Localized Higher Order Structures of mAbs and ADCs Investigated by MS-based Protein Footprinting

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6th International Symposium on the Higher Order Structure of Protein Therapeutics
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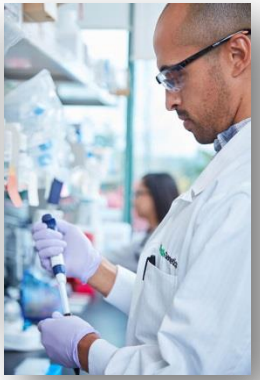
Seattle Genetics: Emerging global multi-product oncology company

- A biotechnology company focused on developing and commercializing a new generation of targeted, empowered antibody-based therapies with the goal to change the foundation of treatment for patients with cancer
- ADCETRIS® (brentuximab vedotin)
  - In collaboration with Takeda Pharmaceutical Company, is commercially available in more than 65 countries
  - Broad clinical development program in CD30-expressing lymphomas
- Vadastuximab talirine (SGN-CD33A; 33A)
  - Pivotal phase 3 CASCADE trial for AML
- Enfortumab vedotin (ASG-22ME)
  - Regulatory discussions planned for registrational trials in urothelial cancer
- Deep pipeline provides opportunities across hematologic malignancies and solid tumors
- Company facts
  - Founded in 1998
  - Headquartered in Bothell, WA
  - Publicly traded (Nasdaq: SGEN)
  - >900 employees



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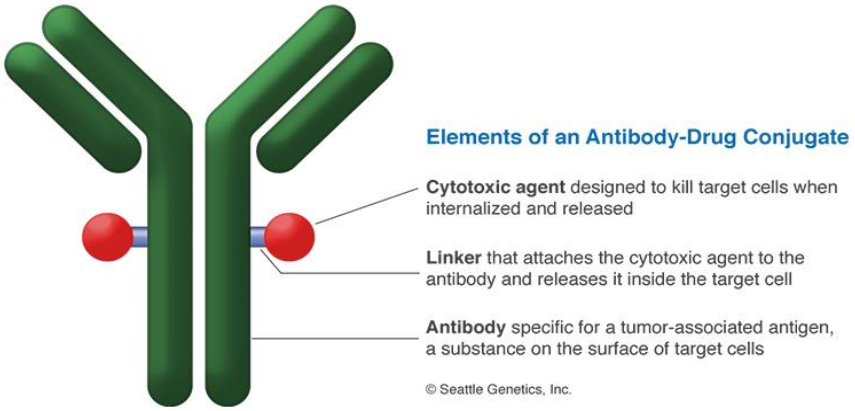
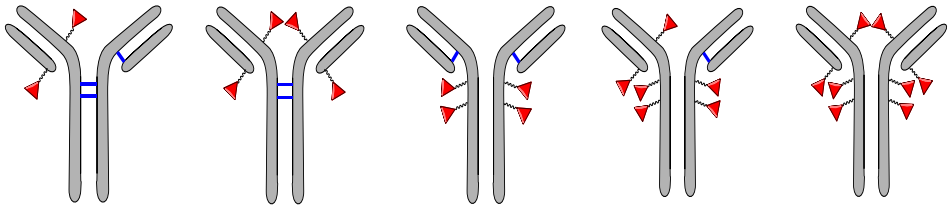
Antibody-Drug Conjugate (ADC) Technology: Empowering Antibodies



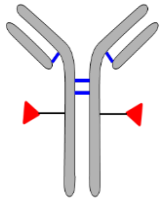
ADCs combine the targeting ability of antibodies with the potency of cytotoxic drugs

Two types of ADCs in SGEN pipeline:



I. **Interchain cysteine linked ADCs**

(av. ~4 drugs per mAb)



II. **Engineered cysteine linked ADCs**

(homogeneous, 2 drugs per mAb)

Doronina, S. O. et al. Nature Biotech, 2003
Kung Sutherland, M. S. et al. Blood, 2013

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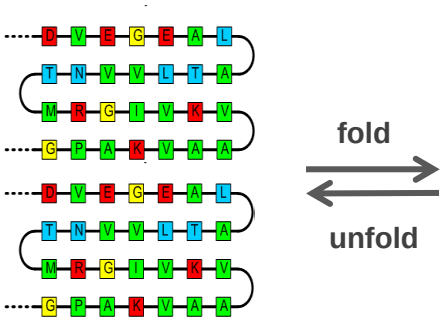
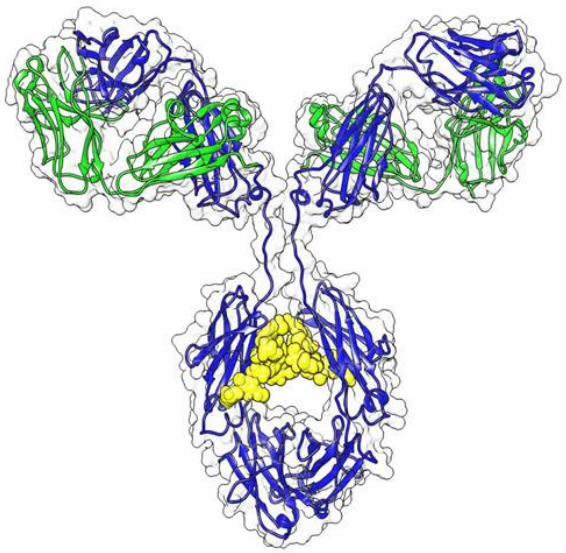
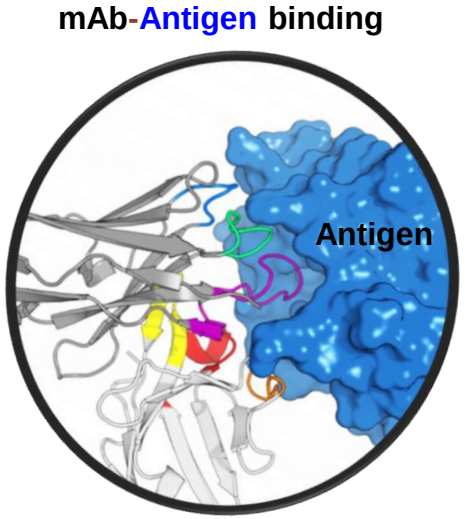
Higher Order Structure (HOS) is an Important Quality Attribute of mAbs and ADCs

- HOS impacts biological function, stability and safety
- Manufacturing process and storage condition may impact HOS







Primary structure
(~1300 amino acids)

Higher order structure
(Conformation)

Function

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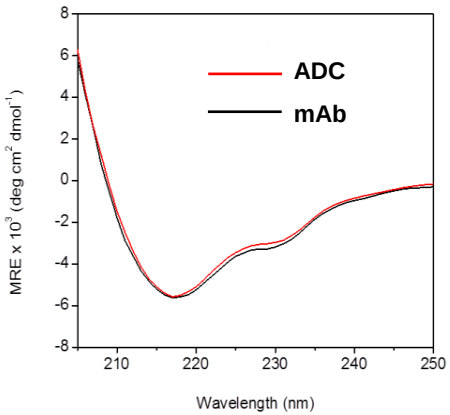
Tools for HOS Characterization of mAb/ADCs

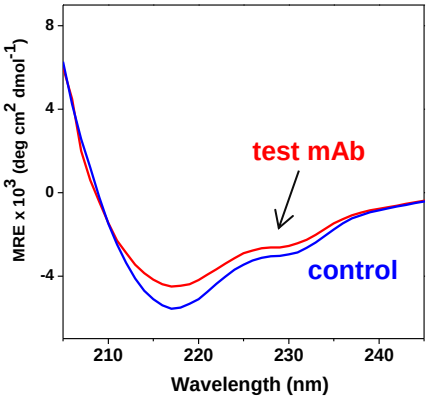
- **Gold standard: X-ray crystallography and NMR**
  - Very challenging to apply to mAb/ADCs in industry
- **Spectroscopic techniques (Circular Dichroism, FTIR, Fluorescence, etc.)**
- **Thermal analysis (DSC)**
- **Bioassay (cell based binding, potency assay)**
  - Well-established in industry, but not suited to identifying local differences

Any local difference?



