

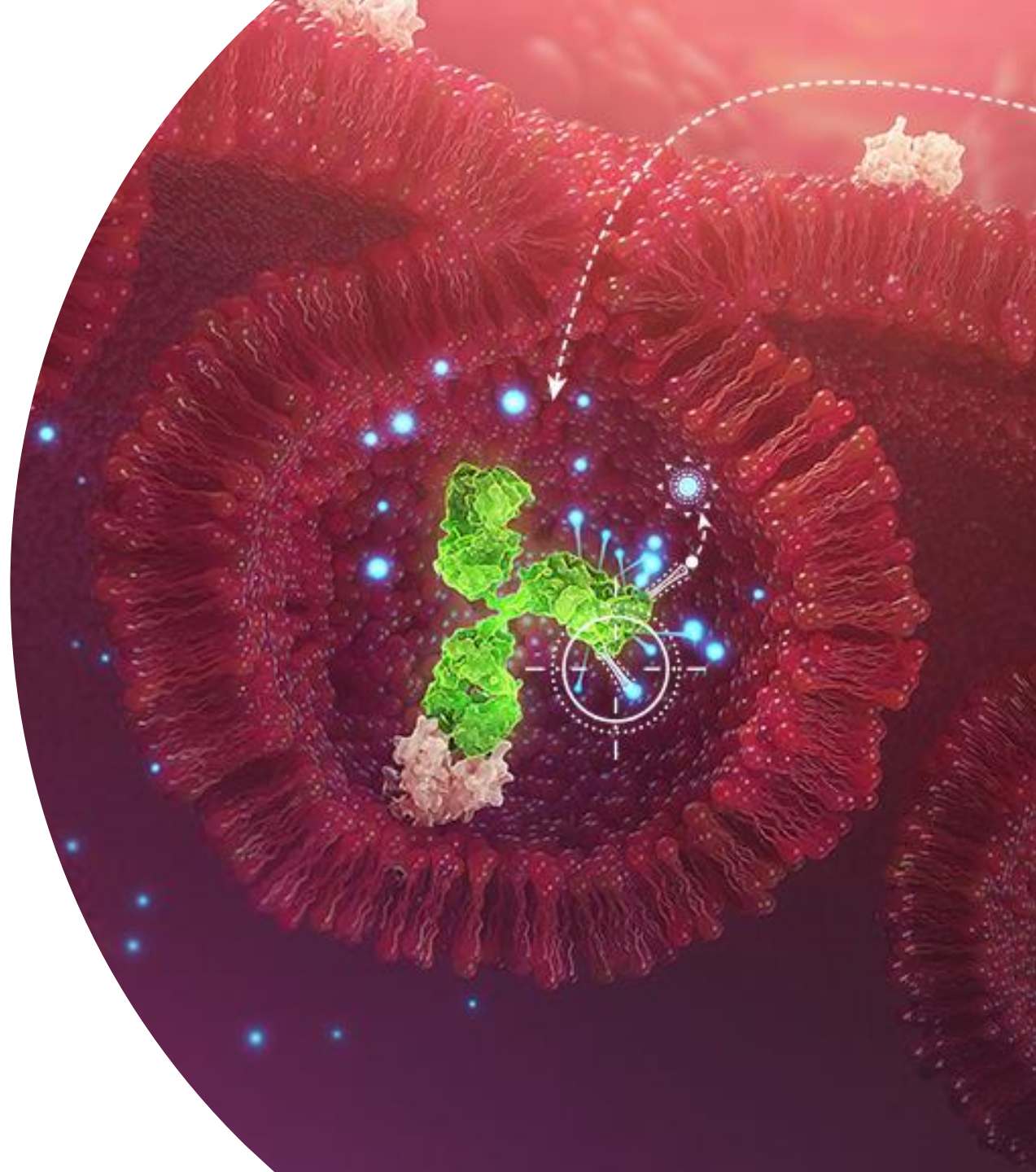


Honing Analytical Methods for Next Generation ADCs

Chunlei Wang, Jun Kim, Qin Yan,
Jessica Webb, Xiaoyu Chen

CASSS CMC Strategy Forum

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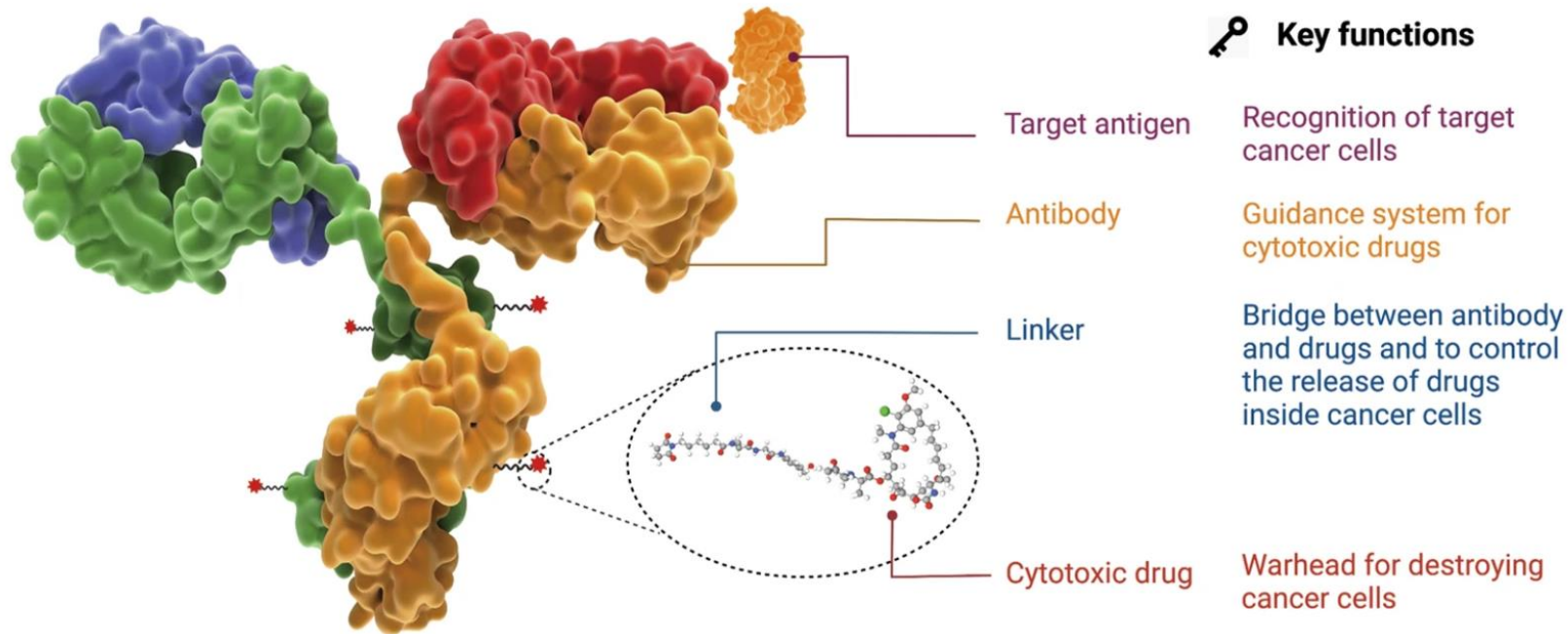


