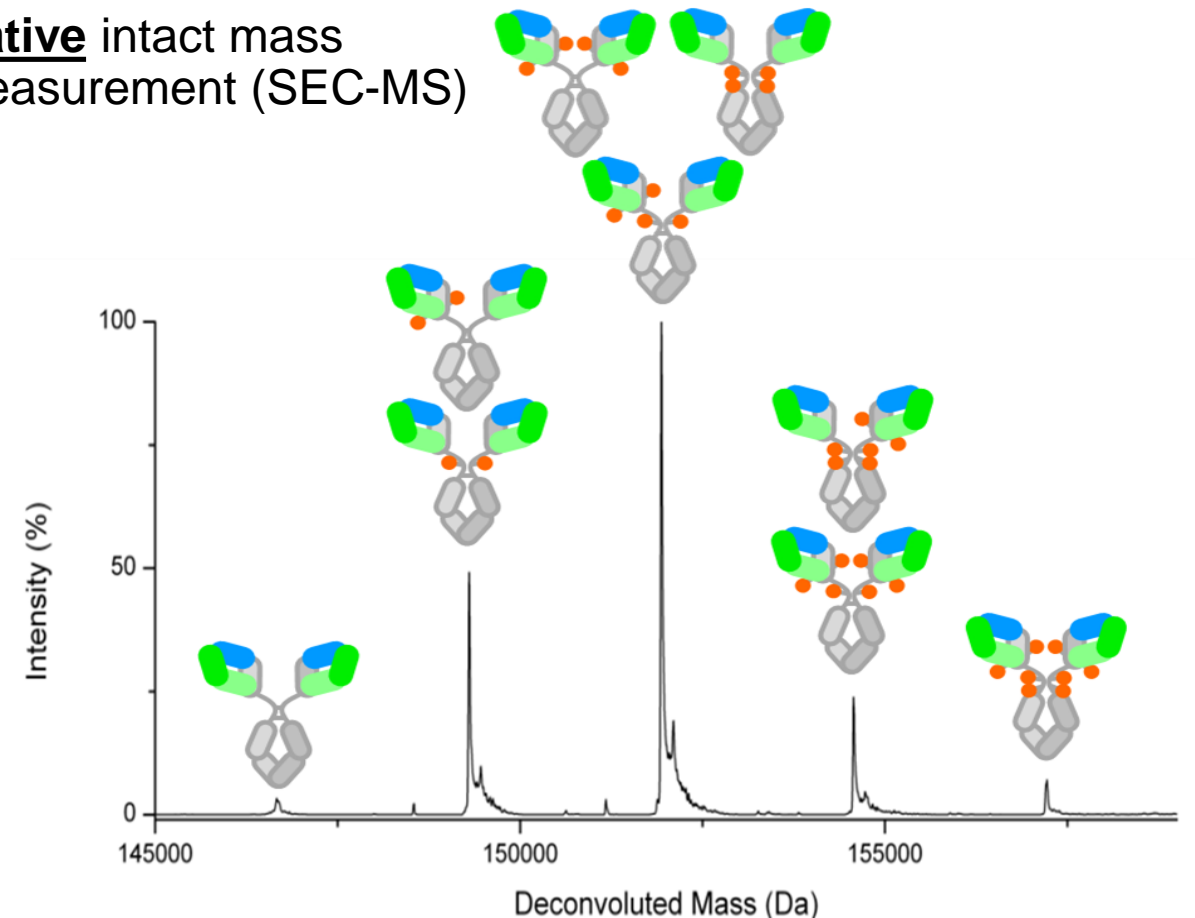H0

H2

H3

LC-MS characterization assay

- Extended characterization and process support

Evolution of MS analytical strategies for intact mass measurement driven by non-covalent interchain cysteine-linked ADCs

Denaturing mass measurement (rpLCMS)

**Native** intact mass measurement (SEC-MS)

Native Intact Mass Determination of Antibodies Conjugated with Monomethyl Auristatin E and F at Interchain Cysteine Residues, John Valliere-Douglass, Bill McFee, Oscar Salas-Solano; A*nal. Chem.* 2012, 84, 6, 2843–2849

Understanding the *in vivo* disposition of individual ADC drug-loaded species is key for developing next-gen modalities
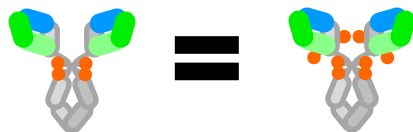
Effects of Drug Loading on the Antitumor Activity of a Monoclonal Antibody Drug Conjugate

Kevin J. Hamblett et al; *Clin Canc Res*, Vol. 10, 7063–7070.

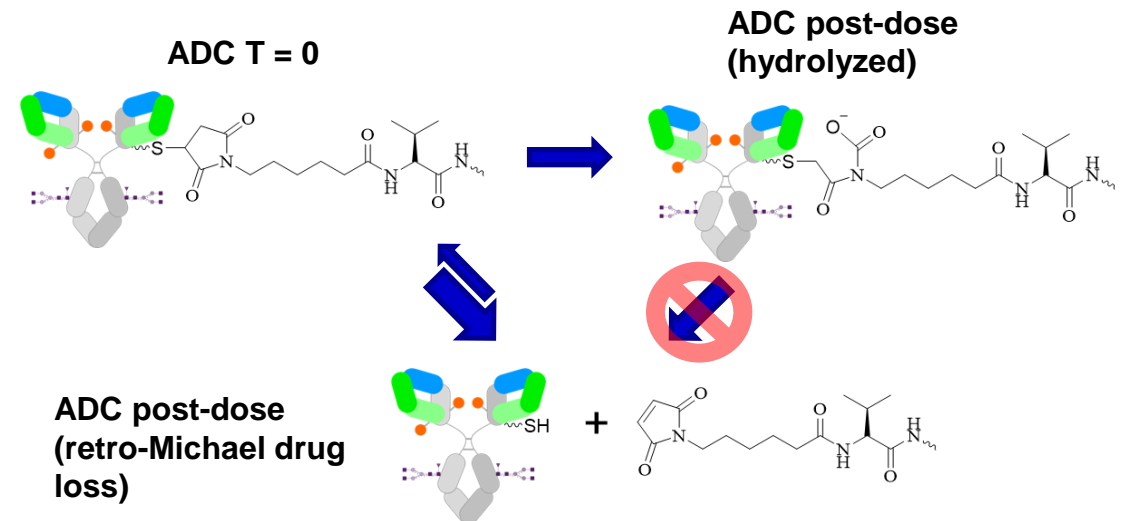- *In vitro* (cell-based assay): cytotoxicity is proportional to drug-load
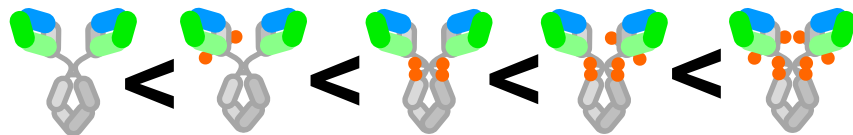
- *In vivo* (tumor xenograft) models: 4-load is equivalent to an 8-load at the same ADC dose

- *Key finding:* higher loaded ADCs are cleared more rapidly

ADCs undergo hydrolysis and retro-Michael chemical transformations *in vivo*
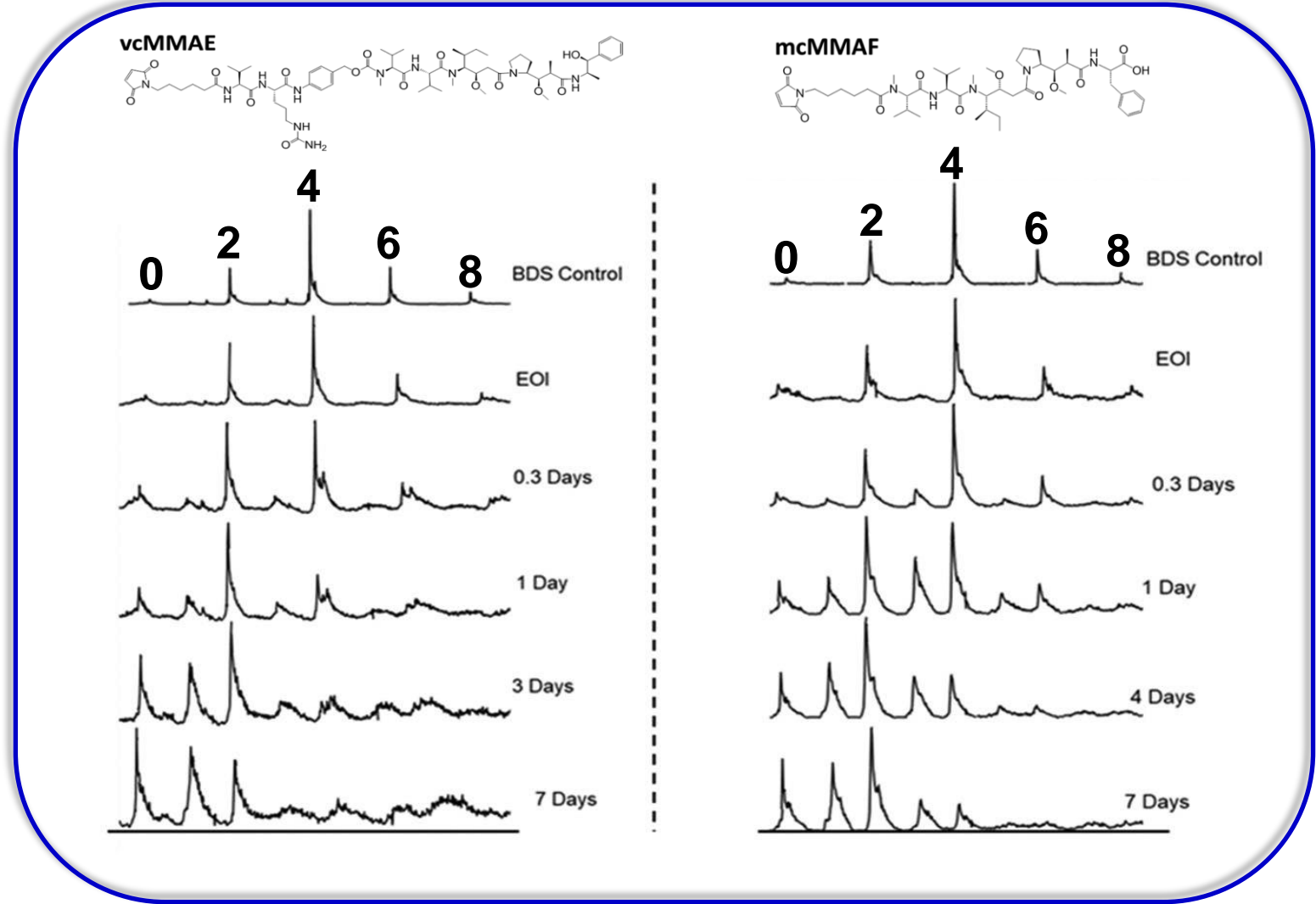
ADC T = 0