CD spectra of interchain cysteine linked ADCs and parent mAbs

Where is the difference?



CD spectra of control (blue) and test mAbs (red color)

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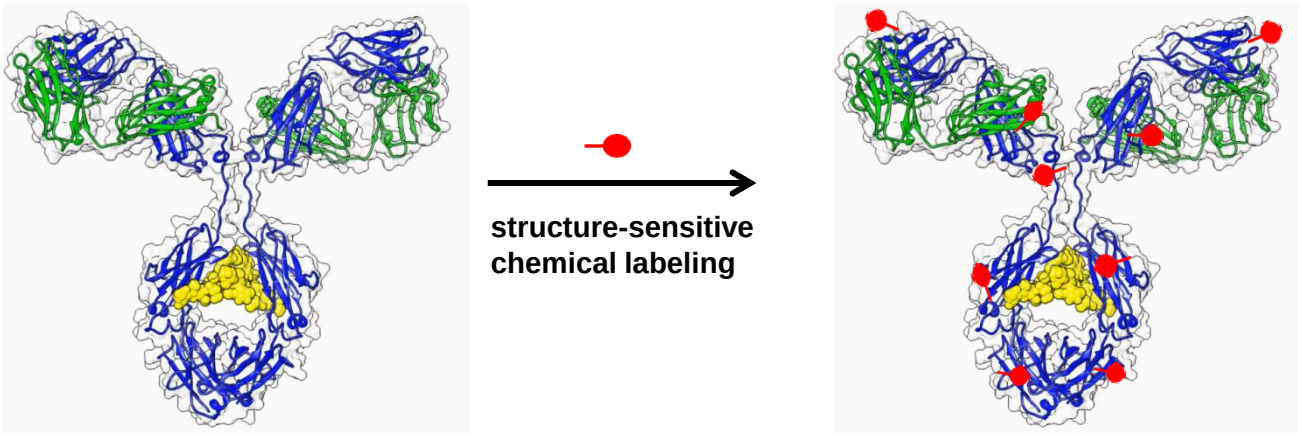
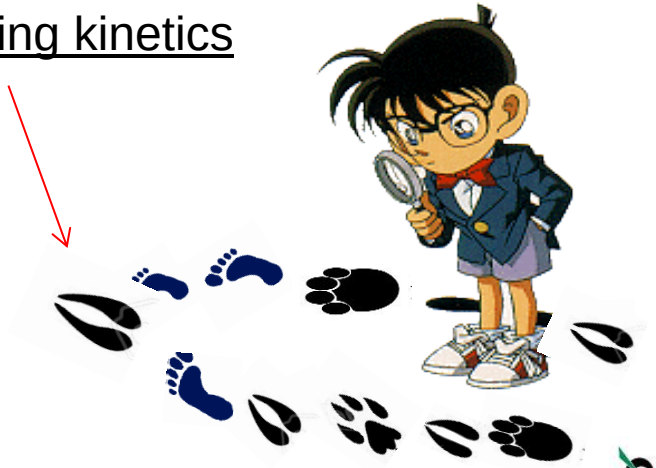
MS-based Protein Footprinting Could Provide Insights to Localized HOS

"Footprinting": Chemical labeling coupled with MS for studying protein HOS



- Individual residues/small regions in proteins may show different labeling kinetics depending on their local HOS.
- MS is used to monitor the labeling kinetics



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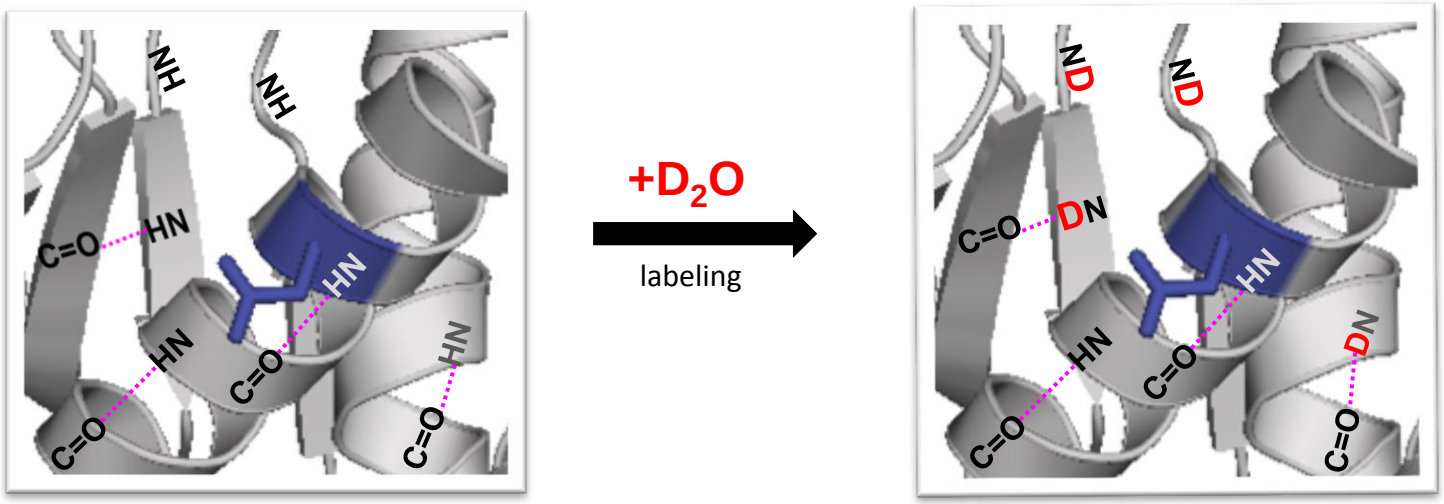
Two Types of Chemical Labeling for mAb/ADC HOS Investigation

I. Isotope labeling (HDX)

II. Covalent labeling



- Monitor isotope exchange kinetics of amide hydrogen
- Powerful tool to assess backbone HOS and structural fluctuation/flexibility
- Labile labeling, limited applications for some proteins with non-MS compatible components; difficult data analysis