Outline

- **Introduction (ADCs, landscape, quality attributes)**
- **Analytical methods and new challenges**
 - Free Drug
 - Charge Variants
 - Drug Antibody Ratio
- **Conclusions**



Antibody Drug Conjugates

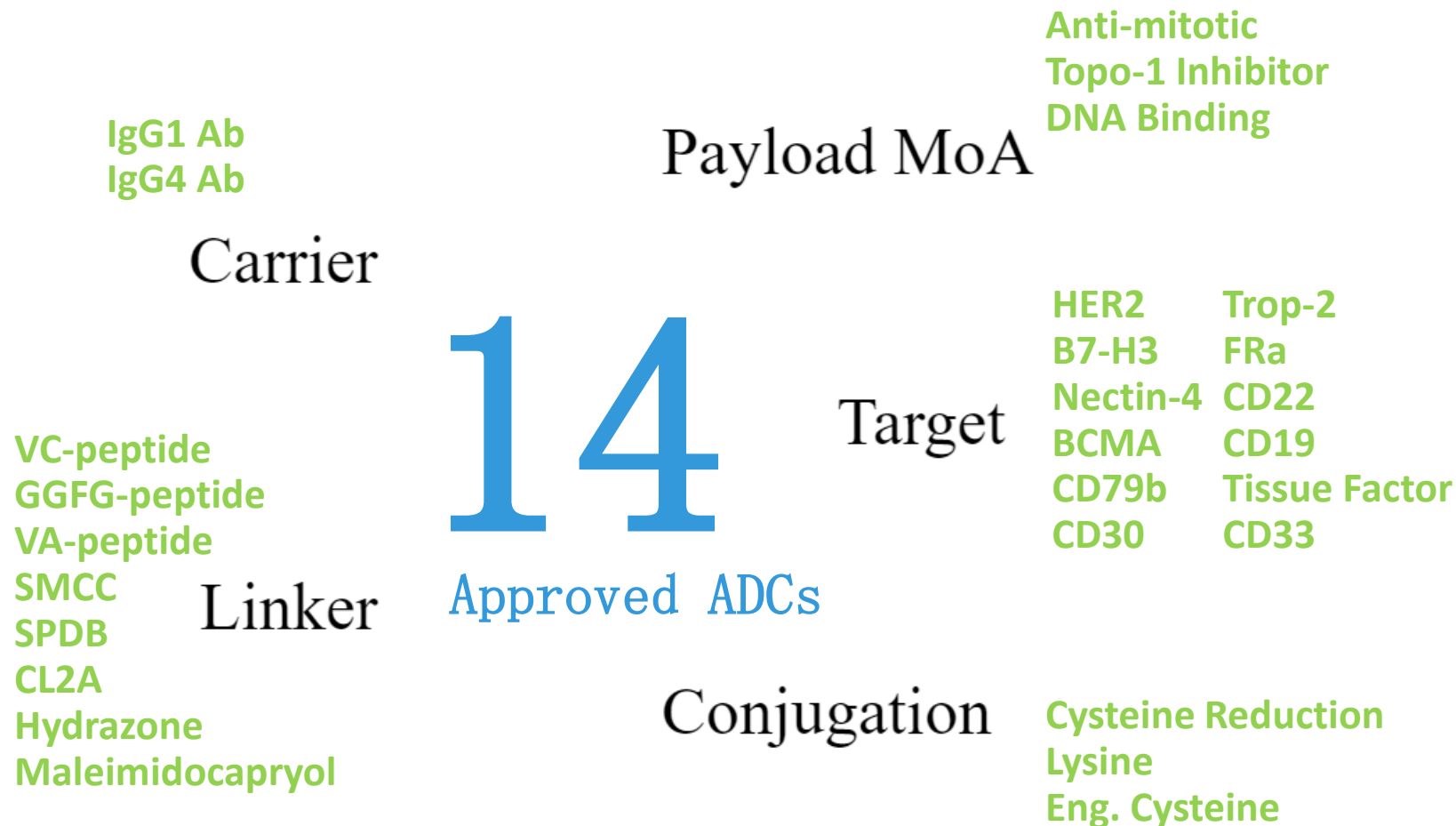


[Signal Transduct. Target. Ther. \(2022\) 7:93](#)

ADCs combine the tumor-targeting properties of the antibody moiety with the potency of cytotoxic agents.



Design of Approved and Clinical-Stage ADCs

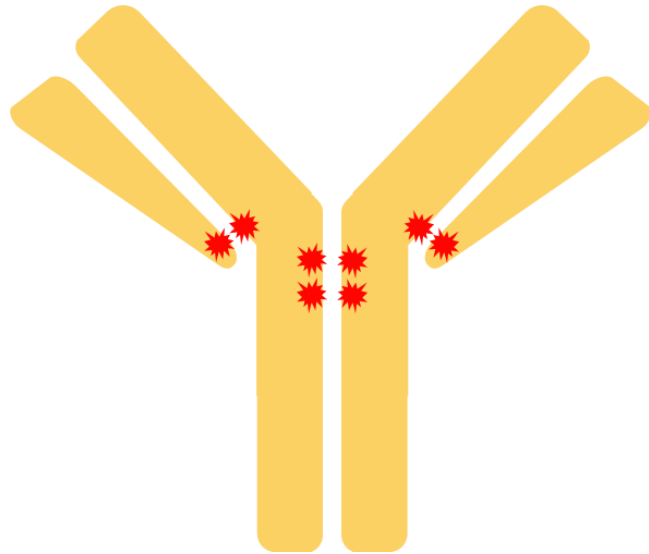


Reconstructed from: [Nat. Rev. Drug Discov. \(2024\)](#)

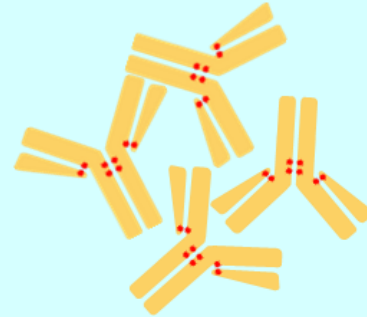
“[W]e anticipate companies will build diverse collections of components to enable ‘plug-and-play’ development tailored to specific targets and indications.”