ADC post-dose (hydrolyzed)

ADC post-dose (retro-Michael drug loss)

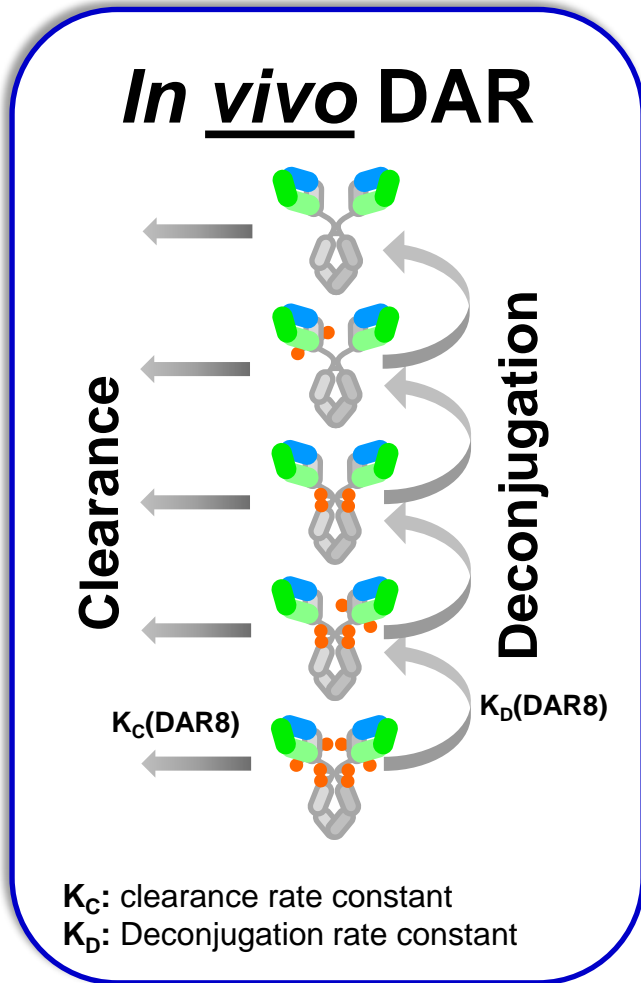
- *Key question:* is retro-Michael drug-loss driving "apparent clearance of higher-loaded forms?

Native SEC-MS provides mechanistic insights into the impact of deconjugation on *in vitro* changes in DAR
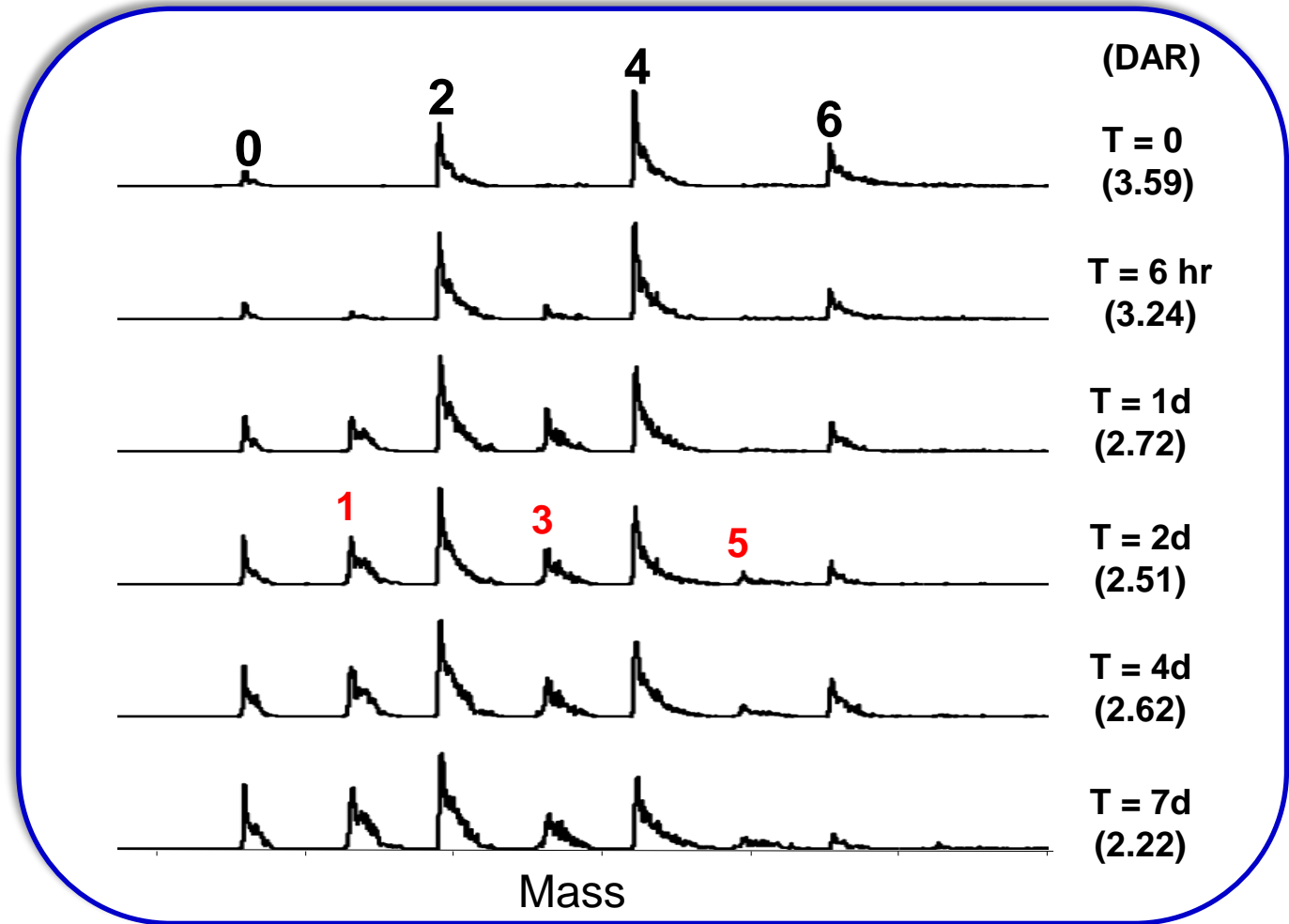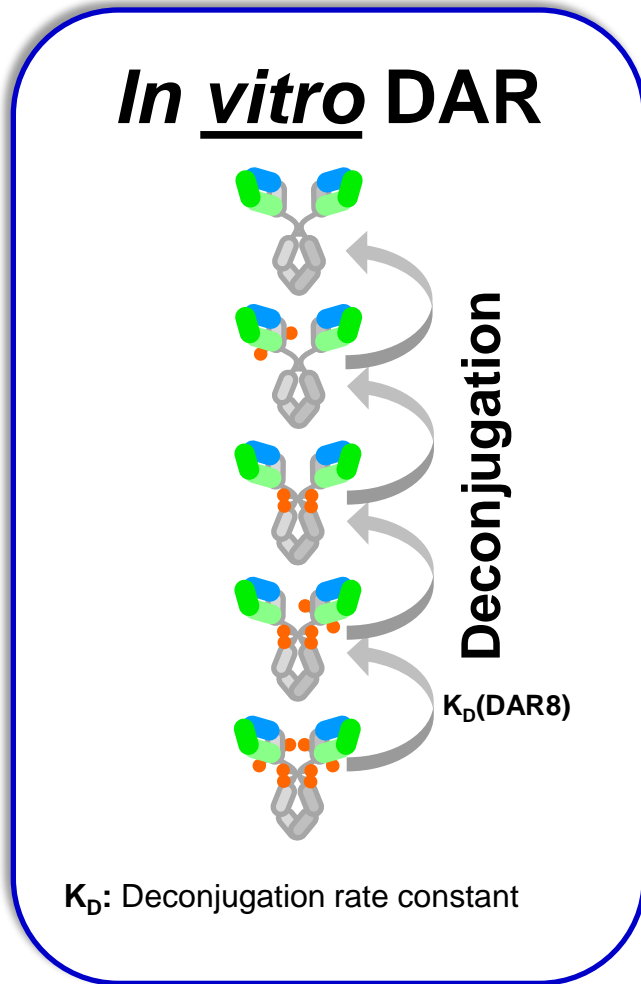
Measurement of in Vivo Drug Load Distribution of Cysteine-Linked Antibody−Drug Conjugates Using Microscale Liquid Chromatography Mass Spectrometry, Shawna Mae Hengel, Russell Sanderson, John Valliere-Douglass, Nicole Nicholas, Chris Leiske, and Stephen C. Alley; A*nal. Chem.* 2014, 86, 3420−3425.