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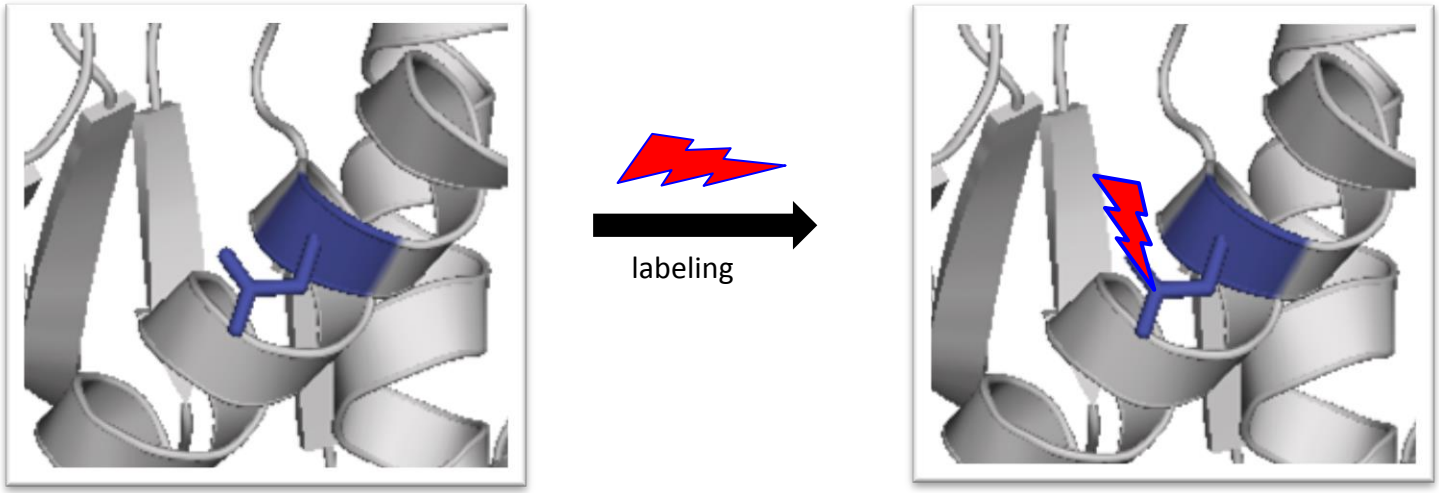
Two Types of Chemical Labeling for mAb/ADC HOS Investigation

I. Isotope labeling (HDX)

II. Covalent labeling



- Monitor labeling kinetics between side chains and labeling agents
- Interrogating side chain accessibility, complementary to HDX
- Stable labeling, ideal for proteins with non-MS compatible components
- Different covalent labeling strategies (e.g. $OH^{\cdot}$)

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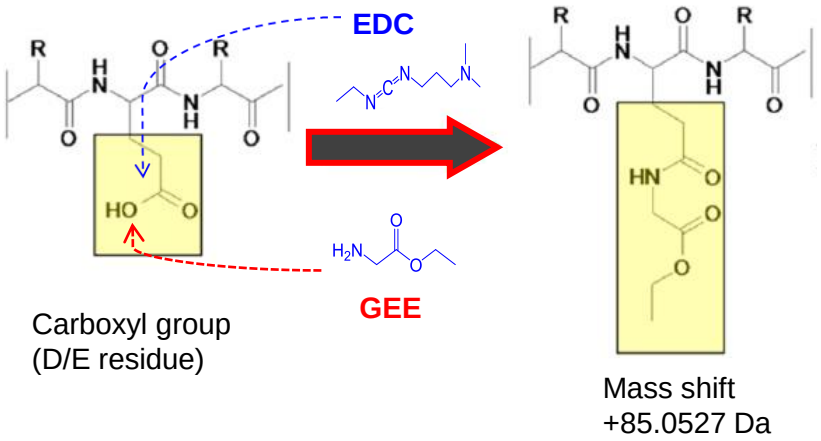
Carboxyl Group Footprinting (CGF):
The covalent labeling is targeted on two amino acids : D & E

Why choose CGF

- 10% of residues are D/E in most mAbs, providing reasonable coverage
- Simple labeling chemistry, no special equipment needed
- Highly specific labeling, can be performed at different buffer conditions; straightforward data analysis



First-order reaction, labeling kinetics reports side chain solvent-accessibility

EDC: 1-ethyl-3-(3-dimethylaminopropyl carbodiimide)
GEE: Glycine ethyl ester

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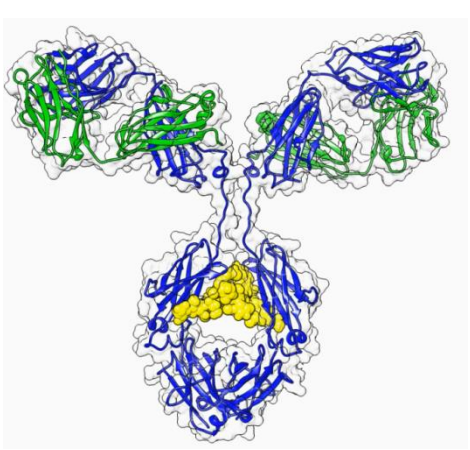
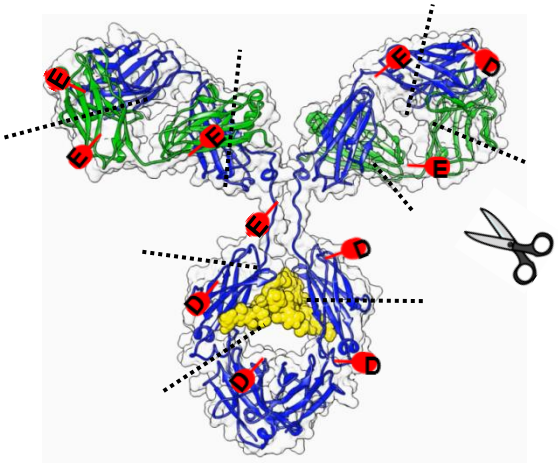
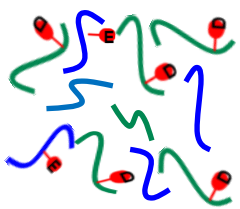
Localized HOS Investigation by Site-specific CGF
Workflow



EDC
GEE
(2, 5, 8 min)

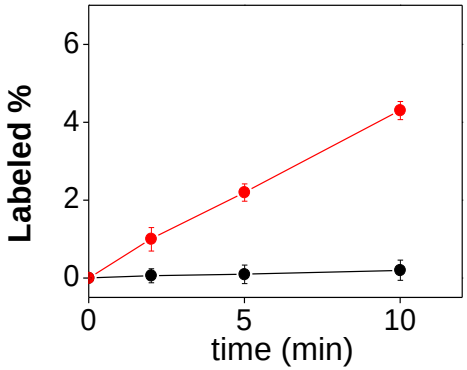


peptides



MS-based Peptide mapping

Monitor labeling kinetics of each D/E residue



- Labeling Rate Constant (RC) is used to report labeling kinetics
- The higher the RC of a D/E residue, the more solvent-accessible
- Well-suited for localized HOS interrogation across mAb/ADC samples