Selected Quality Attributes of ADCs and Common Methods

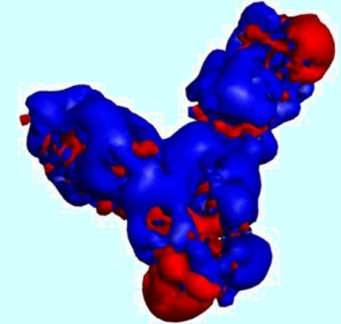


Size Variants



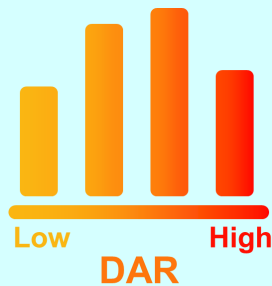
SEC, CE-SDS, AUC

Charge Variants



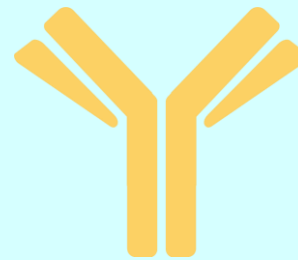
IEC, icIEF, cIEF, CZE

Drug Antibody Ratio



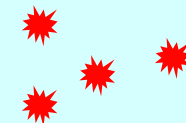
UV, HIC, RP, icIEF, MS

Unconjugated mAb



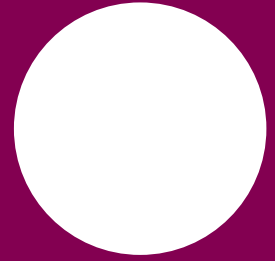
HIC, icIEF, MS

Free Drug

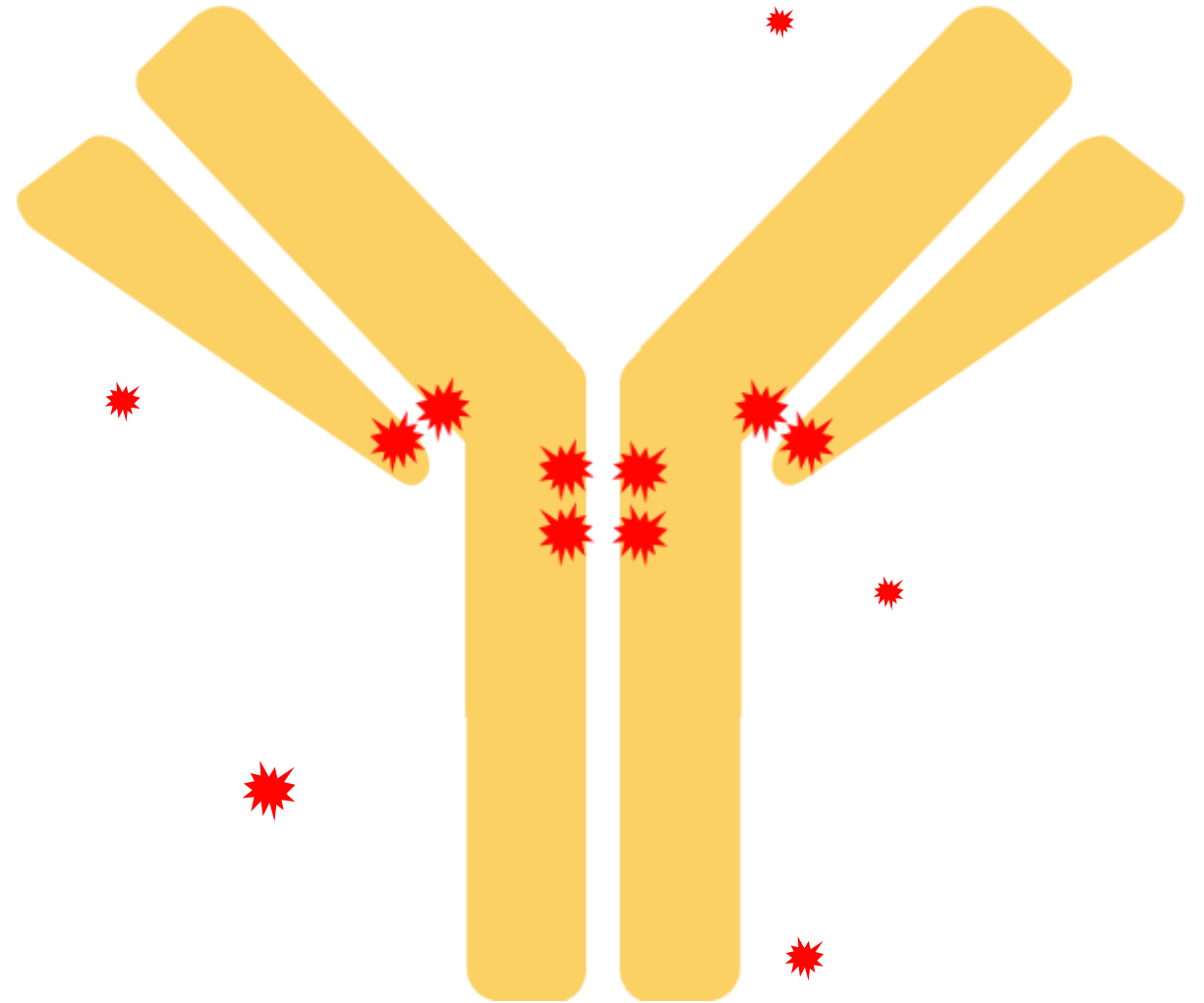


RP, 2DLC-MS





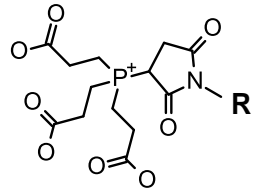
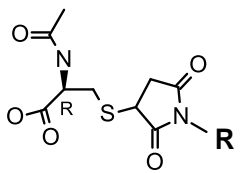
Free Drug



Free Drug – Source and Methods

Source of Free Drugs

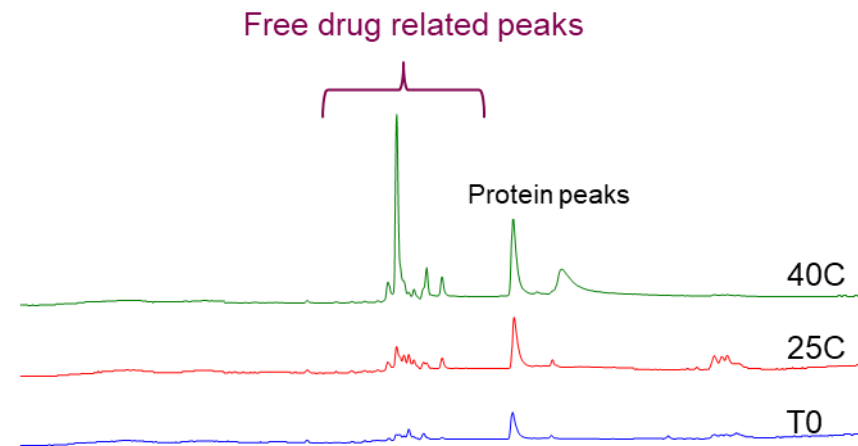
- Process
 - Byproducts (with reduction and quenching reagents)
 - Impurities from payload



- Storage and Stability
 - Deconjugation
 - Linker fragments

Common Method

- Free drug species (small molecules) analyzed after protein precipitation
 - Detects a group of peaks
 - Delicate balance btw recovery and protein removal
 - Safety and exposure

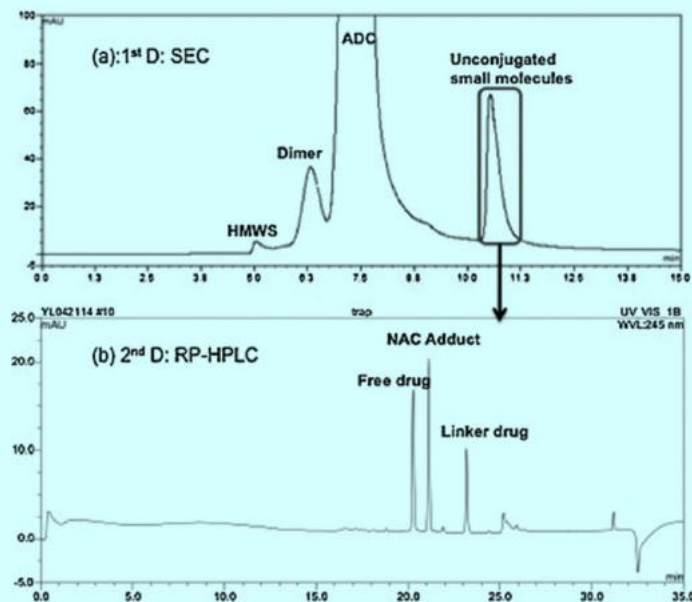


A method with simplified sample preparation steps is highly desirable.