Native SEC-MS provides mechanistic insights into the impact of drug-linker properties on *in **vivo*** clearance of drug-loaded species

In *vivo* DAR

K_C: clearance rate constant
K_D: Deconjugation rate constant

K_C(DAR8) K_D(DAR8)

vcMMAE   mcMMAF

0 2 4 6 8   BDS Control
EOI
0.3 Days
1 Day
3 Days
7 Days

0 2 4 6 8   BDS Control
EOI
0.3 Days
1 Day
4 Days
7 Days

A modelling approach to compare ADC deconjugation and systemic elimination rates of individual drug-load species using native ADC LC-MS data from human plasma, Shawna Mae Hengel et al. Manuscript in press

Refinements on the interchain cysteine-linked vedotin platform to improve pharmacokinetics and therapeutic index

- Immunohistochemistry experiments demonstrated that non-specific clearance of higher loaded ADCs occurred through selective uptake by Kupffer cells in the liver

- Mitigate DL-dependent clearance through linker design

  - In vivo potency for higher loaded Vedotin ADCs with hydrophilic linkers was proportional drug-load (in contrast to Hamblett *et al* observations based on hydrophobic 1st gen ADCs)

  - Led to 2nd gen vedotin-based ADCs that incorporated PEG and/or charged chemical groups into the linker and were conjugated to homogeneous 8-loads

Reducing hydrophobicity of homogeneous antibody-drug conjugates improves pharmacokinetics and therapeutic index, Robert P Lyon *et al*; Nat Biotech, 2015, 33(7), 733-736

Past and present ADC pipeline is comprised of diverse forms of interchain and engineered cysteine-linked ADCs
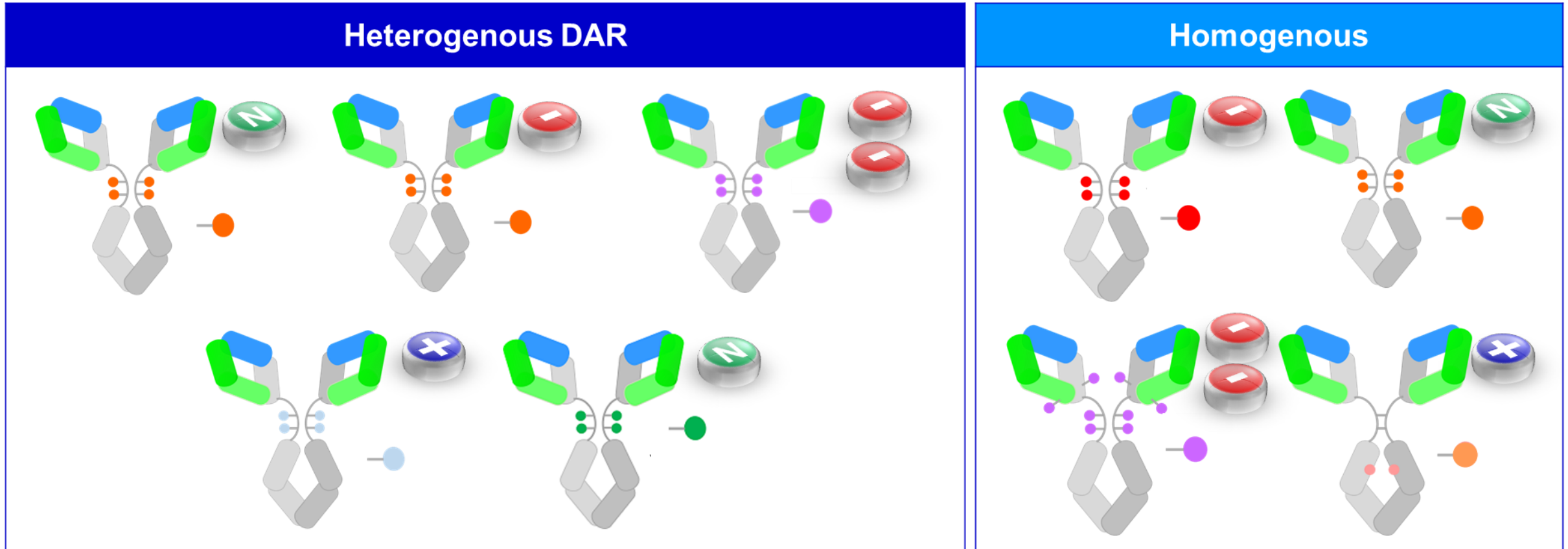
● ● = Auristatin Based   ● = IO   ● = Camptothecin Based   ● = Pyrrolobenzodiazepine-Dimer Based

= Positive charge   = Neutral   = Negative charge

New generation hydrophilic ADCs are poorly resolved by conventional chromatographic methods

Keeping pace with the pipeline requires the development of chemotype-agnostic analytical MS methods

Hydrophobic Interaction Chromatography

Hydrophilic Average DAR4
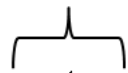
Hydrophobic Average DAR4
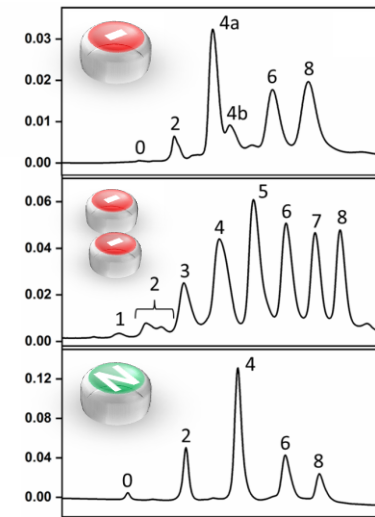
Reduced Revered-Phase

L0

L1

H1

H0

H2

H3
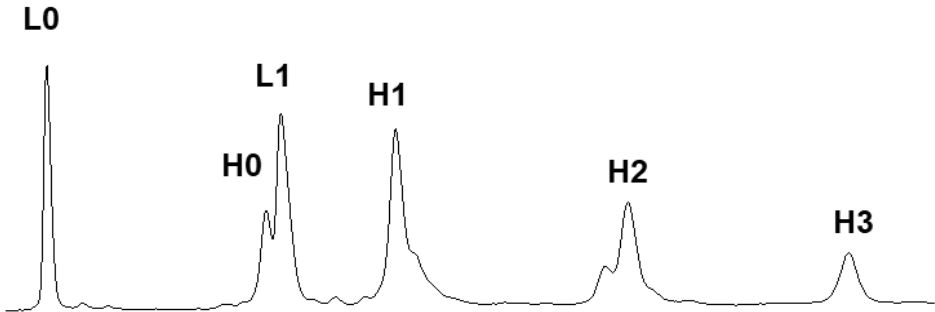
H0, H1, H2, H3
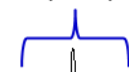
L0, L1
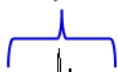
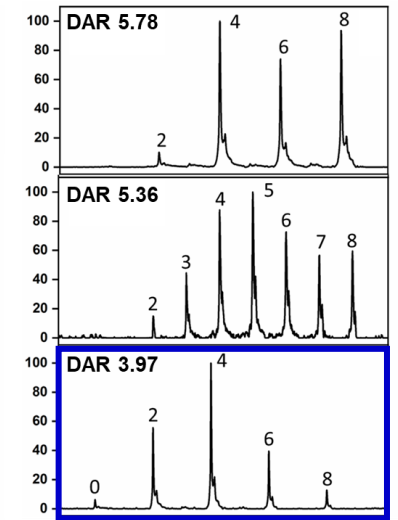# Leveraging native SEC-MS as a chemotype-agnostic DAR assay for a diverse ADC pipeline

Vedotin ADC HIC vs SEC-MS for DAR determination
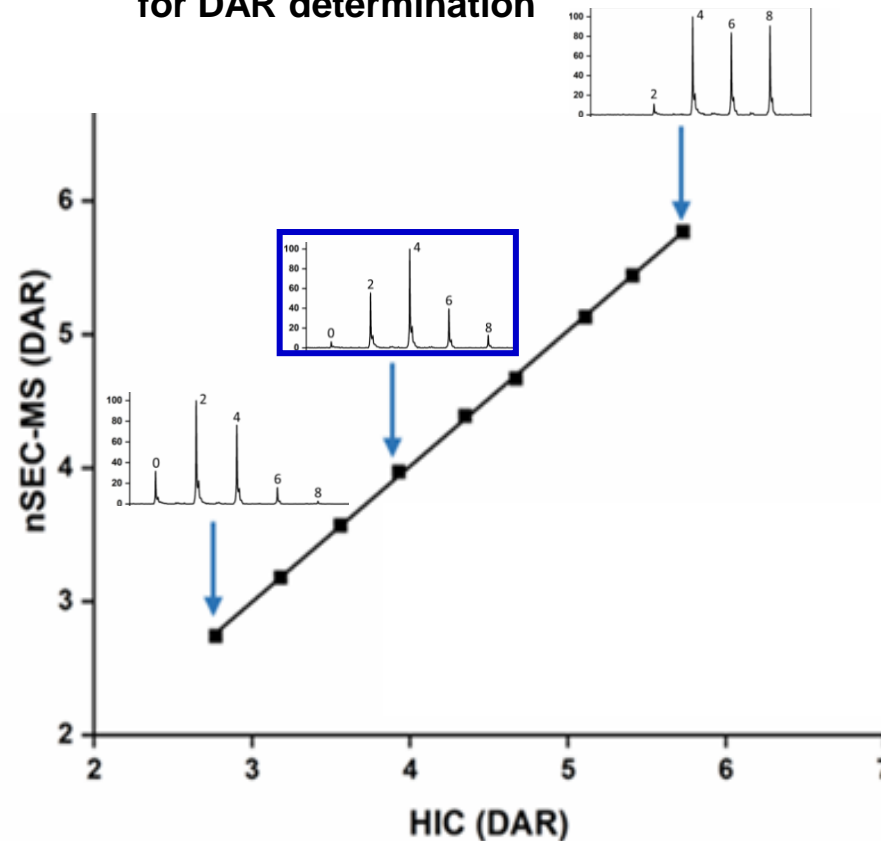

Various ADCs (HIC)   **Native SEC-MS**

Native size-exclusion chromatography-mass spectrometry: suitability for antibody–drug conjugate drug to-antibody ratio quantitation across a range of chemotypes and drug-loading levels; Jay Jones, Laura Pack, Joshua H. Hunter and John F Valliere-Douglass; mAbs, 2020, 12(1).