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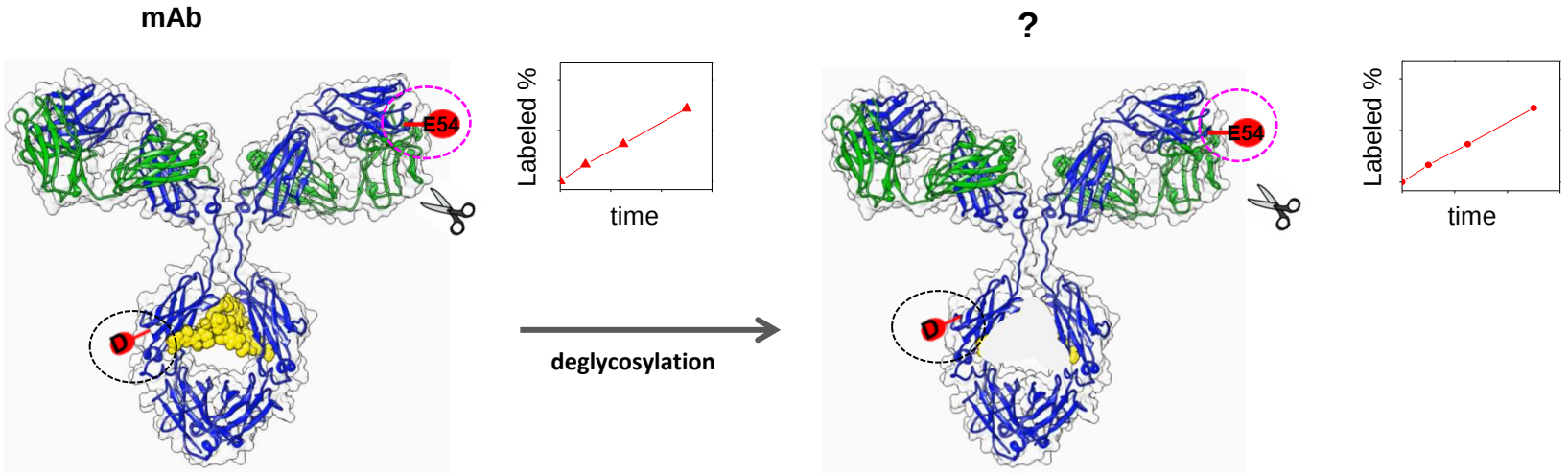
Localized HOS Investigation by Site-specific CGF
Case study-1: mAb vs. deglycosylated mAb



- Spectroscopic methods cannot detect significant HOS differences, how about the new CGF?

CGF Workflow (for a residue level of resolution)

Chemical labeling (targeted on D/E) → **Trypsin Peptide mapping** → **Monitor labeling curve of each D/E, determine RC** → **Site-specific RC comparison between samples**

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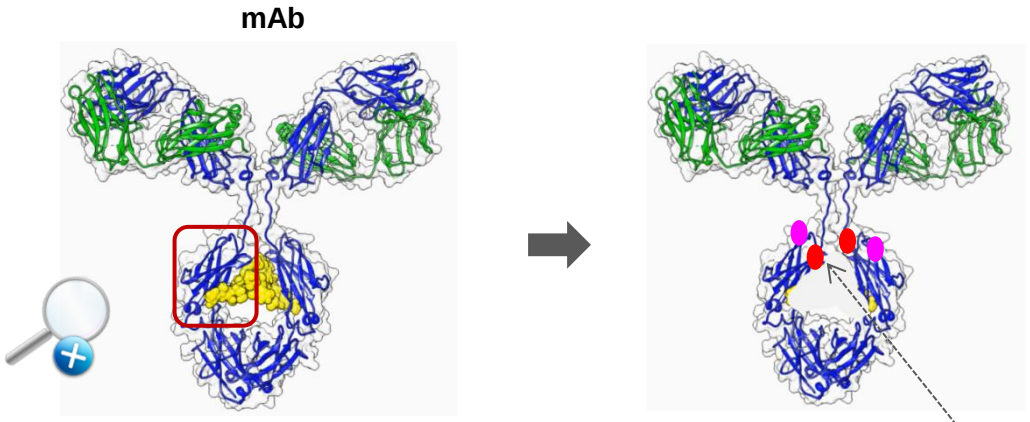
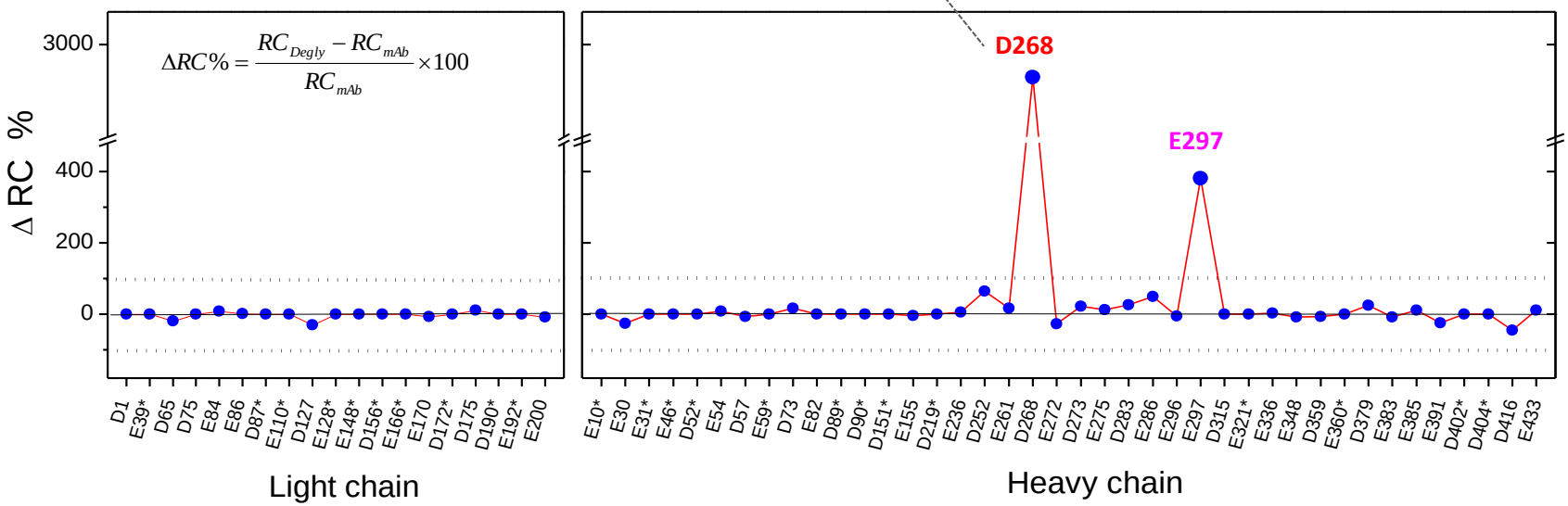
Localized HOS Investigation by Site-specific CGF

Case study-1: mAb vs. deglycosylated mAb



D268 and **E297** become much more solvent accessible after removing the glycan molecules