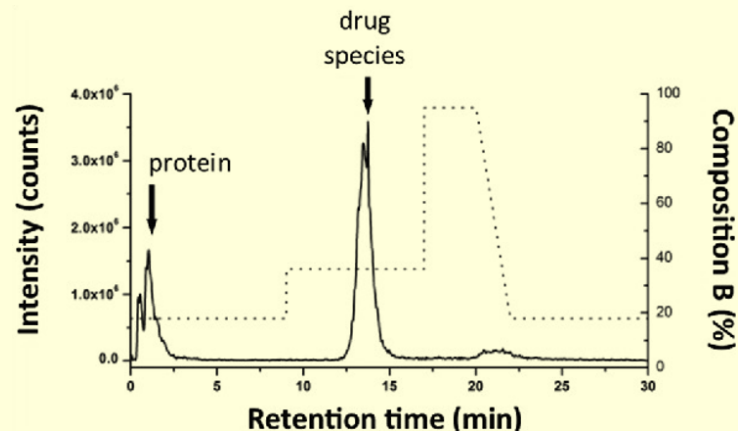
On column separation between free drugs and protein peaks

Size



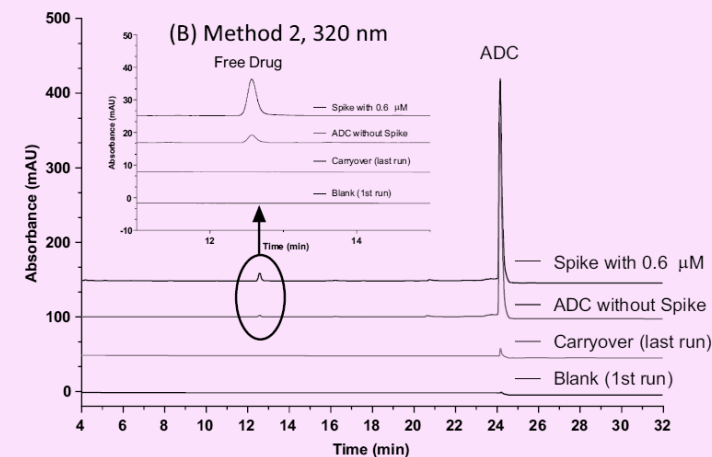
Journal of Chromatography A, 2015, 1393, 81-88

Charge



mAbs, 2016, 8, 306-317

Hydrophobicity

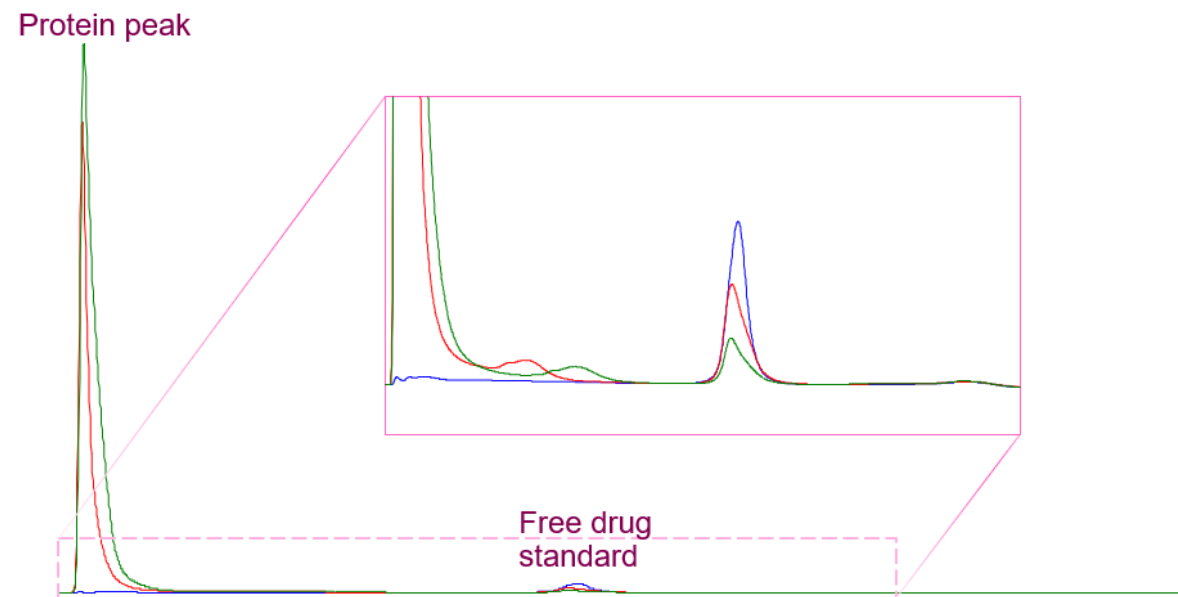
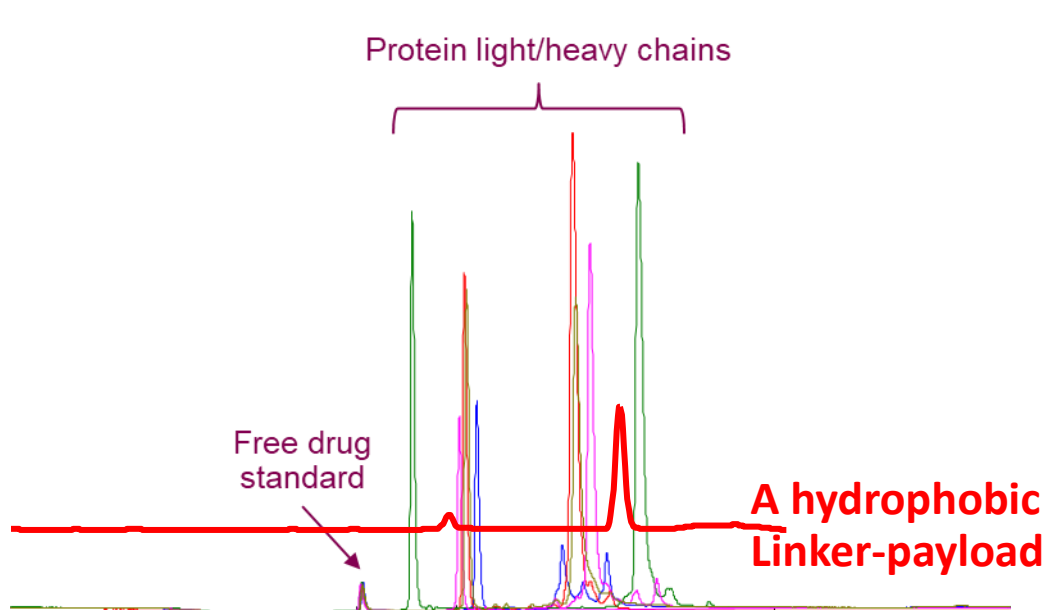


Journal of Chromatography B, 2019, 1161, 51-59

Multiple physicochemical properties can be exploited to afford online resolution of free drugs from proteins.