Native MS is sensitive enough to quantitate minor species that are controlled by release assay specifications
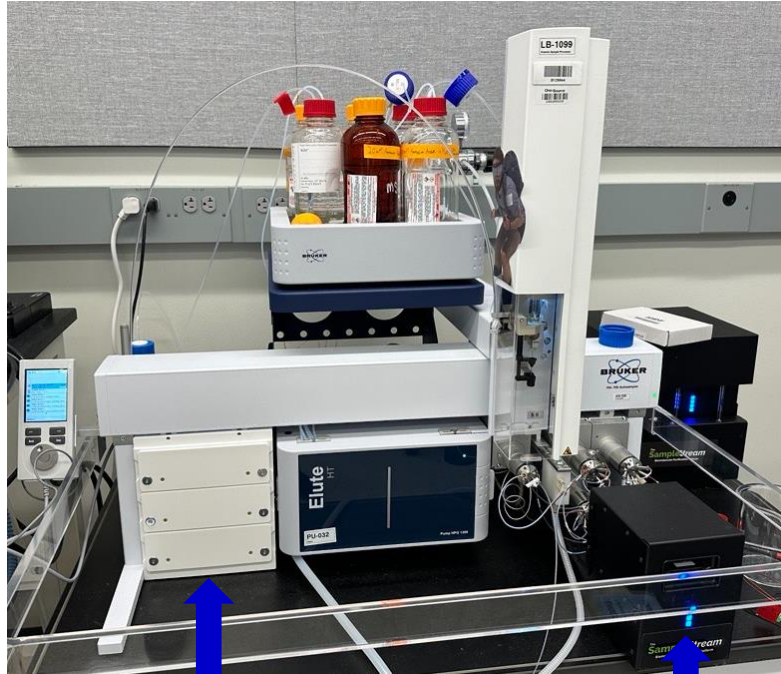
Vedotin 4-load (HC)
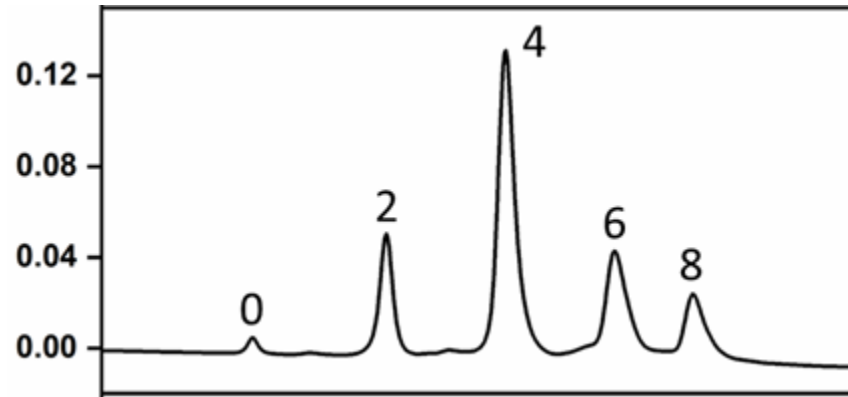
Vedotin 4-load (Native MS)
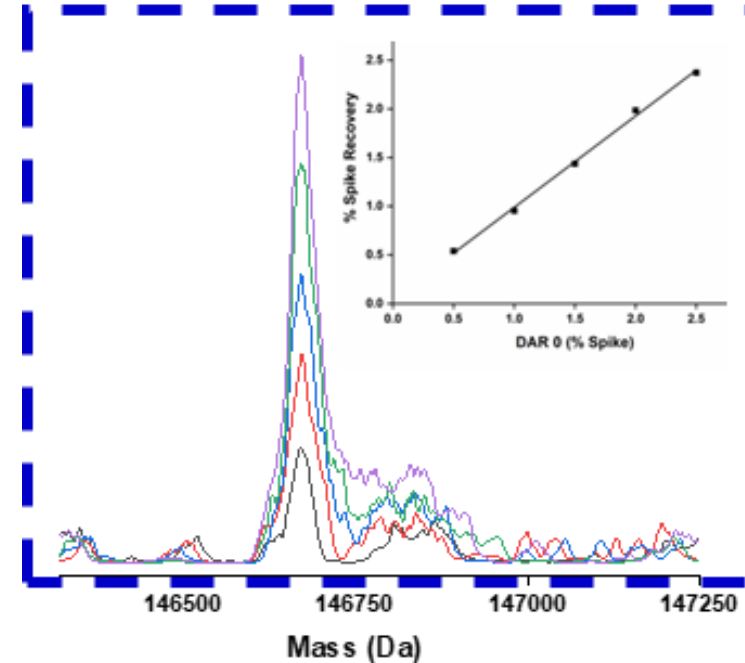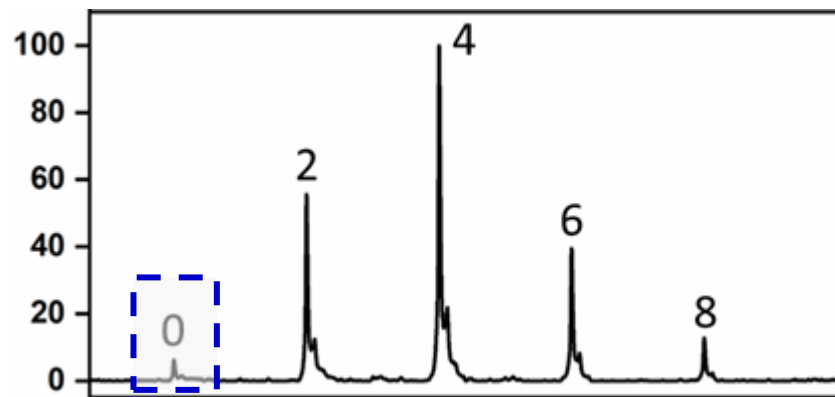
Quantitative recovery and linearity of minor species

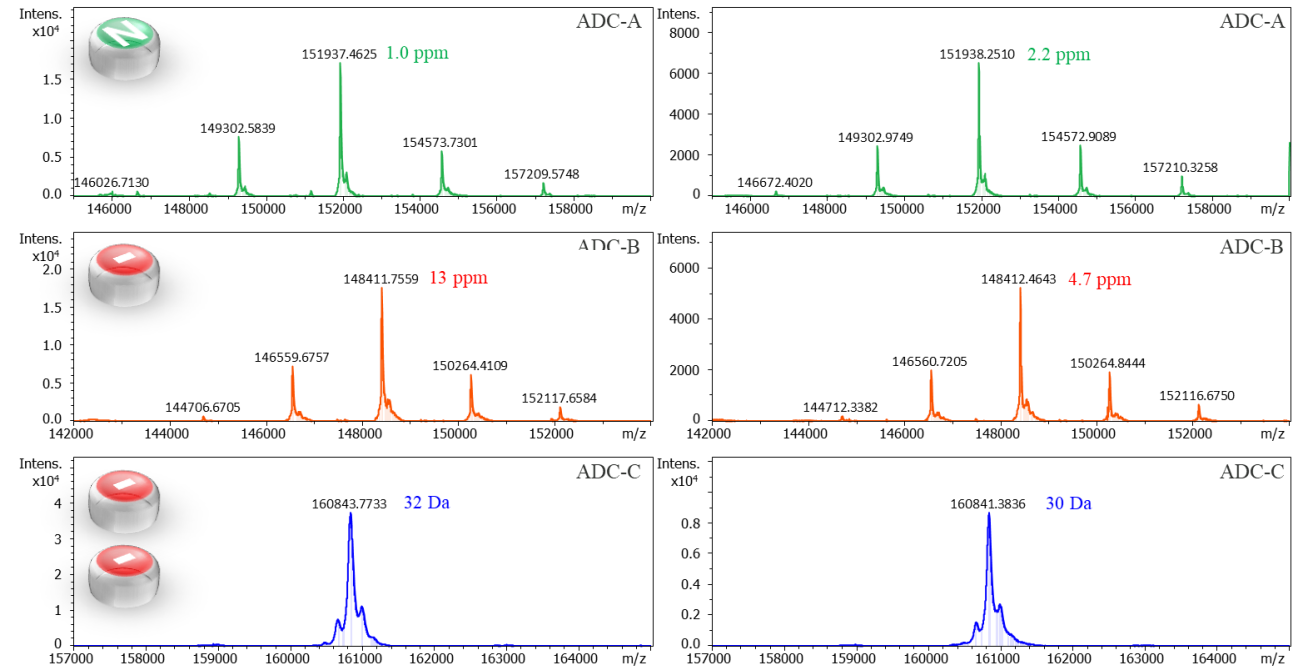# Using a novel interface to improve throughput and ruggedness of native MS workflows for ADCs

Integrated Protein Technologies – SampleStream

Autosampler

Regenerated cellulose membrane chip


- **Novel Interface for High-Throughput Analysis of Biotherapeutics by Electrospray Mass Spectrometry,** Hae-Min Park, Valerie J. Winton, Jared J. Drader, Sheri Manalili Wheeler, Greg A. Lazar, Neil L. Kelleher, Yichin Liu, John C. Tran and Philip D. Compton, *Anal Chem,* 2020, 92, 2186-2193.
- **Automated high-throughput buffer exchange platform enhances rapid flow analysis of antibody drug conjugates by high resolution mass spectrometry,** Yun Yang, Romesh Rao, John Valliere-Douglass and Guillaume Tremintin; *Journal of Chromatography B,* 1235 (2024) 124007.

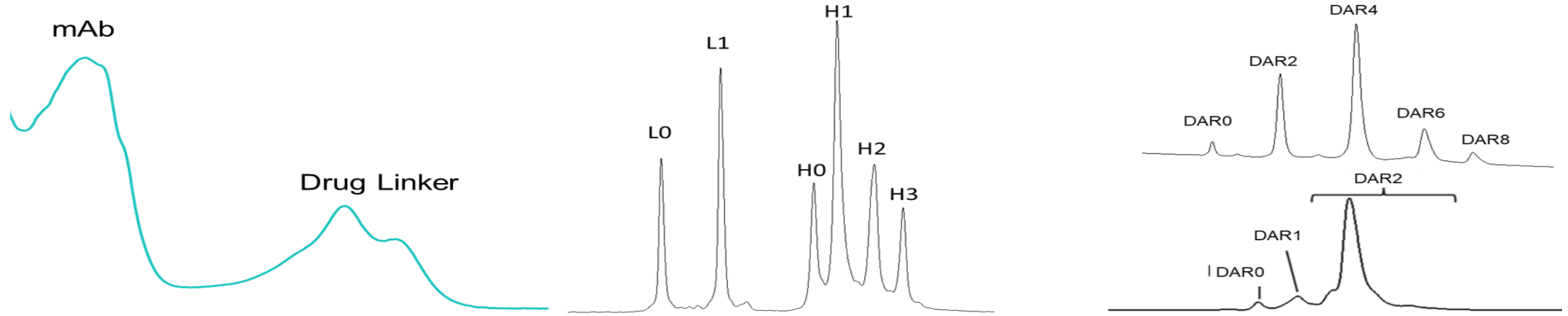# Developing the future platform for high-throughput native-MS analysis of ADCs