Rate constant difference (ΔRC%) plot of mAb and deglycosylated mAb

Lucy Y. Pan, et al. mAbs, 2017

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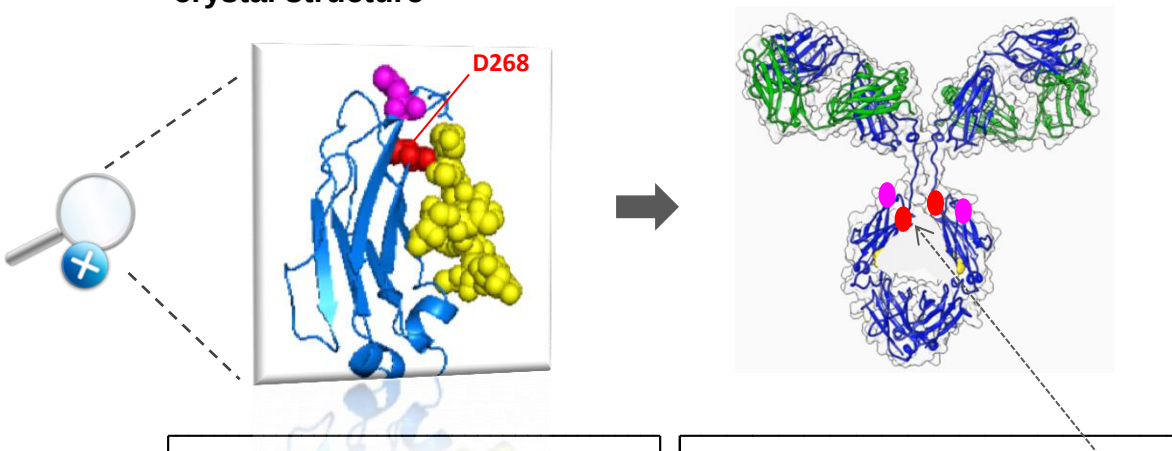
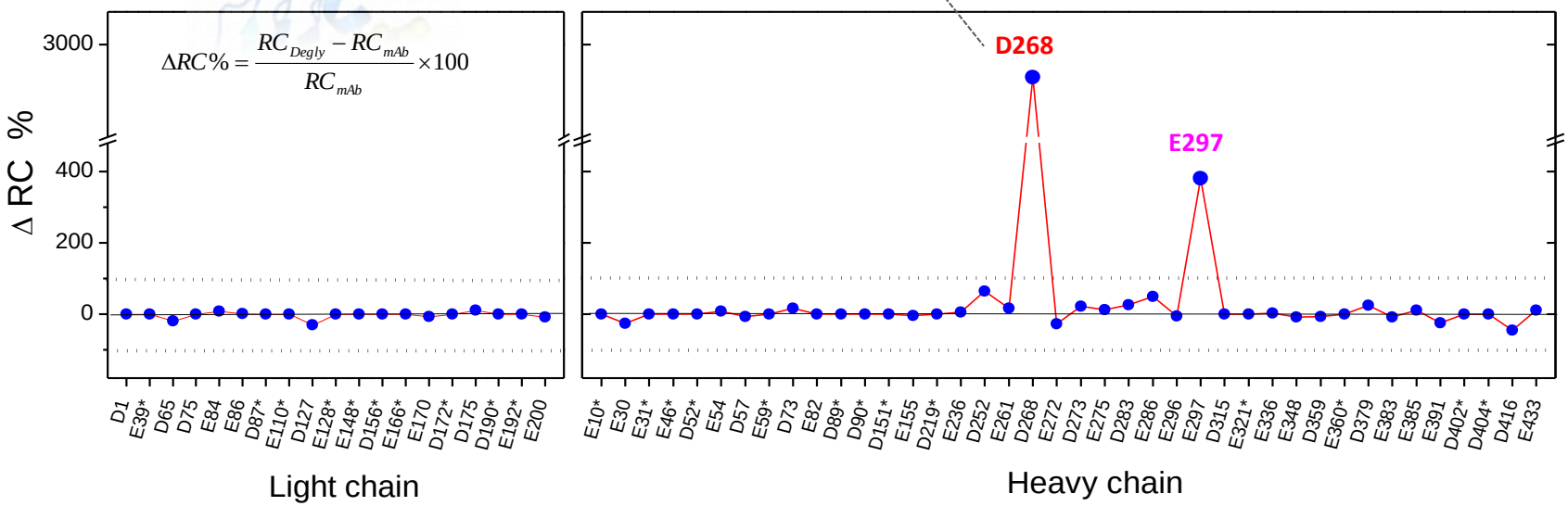
Localized HOS Investigation by Site-specific CGF
Case study-1: mAb vs. deglycosylated mAb

Zoomed-in view of model Fc crystal structure



D268 and **E297** become much more solvent accessible after removing the glycan molecules



Rate constant difference (ΔRC%) plot of mAb and deglycosylated mAb

Lucy Y. Pan, et al. mAbs, 2017

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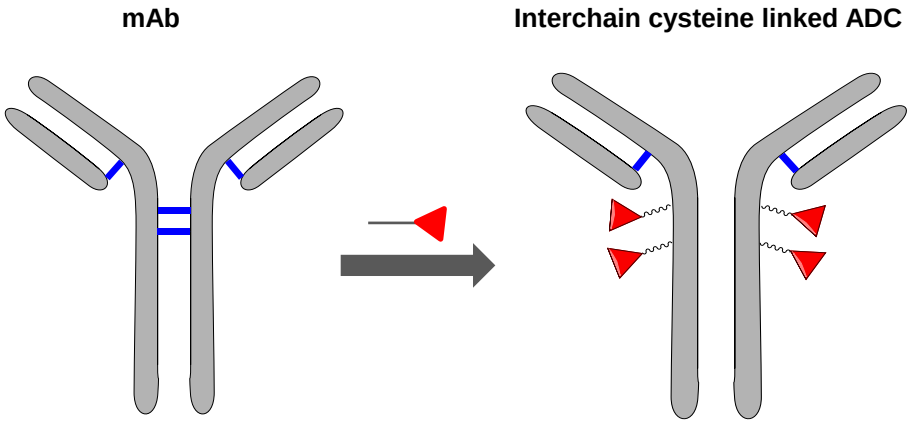
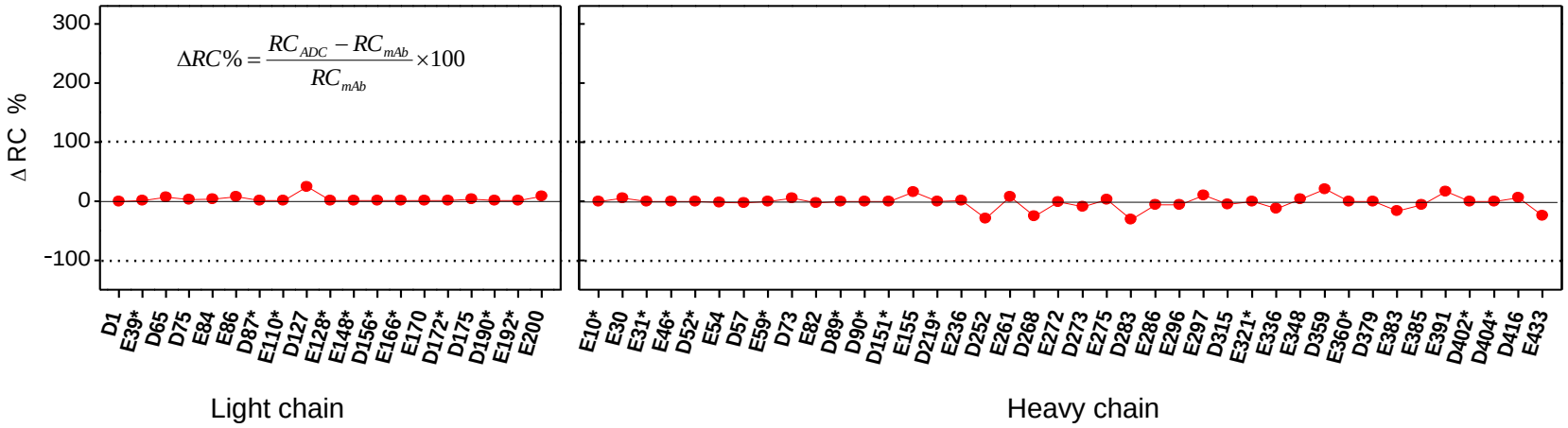
Localized HOS Investigation by Site-specific CGF
Case study-2: ADC vs. mAb