Gamut of Payload Hydrophobicity

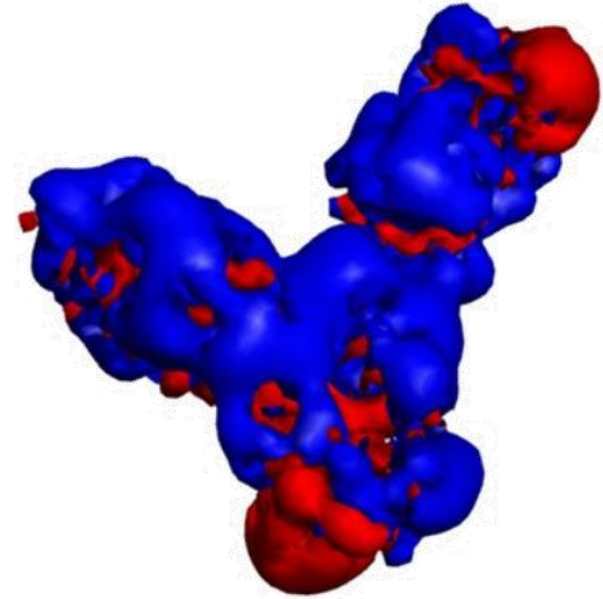


- **RP** has been working well for both site-specific and interchain-cysteine conjugated ADCs
- Linker-payloads with increased hydrophobicity abate the resolution between free drugs and protein components

- **Mixed-mode separation (based on charge and hydrophobicity)** on a single column showed promise to be a routine free drug assay.
- Best for hydrophobic payloads – complimentary to the RP.

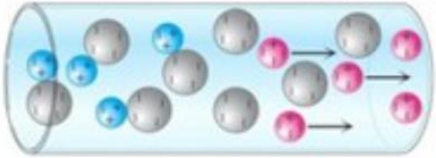


Charge Variants

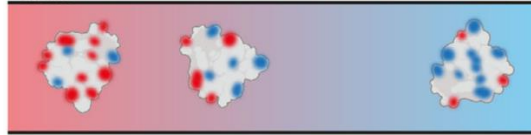


Comparing Methods for Protein Charge Variants

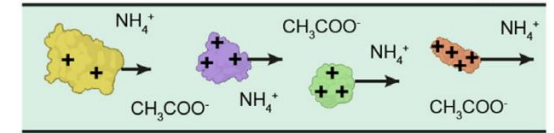
Ion Exchange Chromatography



Isoelectric Focusing



Capillary Zone Electrophoresis



No sample preparation needed;
Options for extensive optimization;
On HPLC platform;
Easy fraction enrichment

High resolution;
Fast run time (icIEF);
Can be the platform method

No sample preparation needed;
High resolution



Not as high resolution;
Potential 2nd interactions;
Less likely to be platform;
Column lot-lot variance

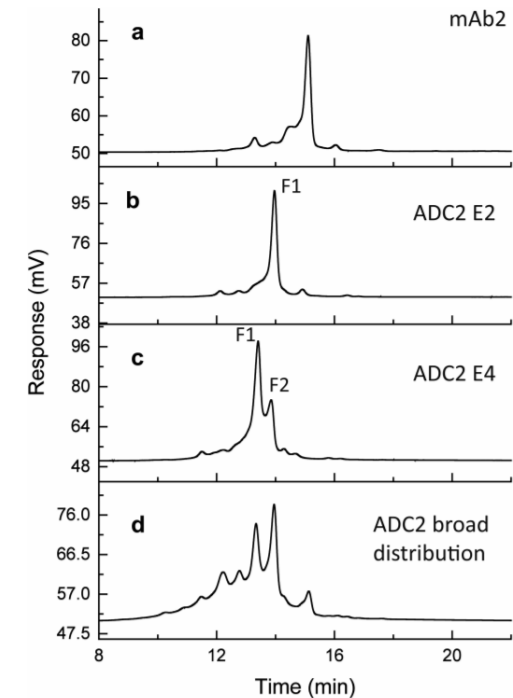
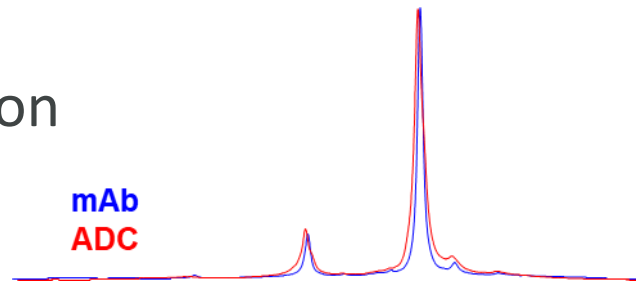
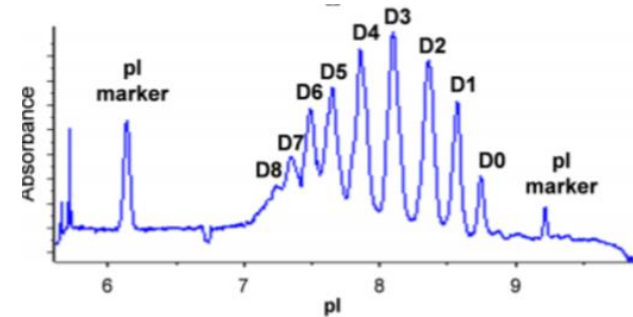
Sample preparation needed;
Peak characterization/ID;
Reagent lot-lot variance

Peak characterization/ID;
Capillary and Reagent lot-lot
variance



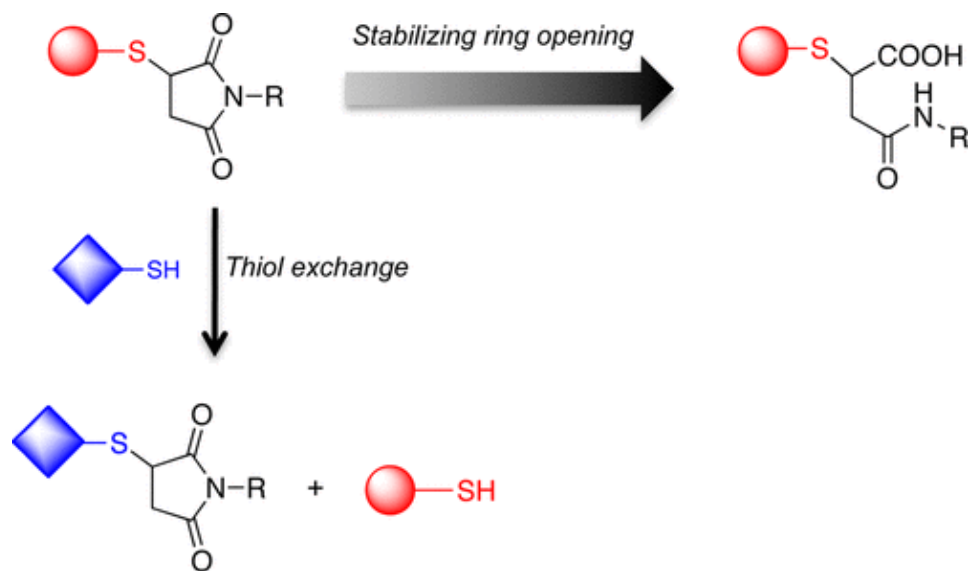
Impact of Conjugation on Charge Profiles (*neutral payload*)