SampleStream and Bruker MaXis II Q-TOF
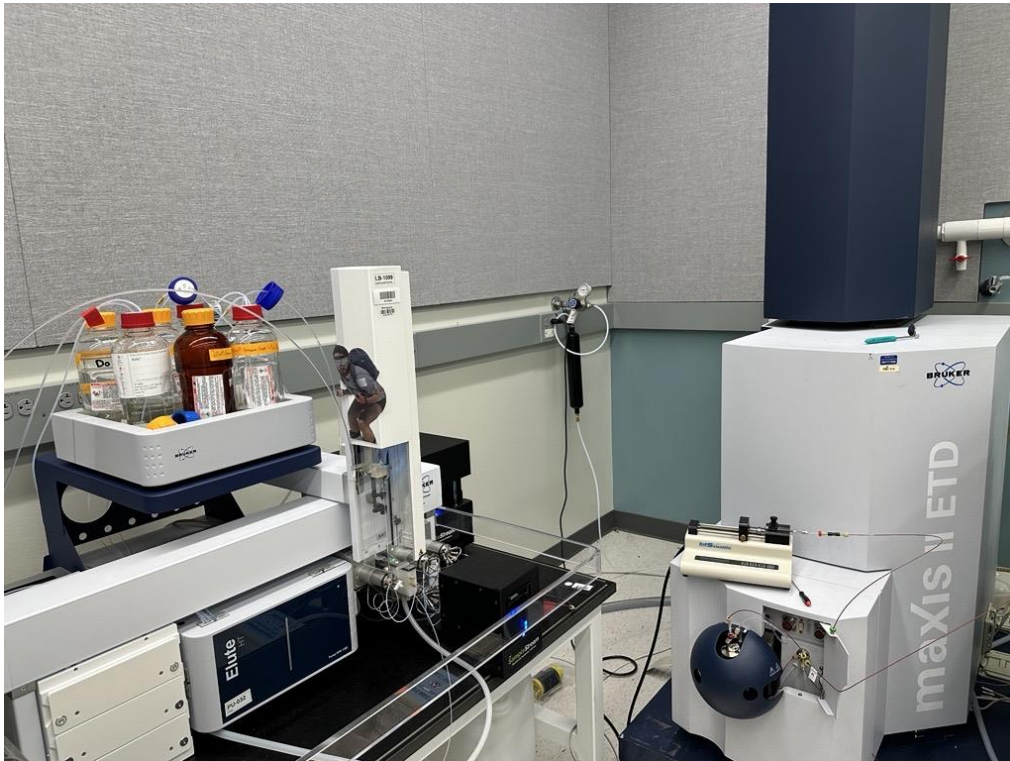
Sample	Drug Linker		Sample Stream-MS	SEC-MS
A	1		4.07	4.05
B			3.92	3.91†
C	2		4.03	4.08
D	3		7.9	7.86†
E			7.84	7.84
F	4		3.84	3.92†
G	5		3.8	3.79
H	6		4.04	4.04

†HIC Data

DAR analytical release assay strategy is driven by ADC format and linker properties

Attribute	DAR by UV	Reduced Denatured Reversed Phase	Native (Reverse Phase or Hydrophobic Interaction)
DAR	🟢	🟢	🟢
Distribution	🔴	🟡	🟢
%DAR0	🔴	🔴	🟢

- **Prior to development of the DAR release assay:** the native MS method supports process development and process to product understanding
- **During DAR assay development:** the native MS method supports attribute to assay understanding and supports assessment of DAR release assay accuracy

Leveraging MS to uncover process-to-product relationships and support release assay development and understanding

Process and product related impurities
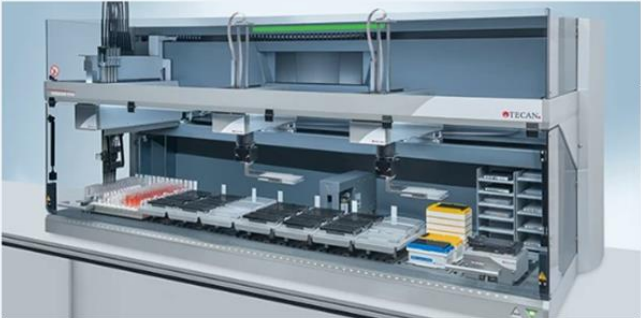
- Cysteine modifications impact DAR, MS is critical for routine monitoring of cysteine modifications

- MS is used to understand how conjugatable impurities manifest as ADC product variants

Degradation pathways

- Charge variant assay characterization
  - Mass spec can be leveraged to understand the composition of charge variants formed in liquid and lyophilized stress conditions

MS for process support is enabled by automation and *most molecular attributes per assay* philosophy
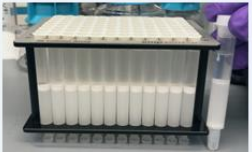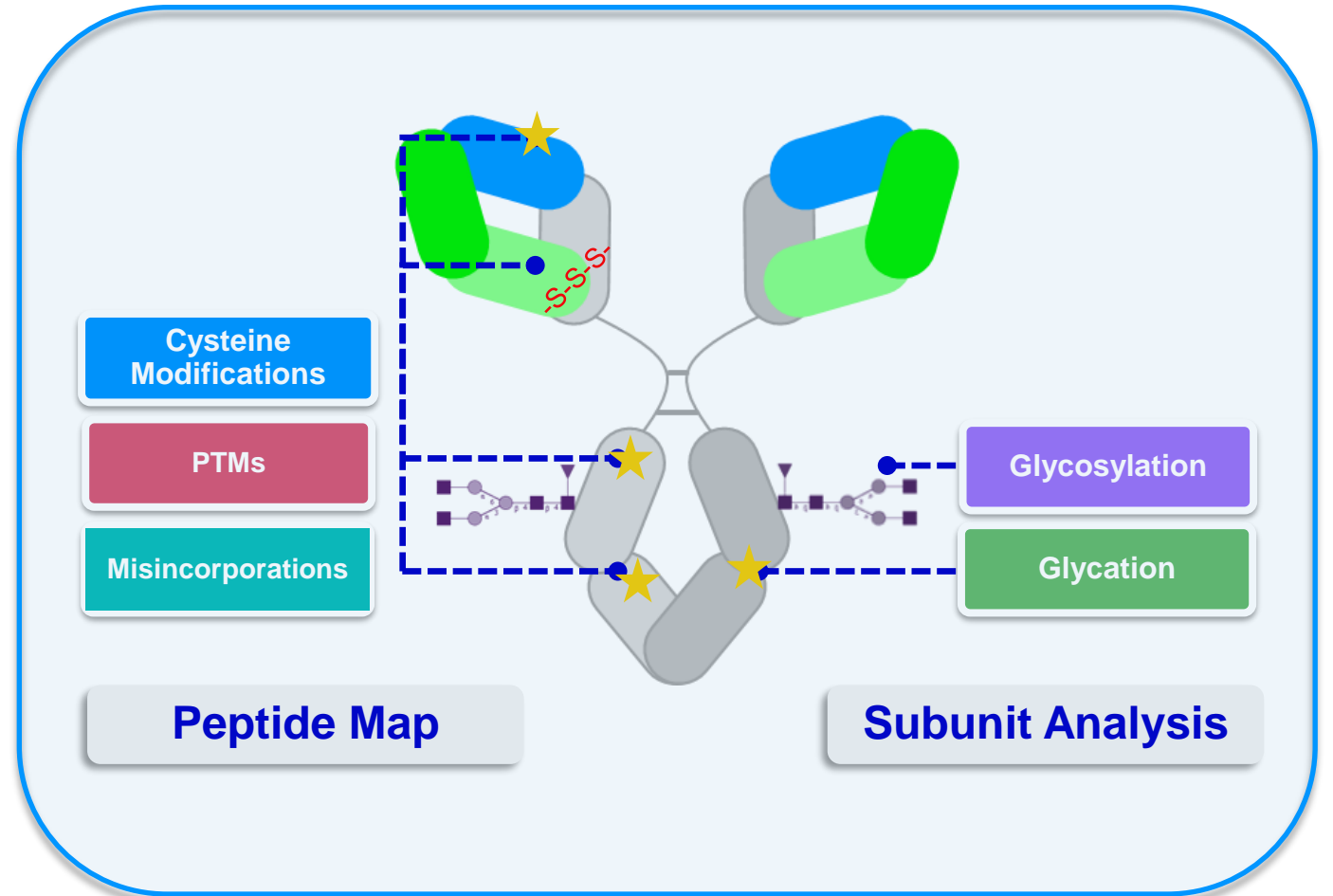
ADC conjugation process and DAR can be impacted by redox active HCP impurities in the parent mAb

May be of elevated concern for specific ADCs depending on the ADC modality and conjugation process

- Oxidases and reductases may impact the DAR of cysteine conjugates if the HCPs are not adequately cleared through the mAb purification process
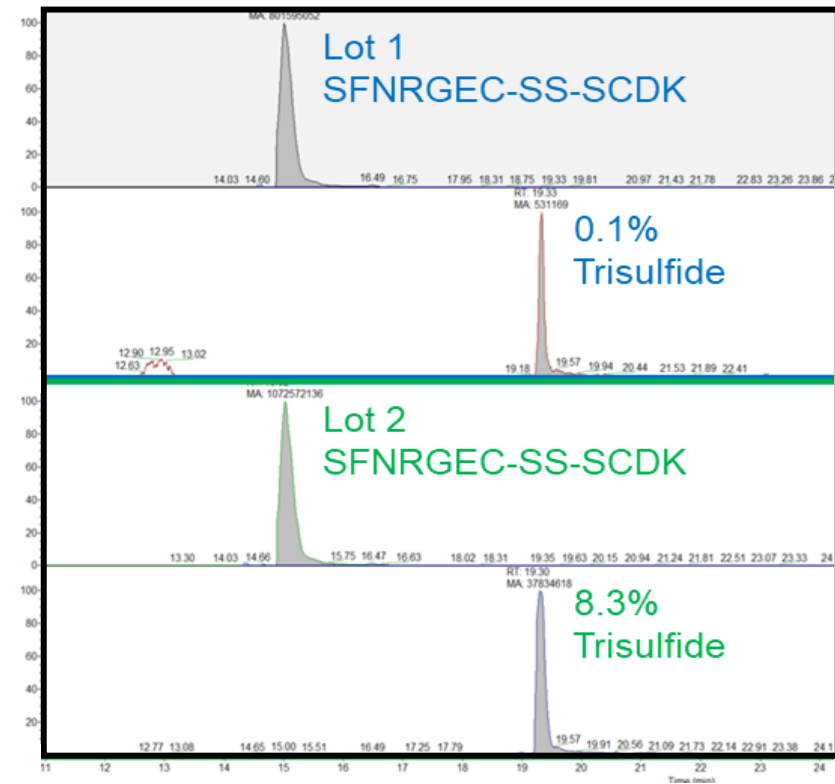- Protease cleavable linkers may be substrates for enzymatic cleavage by HCPs (Cathepsin or glucuronidase-like activity)