All D/E residues show similar RC, suggesting similar side chain confromation in the ADC and mAb.



Rate constant difference (ΔRC%) plot of ADC and parent mAb

Lucy Y. Pan, et al. mAbs, 2017

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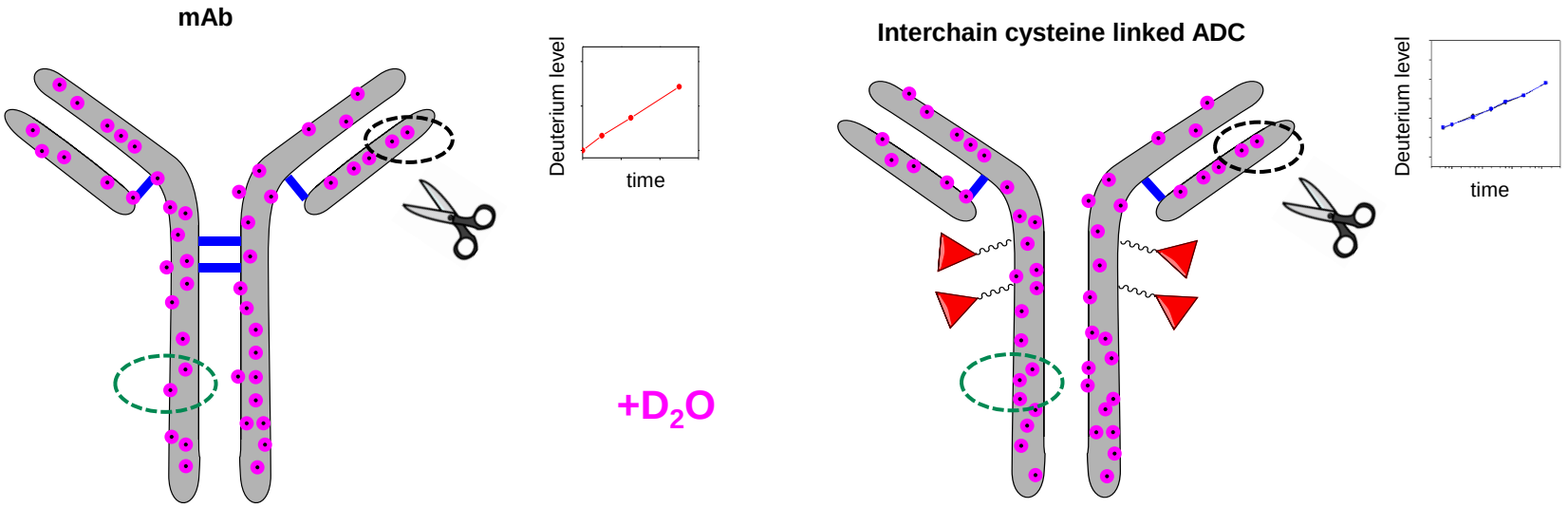
Localized HOS Investigation by HDX MS
Interchain cysteine linked ADC vs. mAb



HDX Workflow (for a peptide level of resolution)

D_2O labeling → **Pepsin digest (only few minutes at 0°C, pH 2.5)** → **Monitor deuterium uptake kinetics of individual peptides** → **Compare deuterium uptake for each peptide**

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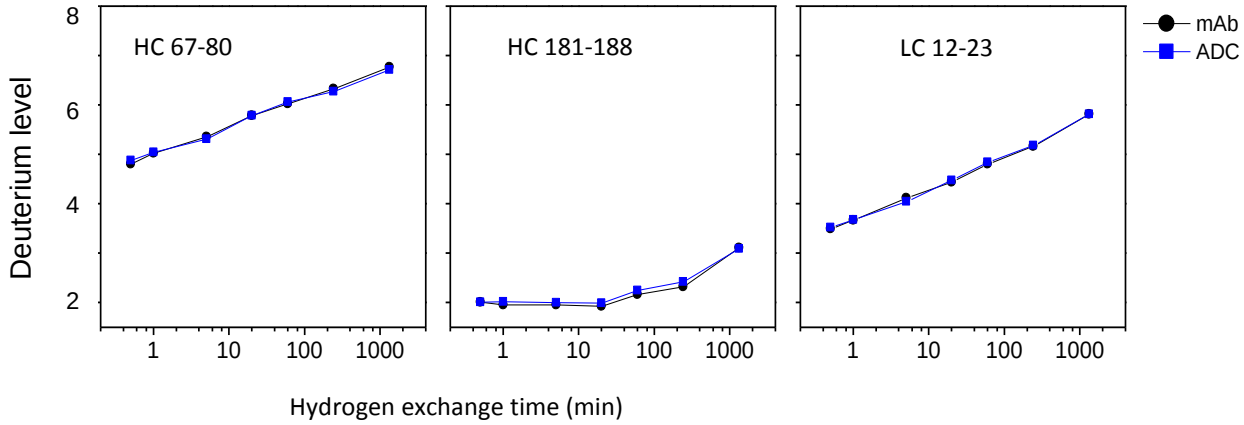
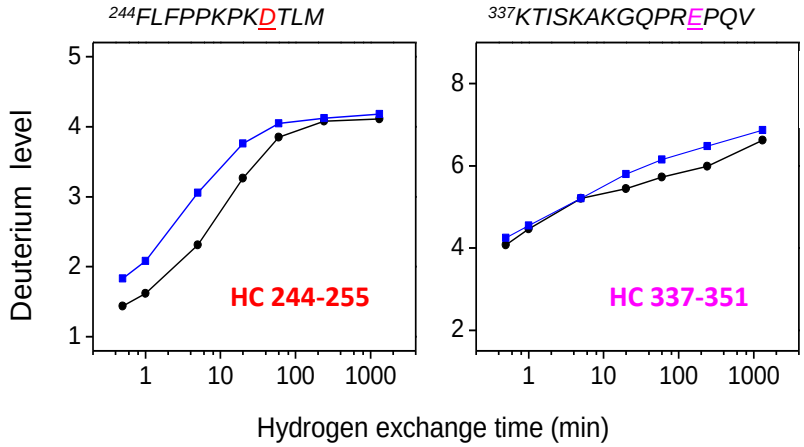
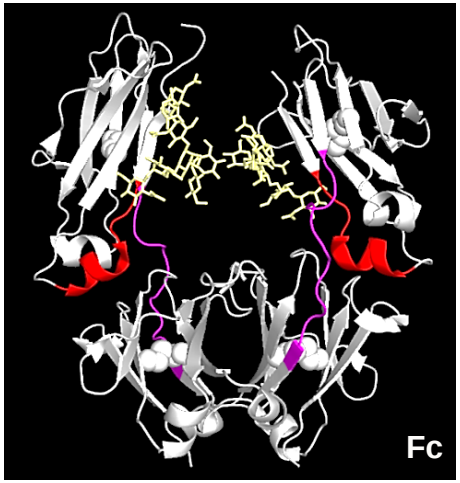
Localized HOS Investigation by HDX MS
Interchain cysteine linked ADC vs. mAb