- Lysine conjugation
 - Loss of positive charge upon conjugation
- Site-specific cysteine conjugation
 - No impact of charge profile
- Hinge cysteine conjugation (profile shifts to acidic)
 - Distribution of DAR – complex profiles
 - Uniform DAR – similar profile to mAb



Thio-Succinimide Hydrolysis and Charge Variants

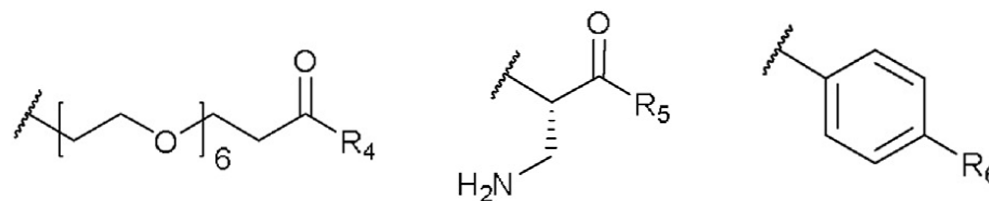
Hydrolysis Improves in vivo stability



Bioconjugate Chemistry, 2015, 26, 145-152

Conditions that promote ring opening

- Local positively charged residues on mAbs
- Electron withdrawing N-substitutes



Drug Discovery Today: Technologies, 2018, 30, 27-34

Dilemma

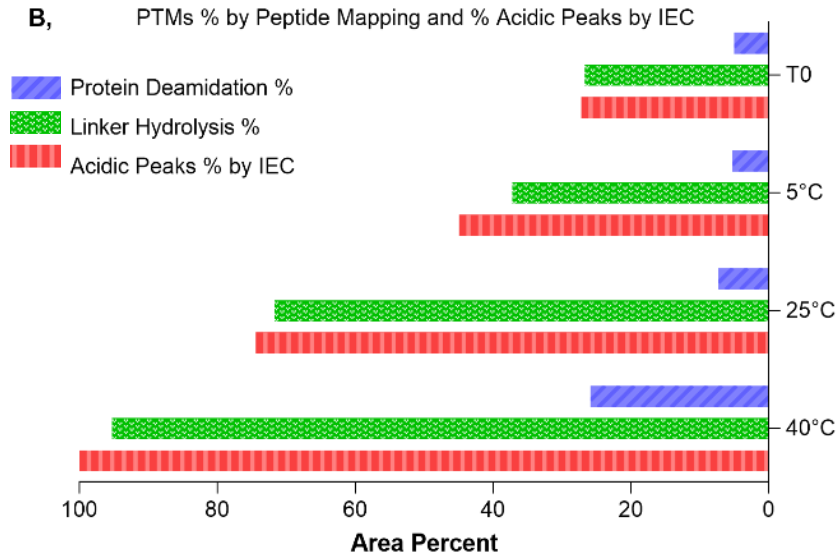
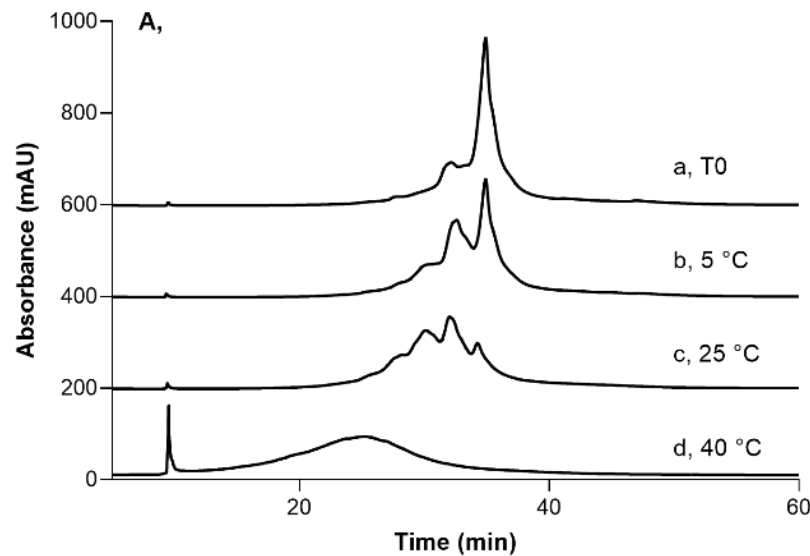
- Hydrolysis pre-conjugation is detrimental
- Cannot decouple pre- and post-conju. hydrolysis

CMC is challenged by conjugation products with various hydrolysis rates.



Challenges with Hydrolysis-Prone Linkers

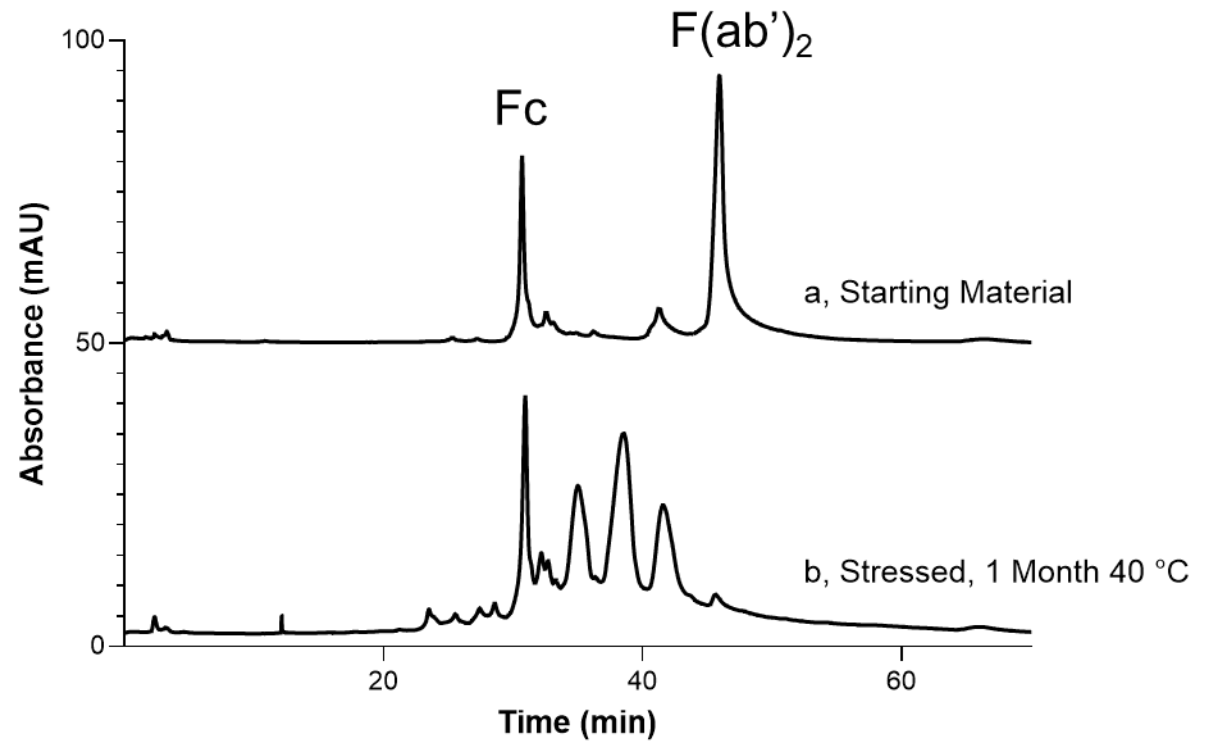
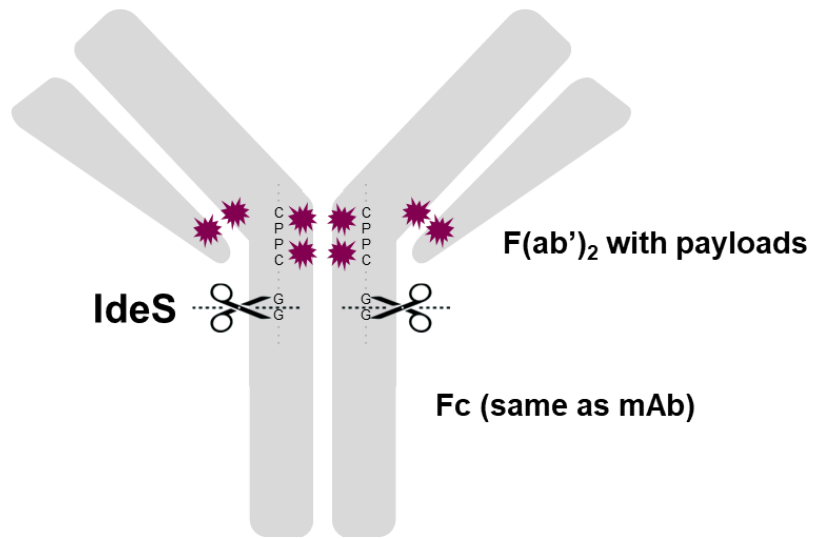
- Large variation of charge variants under desirable process flexibility
- Sample storage condition need strictly managed
- Stability profile is driven by the ring hydrolysis