mAb trisulfides have a direct impact on ADC DAR – non-reduced Lys-C peptide is the front-line trisulfide monitoring method
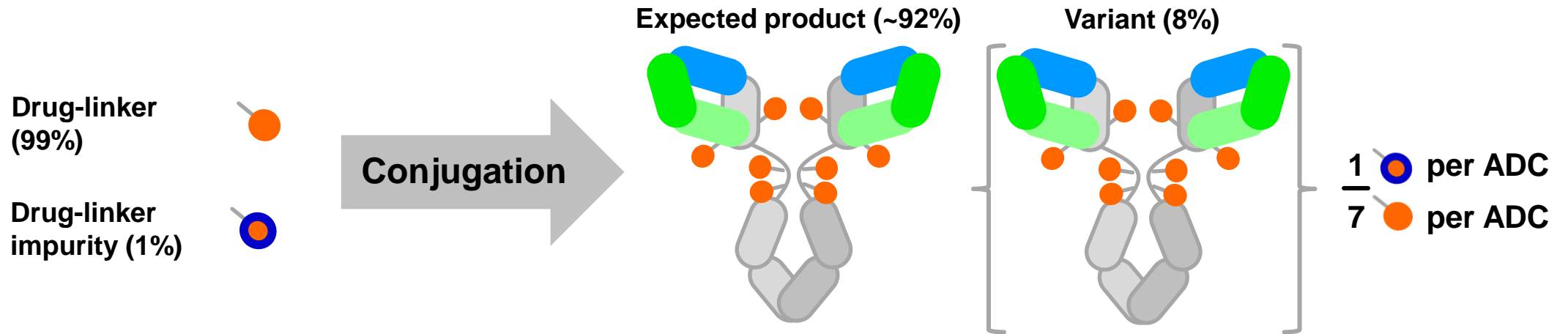
HIC DAR analysis

nr Lys-C peptide map (mAb)

Lot 1
SFNRGEC-SS-SCDK

0.1% Trisulfide

Lot 2
SFNRGEC-SS-SCDK

8.3% Trisulfide
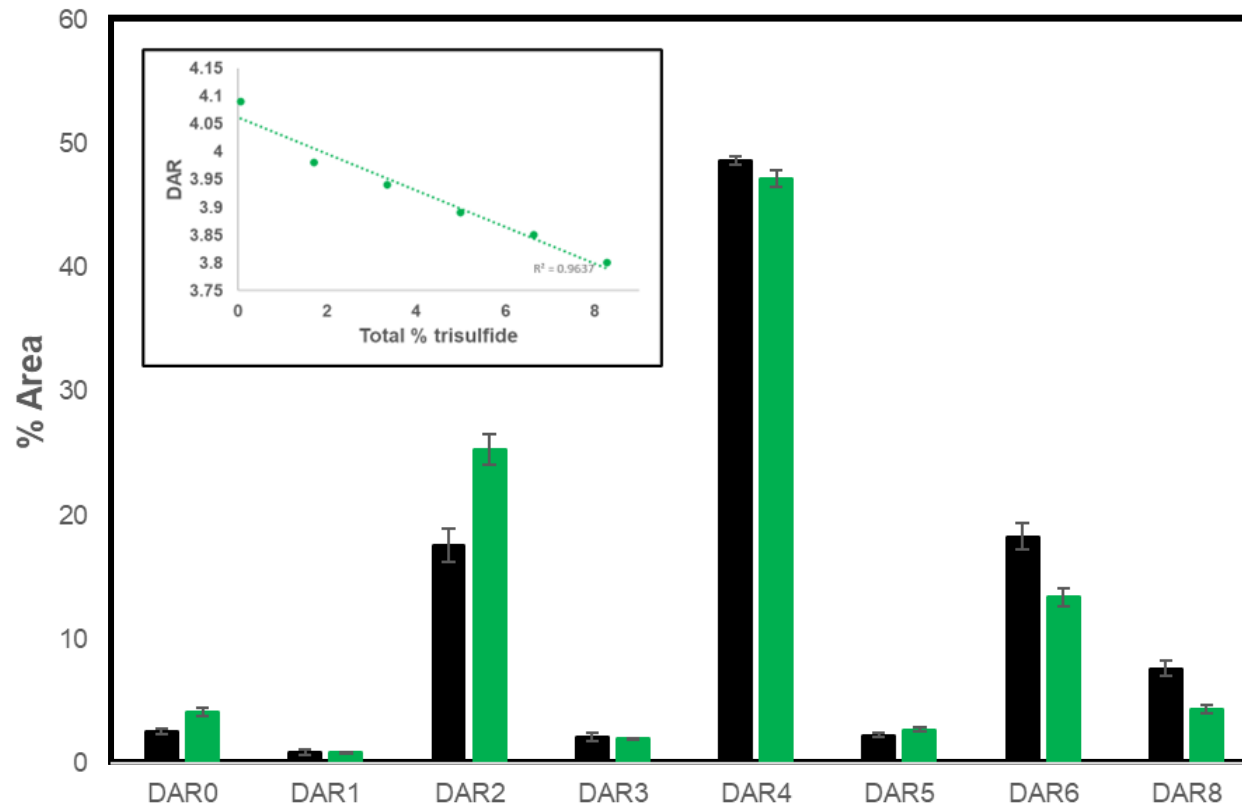
■ DAR: 4.1   ■ DAR: 3.7

Impact of drug-linker conjugatable impurities on large molecule (ADC) analytical product quality

Drug-linker (99%)

Drug-linker impurity (1%)

Conjugation

Expected product (~92%)

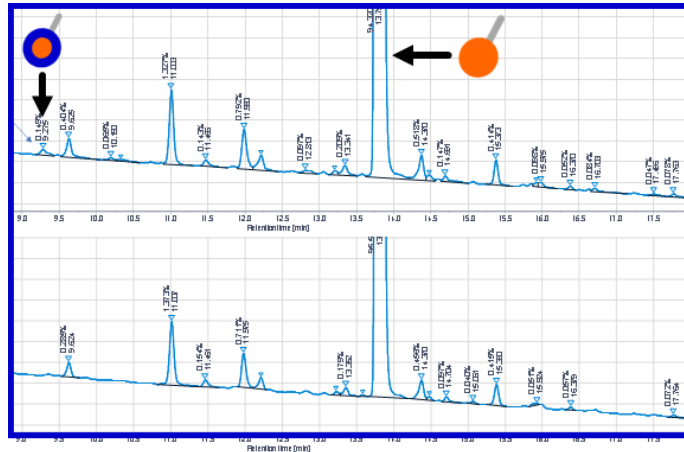**Variant (8%)**

$\dfrac{1}{7}$ per ADC

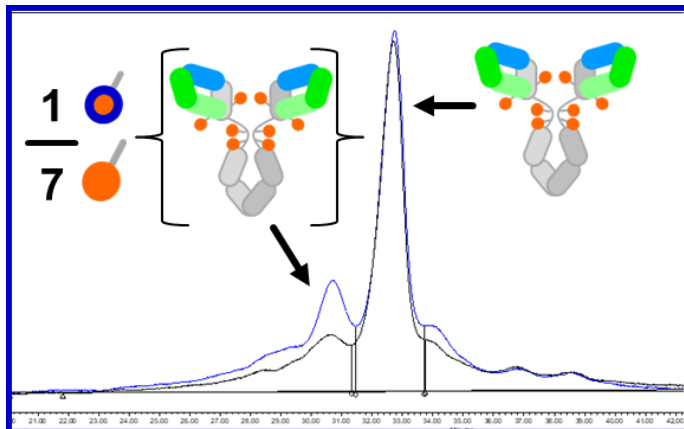per ADC

Key take-home messages

- For ADC of DAR 'N', a conj impurity has 'N' chances to be incorporated into the ADC
- Conjugatable impurities on an ADC present a challenge to large molecule product comparability