- **Most peptides (90% sequence) show similar deuterium uptake in the ADC and mAb**



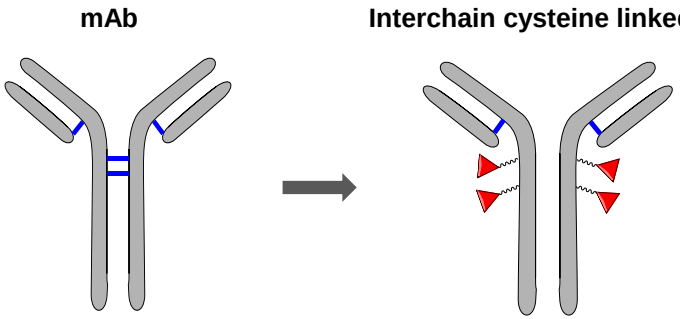
- **2 peptides show increased deuterium uptake in the ADC** ➡ **more solvent-accessible and/or more structurally flexible**

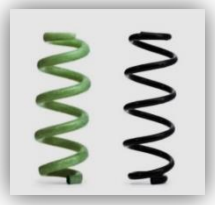




Lucy Y. Pan, et al. Anal. Chem., 2014

Structural Perspective from Complementary Protein Footprinting: ADC vs. mAb





Similar shape
Different strength

- **<u>HDX</u>: assess backbone HOS and structural flexibility**
  - 90% sequence show similar HDX kinetics in the ADC and mAb,
  - Local differences observed at 2 regions (*[244]FLFPPKPK**<u>D</u>**TLM, [337]KTISKAKGQPR**<u>E</u>**PQV)* that become more solvent-accessible and/or more structurally flexible in the ADC
- **<u>CGF</u>: monitor side chain accessibility, report dominant HOS**
  - All D/E residues show similar RC in the ADC and mAb
  - The side chains of D252 and E348 are highly protected in both the ADC and mAb

➡ **More structurally flexible at 2 regions (CH_2-CH_3 interface) in the ADC**

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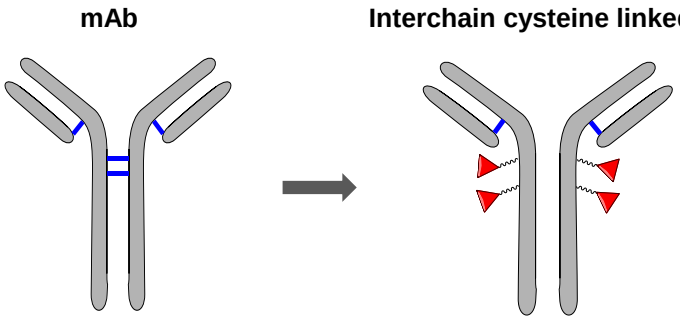
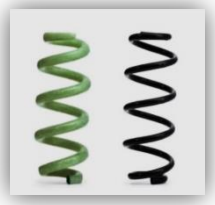
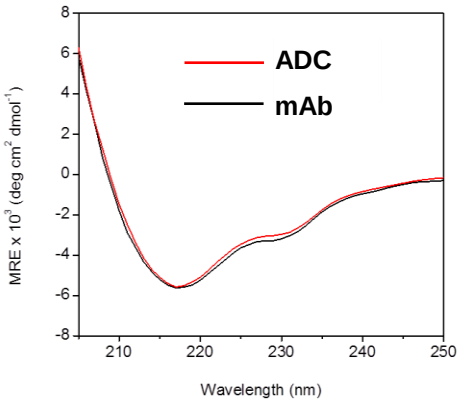
HOS Characterization from Traditional Assays
ADC vs. mAb





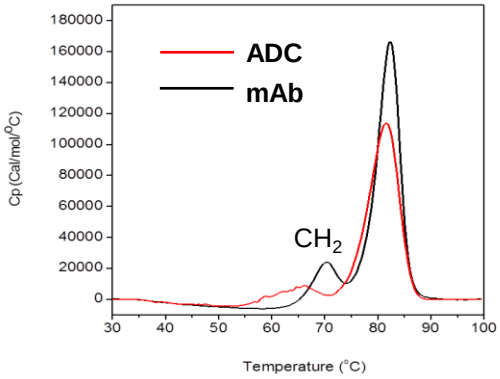
Similar shape
Different strength

CD spectra comparison



The ADC and mAb share similar HOS

DSC thermogram comparison



ADCs show slightly lower thermal stability at CH_2

- **The findings from protein footprinting (CGF, HDX) provide insights into results from traditional assays.**

Lucy Y. Pan, et al. mAbs, 2017

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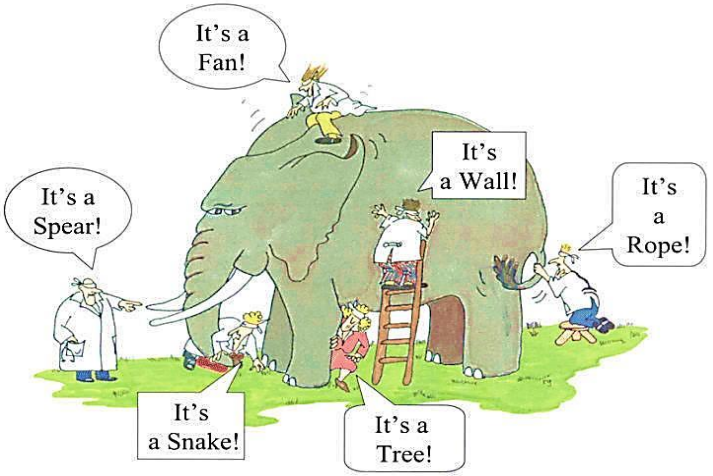
Summary

- CGF is a useful tool to assess localized HOS of mAb/ADCs. It can be readily implemented in industry due to the simple labeling protocol and straightforward data analysis
- CGF is especially good at characterizing mAb/ADC samples with non-MS compatible components
- CGF interrogates side chain conformation and HDX monitors backbone conformation and dynamics. Combining the two complementary footprinting techniques, as well as other biophysical assays, would enable more complete HOS insights to be gained