Charge variants are no longer appropriate measurements of protein-related degradations.



Dissecting the Problem ...

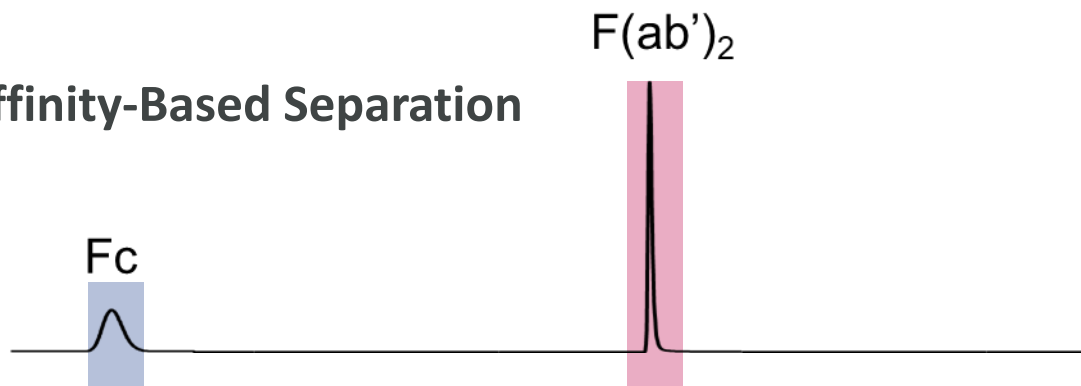


At the Sub-unit level, the hydrolysis peaks appear to be well-resolved by IEC.

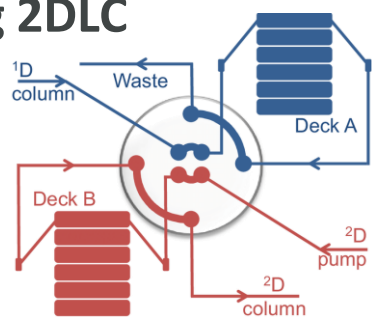


Separation on Another Dimension

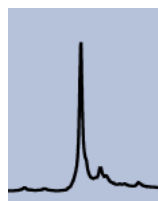
Affinity-Based Separation



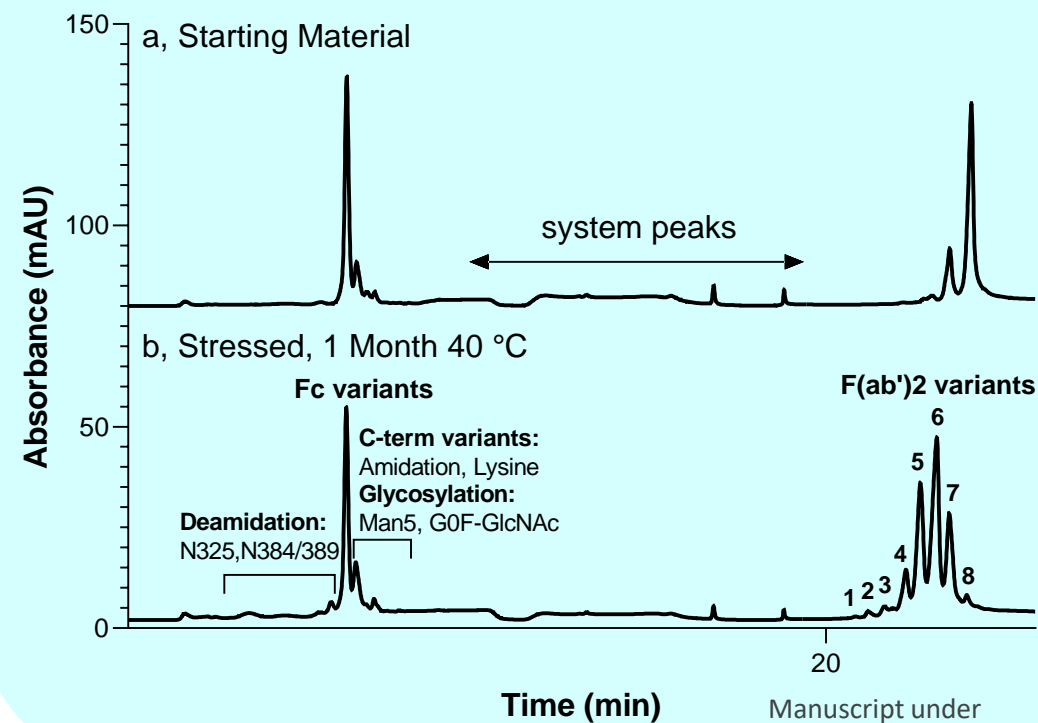
Heart-cutting 2DLC



IEC as the 2D



Affinity-IEC Tandem column

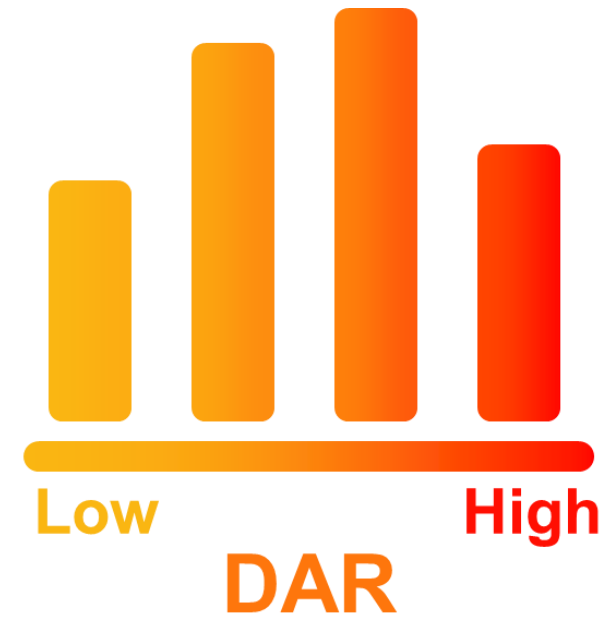


Manuscript under revision.