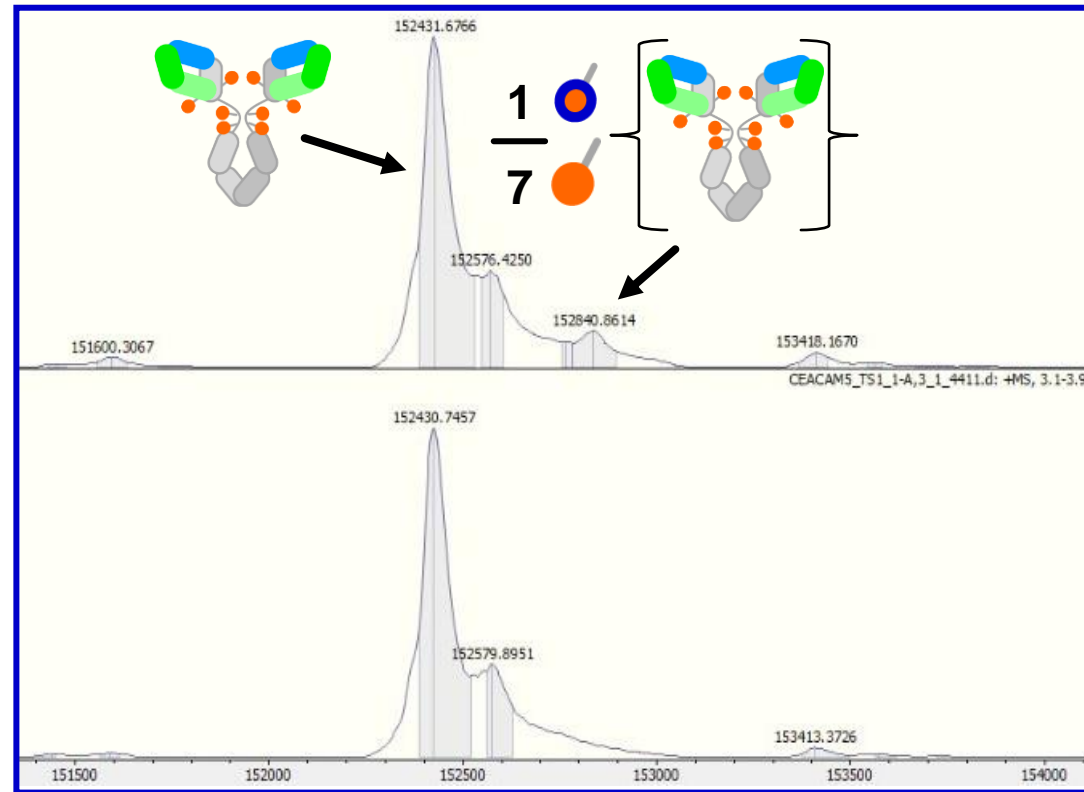# Conjugatable impurities – intact MS is the front-line strategy for detecting conjugatable impurities on the ADC
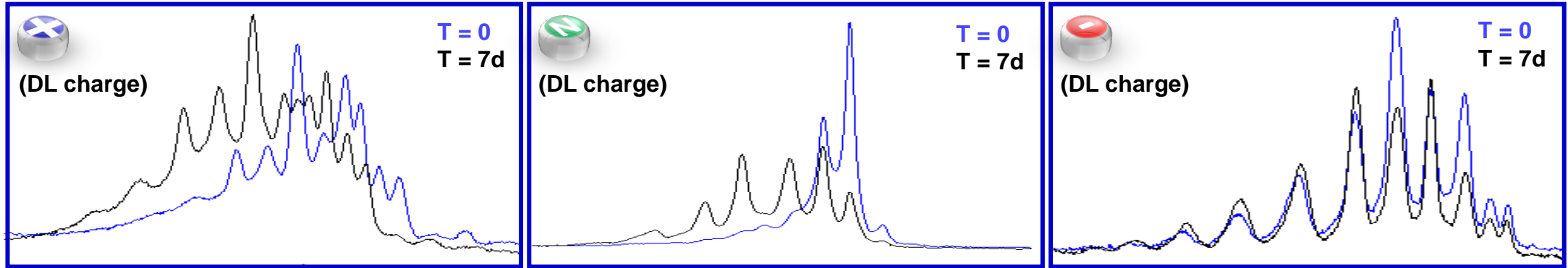
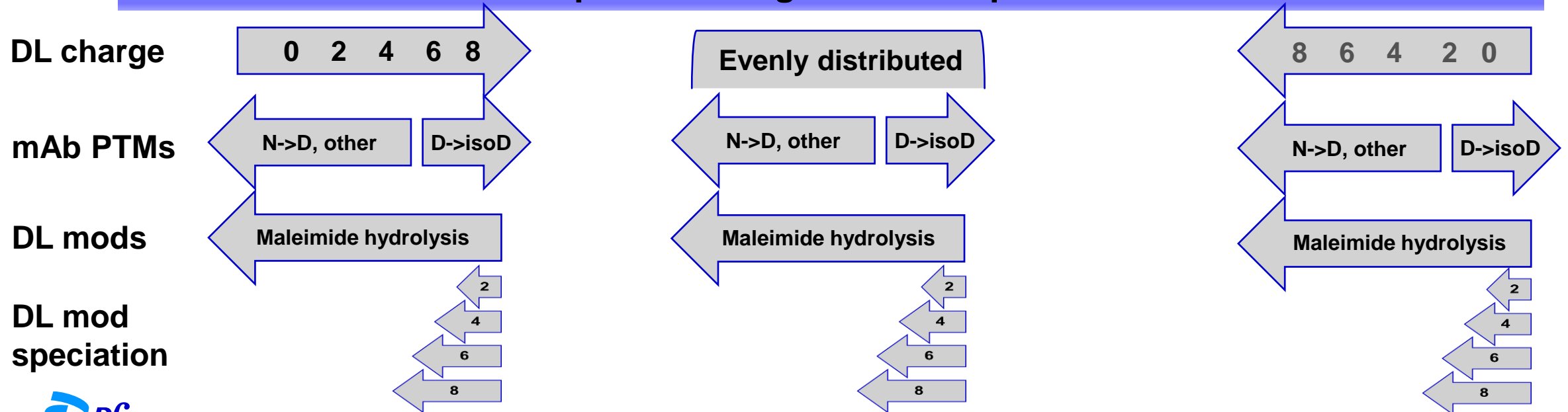**rpHPLC separation of DL**

CEX separation of ADC

Native-intact MS analysis of ADC

icIEF charge variant separations of charged and uncharged ADCs subjected to heat-stress
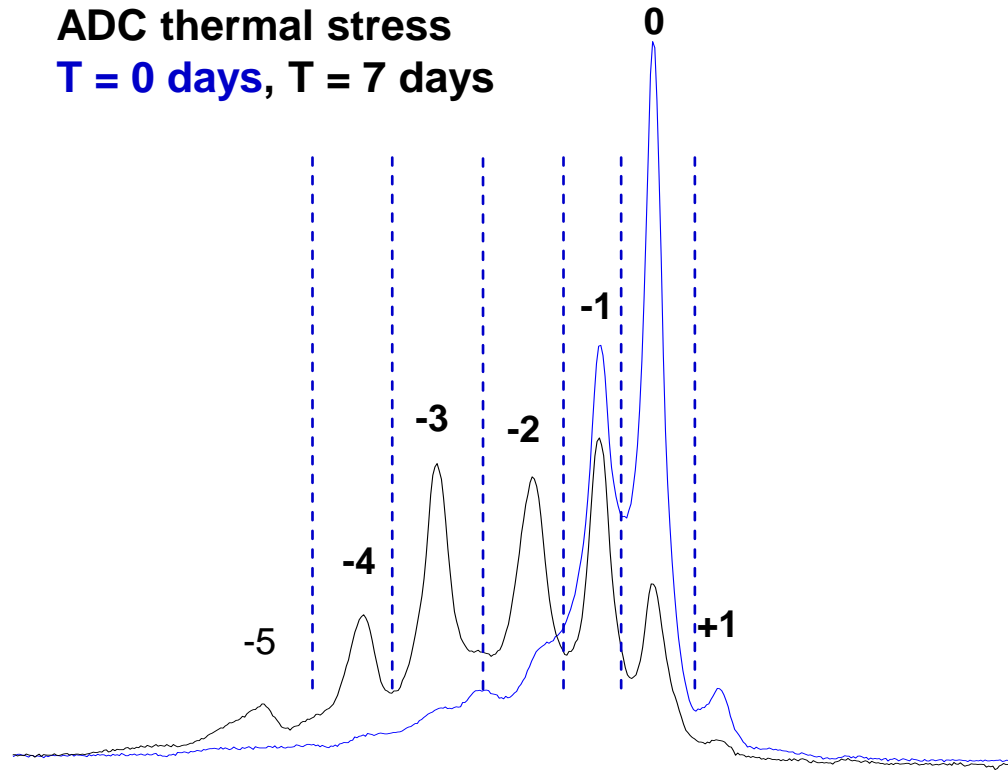
Interpretation of ADC icIEF: mAb Stress Data and MS Characterization on Stressed ADC Supports Greater Understanding of % impact to AV

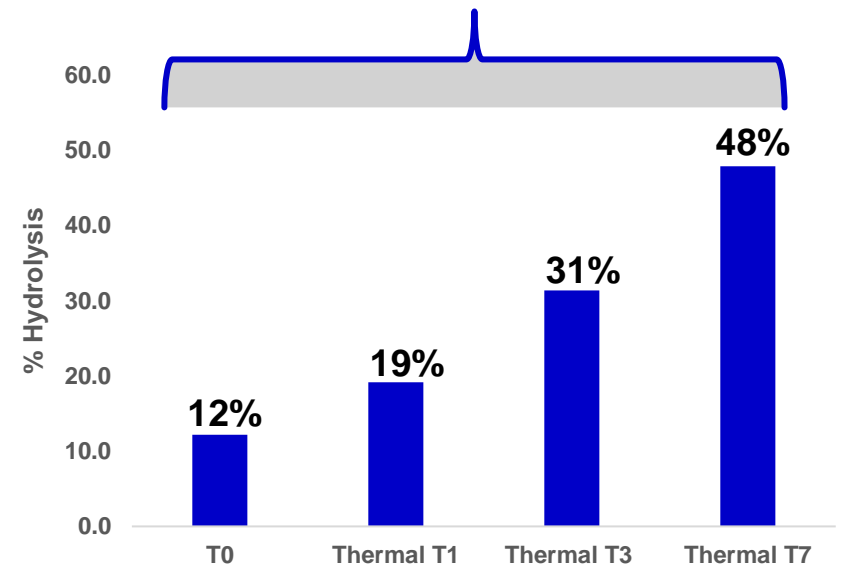**ADC thermal stress**
T = 0 days, T = 7 days

0

-1

-2

-3

-4

-5

+1

$$Average\ charge\ per\ ADC = \sum_{n=-i}^{j} \%UV\ area_n * Charge_n$$

j = number of basic variants
i = number of acidic variants

Drug-linker hydrolysis by red-peptide map

% Hydrolysis

60.0
50.0
40.0
30.0
20.0
10.0
0.0

12% — T0
19% — Thermal T1
31% — Thermal T3
48% — Thermal T7

Quantitation of charge by icIEF

Stress timepoint	Average charge per ADC	%Charge per Drug-linker
T0	-0.44	11.1%
T7	-2.0	50.0%

Uncovering a unique mechanism of lyophilized ADC drug-product charge variant distribution changes occurring during heat stress