Blind men and the elephant



Acknowledgments

Analytical Sciences

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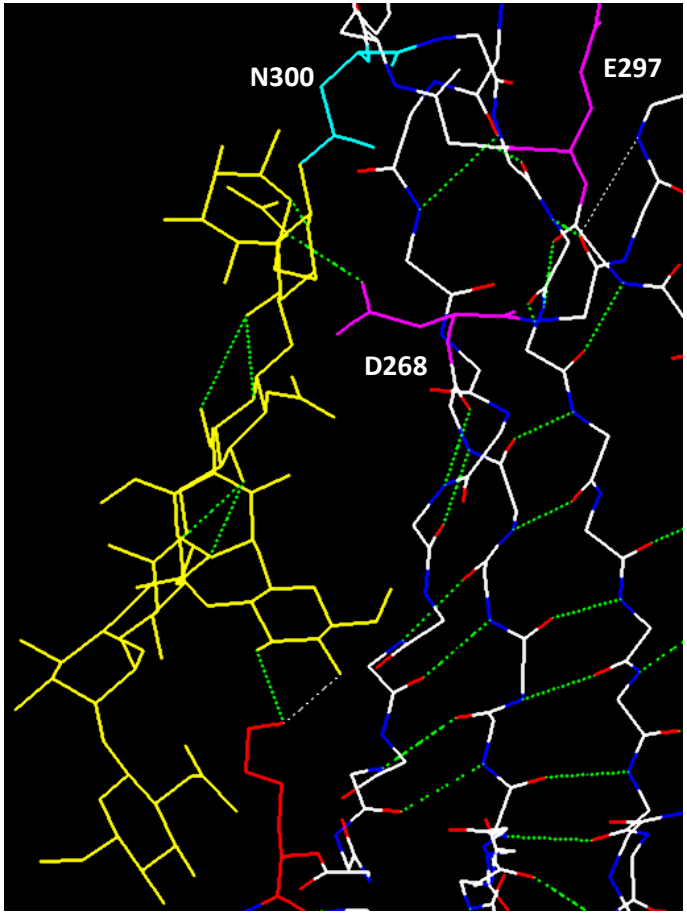
- Scott Henry
- Bill McFee
- Jay Jones
- Romesh Rao
- Nomalie Jaya
- Oscar Salas-Solano

Conjugation

- Ben Burrone
- Damon Meyer

Crystal Structure of a Model Fc Supports the Findings from CGF



CGF data reveal that residues D268 and E297 have significantly enhanced side chain accessibility after deglycosylation

Zoomed-in view of the crystal structure of a model Fc domain (PDB: 3AVE). The backbone amide hydrogen is depicted in blue and carbonyl oxygen is depicted in red. Glycans (Man3GlcNAc4Fuc1) are depicted as yellow sticks. D268 and E297 are highlighted in magenta sticks. Green dotted lines represent stable H-bonds identified by Swiss PDB Viewer.

Lucy Y. Pan, et al. mAbs, 2017

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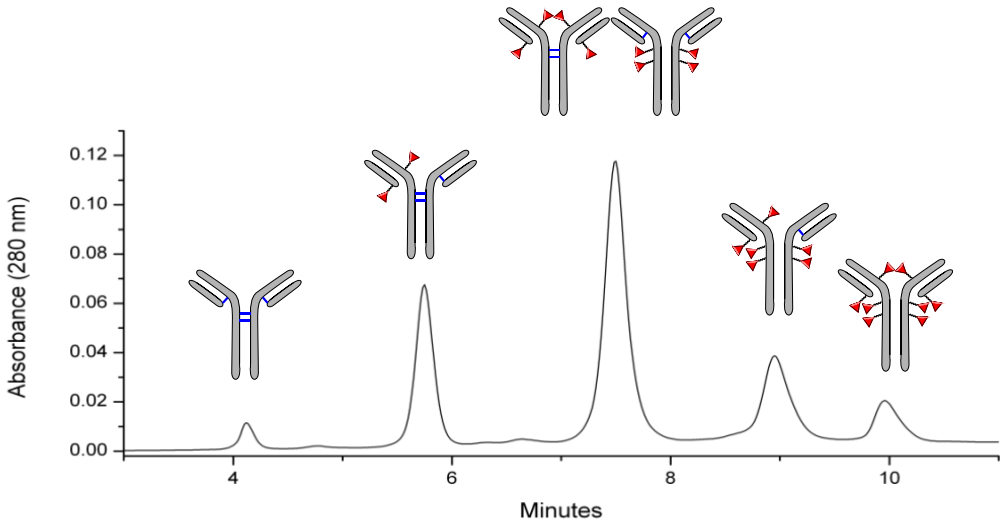
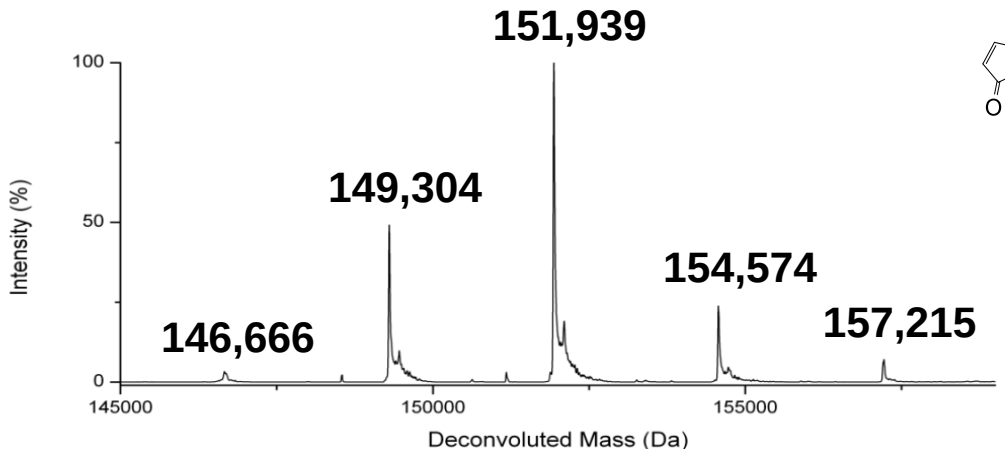
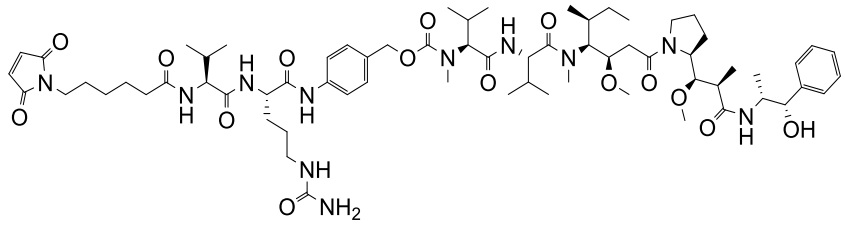
Drug load and Distribution of ADCs are determined by HPLC and MS





~4 drugs/antibody

Drug-linker (vcMMAE)



Lucy Y. Pan, et al. Anal. Chem., 2014

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