Charge variants are completely resolved with Sub-unit analysis on a tandem-column setup.

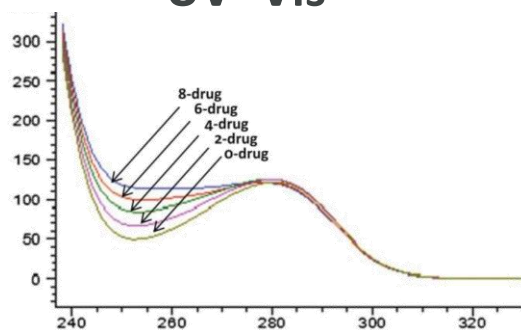


Drug Antibody Ratio



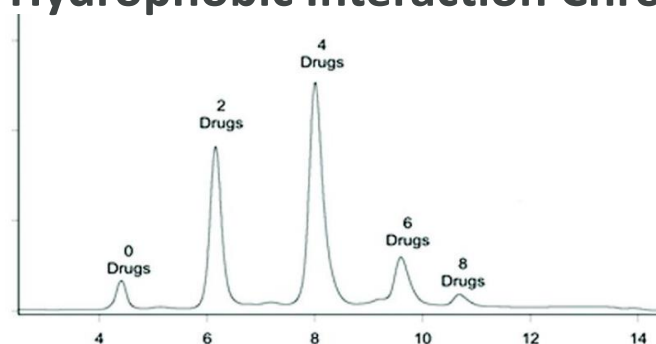
Comparing DAR Methods

UV- Vis



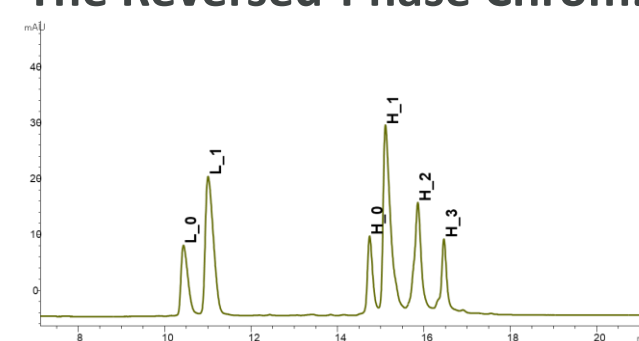
Simple, fast and robust;
No need for method dev

Hydrophobic Interaction Chrom.



No sample preparation needed;
Informative DAR distribution;
Detects unconjugated mAb

The Reversed-Phase Chrom.



High resolution;
MS-compatible solvents for
easy Peak ID



Need unique λ_{max} ;
Potential interferences;
DAR distribution not available

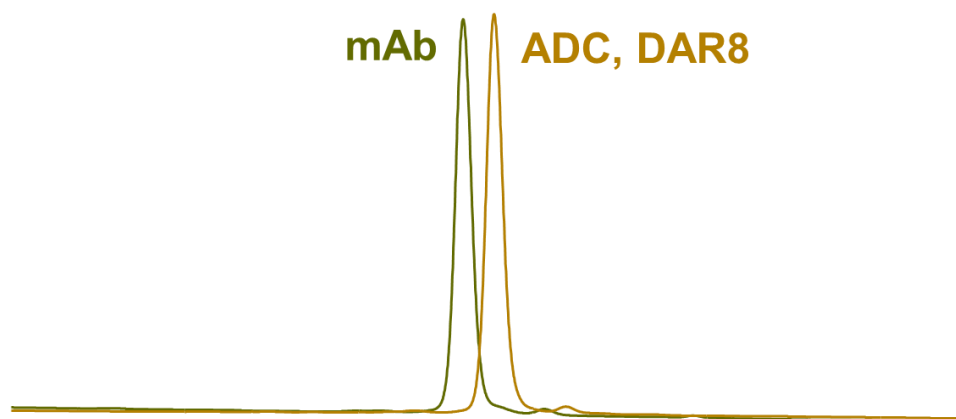
High salt mobile phase;
Complex workflow for Peak ID

No DAR distribution on the
intact level;
Resolution of PTMs, degradants;
Data processing can be
complicated



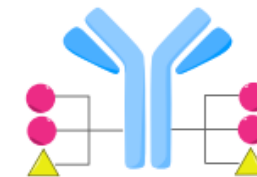
Emerging Challenges on the DAR Method

Hydrophilic Payloads

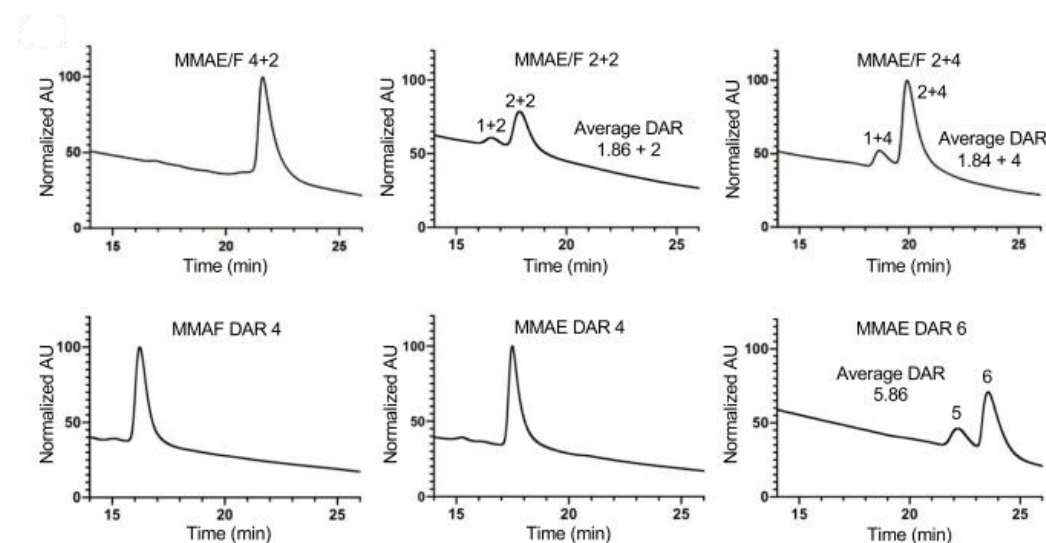


Small change in hydrophobicity after conjugation

Dual-Payloads ADCs



MMAE/F 4+2



Nature Communications, 2021, 12, 3528

Not enough peak capacity to resolve ADCs with complex combinations of dual-payloads



Conclusions

- The increasing number of clinical-stage ADCs showcases a significant diversity in their molecular structures.
- This diversity in physicochemical properties presents a challenge for existing analytical methods.
- Having a one-size-fits-all method becomes less viable.
- With the accumulated knowledge and a deeper understanding of chemistry, the industry is rapidly broadening its analytical toolkit to effectively characterize the new generation of ADCs.



Thank You

- AstraZeneca Colleagues
- CASSS CMC Strategy Forum Organizers and Session Chairs
- Audiences and Attendees



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