vcMMAE-ADC (Histidine/Trehalose)
- 9 months at 50C
- 9 months at 40C
- 9 months at 25C
- 9 months at 5C
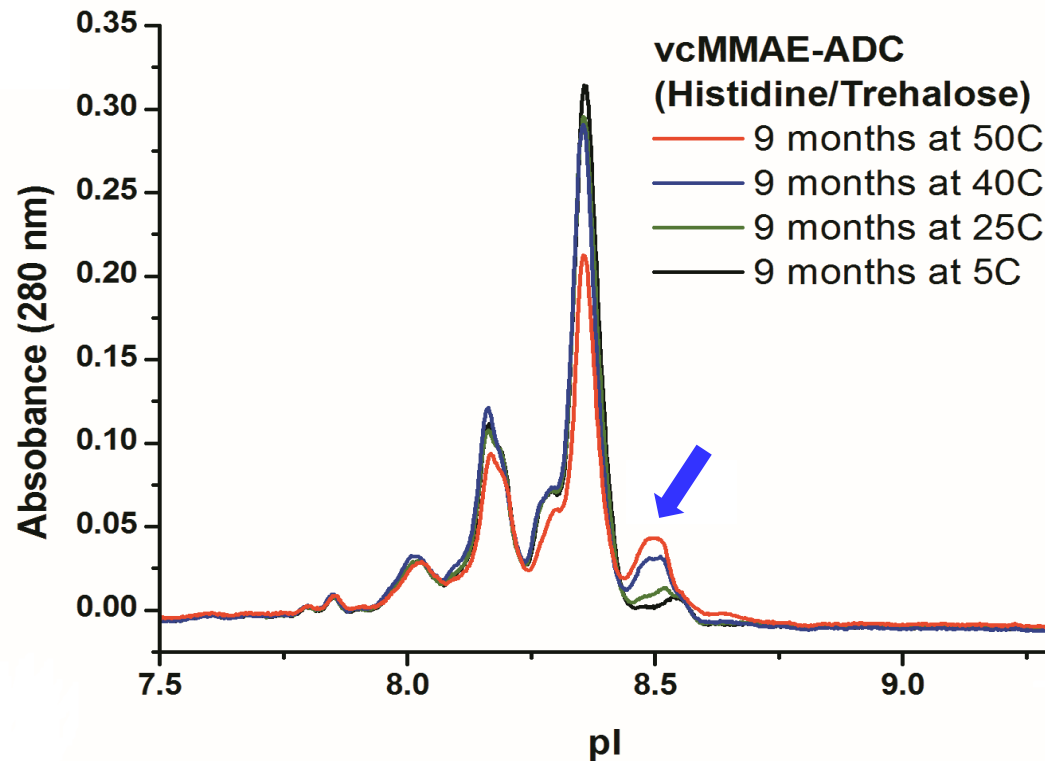
Solid-State mAbs and ADCs Subjected to Heat-Stress Stability Conditions can be Covalently Modified with Buffer and Excipient Molecules, John F. Valliere-Douglass, Patsy Lewis, Oscar Salas-Solano, Shan Jiang; *Pharmaceutical Sciences*, 104:652–665, 2015.
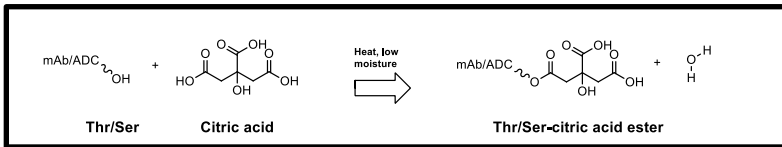

+324 Da mod is consistent with a disaccharide

Trehalose
MW = 342.3
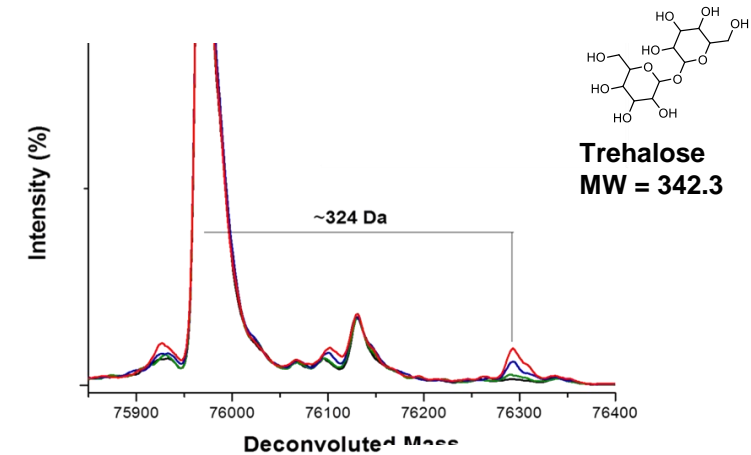
+324 Da mod localized to Glu/Asp residues
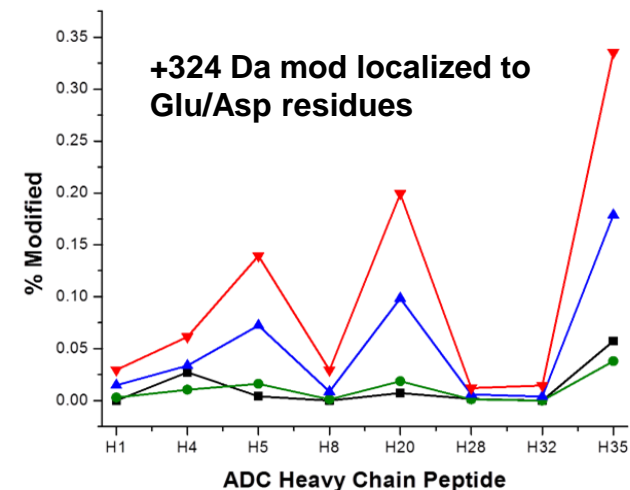
Buffer-product condensation reactions occur under heat stress, force degraded lyophilization conditions and contribute to the formation of charge variants
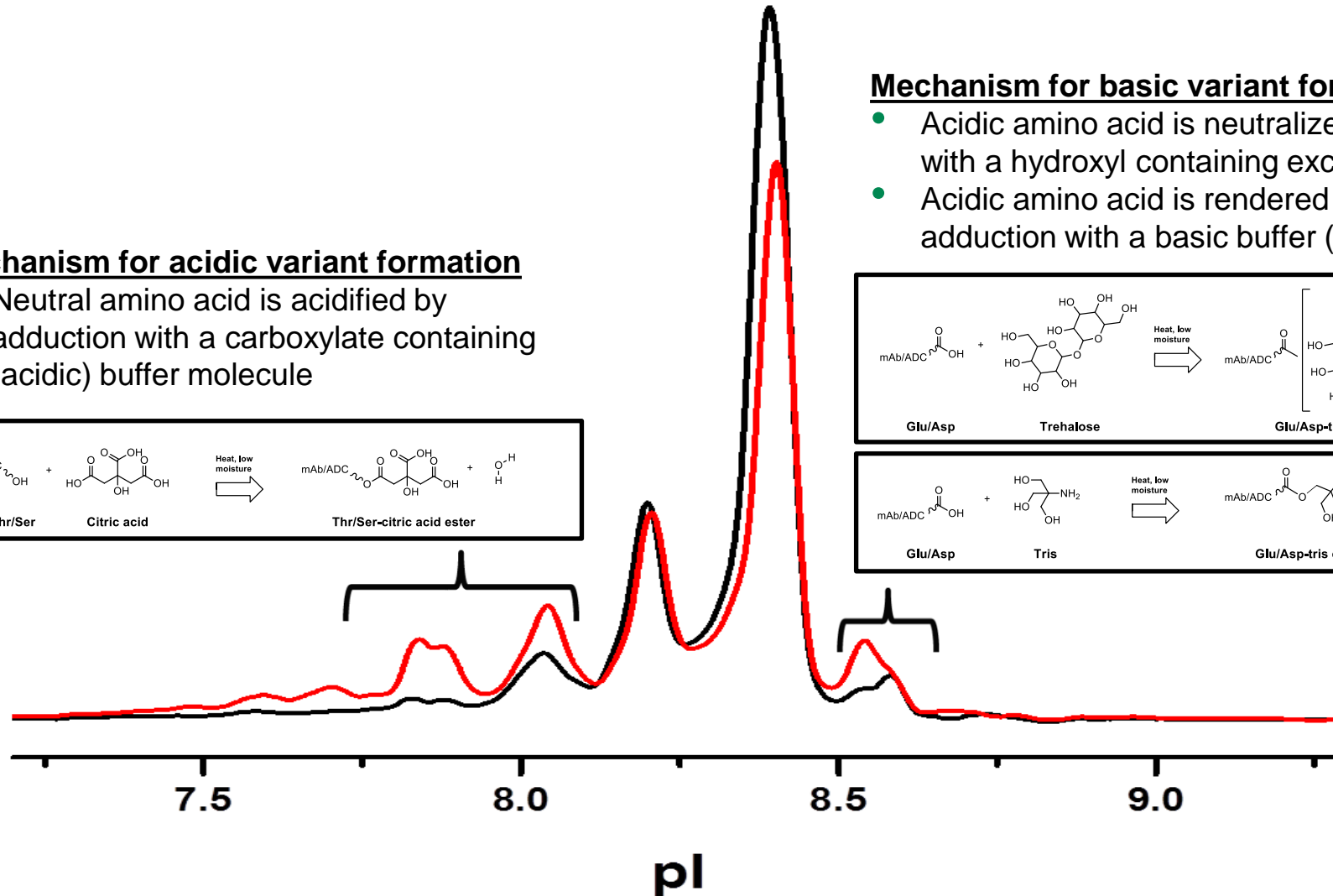

Mechanism for basic variant formation
- Acidic amino acid is neutralized by adduction with a hydroxyl containing excipient
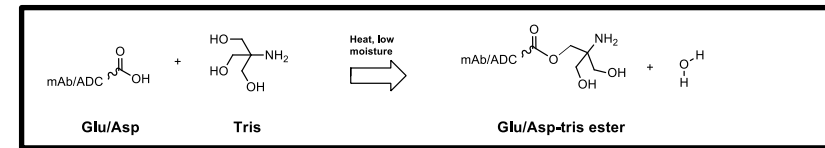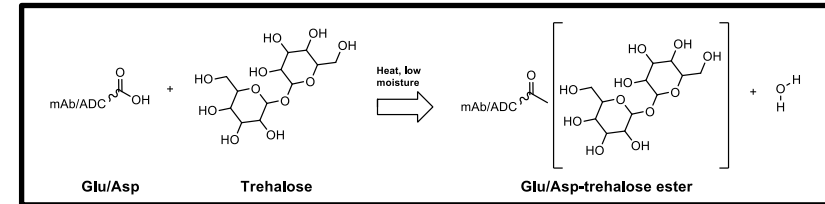- Acidic amino acid is rendered basic by adduction with a basic buffer (tris)

Mechanism for acidic variant formation
- Neutral amino acid is acidified by adduction with a carboxylate containing (acidic) buffer molecule

Conclusions / summary

- Mass spectrometry is a critical tool for developing a comprehensive understanding of ADC attributes and defining process-to-product relationships

- The evolving understanding of the biology of ADCs has led to the development of 2nd gen ADCs that stress the vedotin analytical platform
  - Ahead of analytical assay development, MS is deployed to support next-gen ADC early process development
  - MS is valuable for supporting the development of next-gen ADC release and stability assays

- Automation is increasingly important for making in-depth characterization routine and tractable

Acknowledgements

MS Core members past and present, collaborators and the CASSS-MS organizing committee

MS core alumni
- Lucy Pan
- Jay Jones
- Bill McFee
- Scott Henry
- CJ Waite

Present members
- Romesh Rao
- Kinsey Reed
- Richard Moran

Collaborators
- Shawna Hengel (Pfizer)
- Chris Leiske (Pfizer)
- Guillaume Tremintin (Bruker)
- Yun Yang (Bruker)
- Phil Compton (IPT)