Mechanistic Insights into non-mAb Aggregation Using MS-Cleavable Linker

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Merck & Co., Inc., Rahway, NJ, USA

24 Sep 2025

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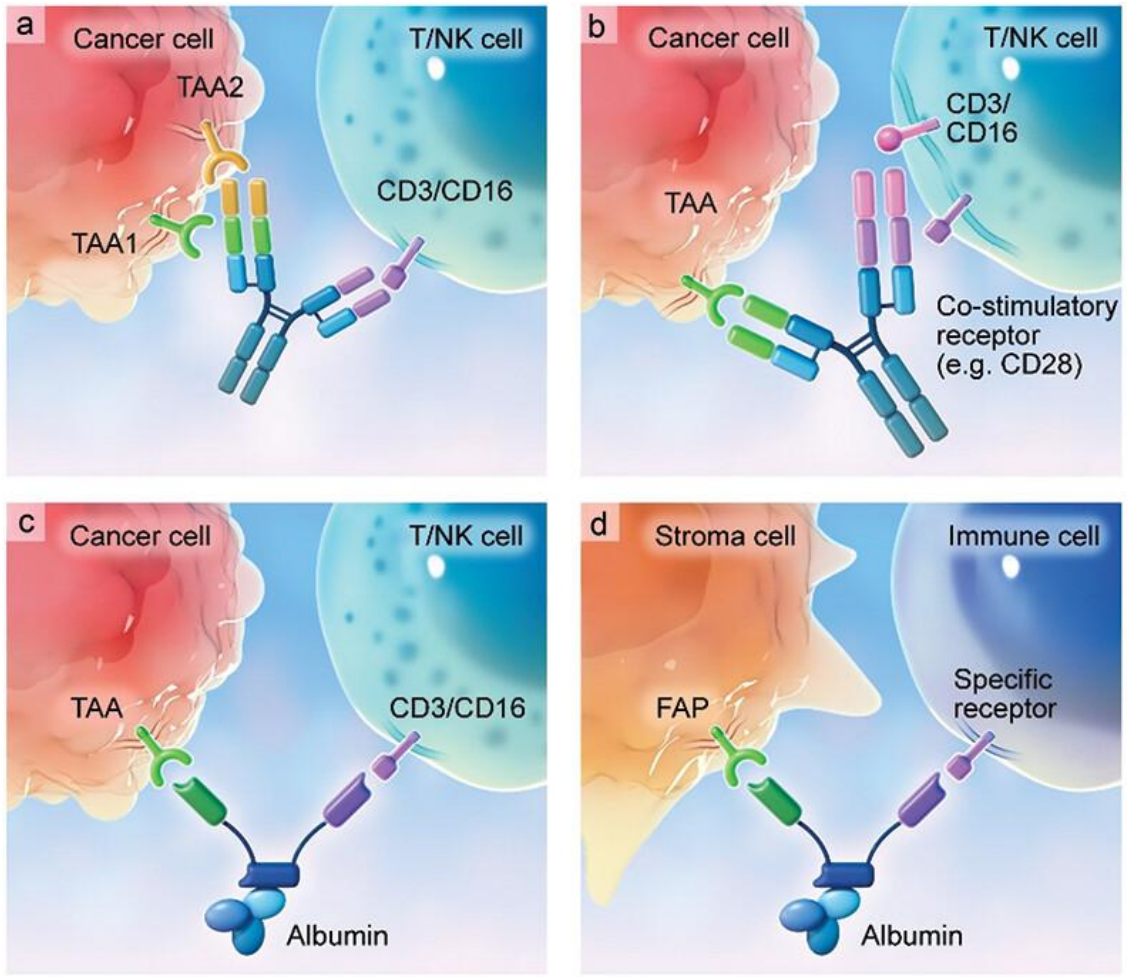
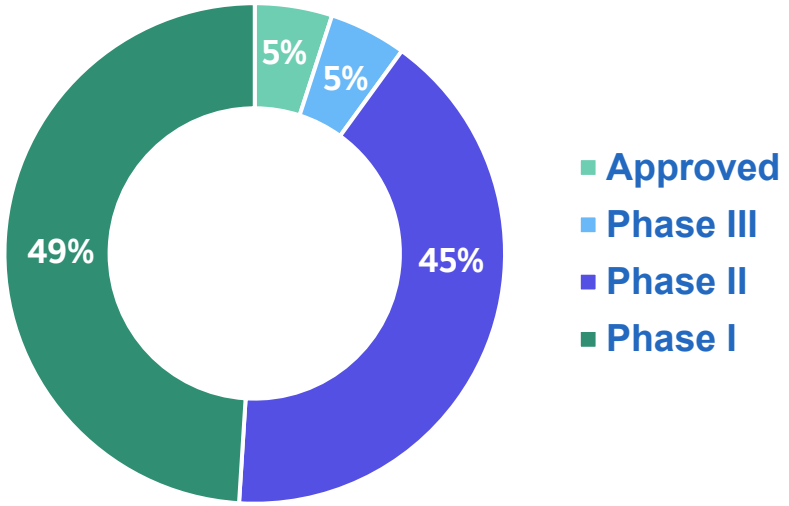
The Next Big Thing in Tumor Therapeutics

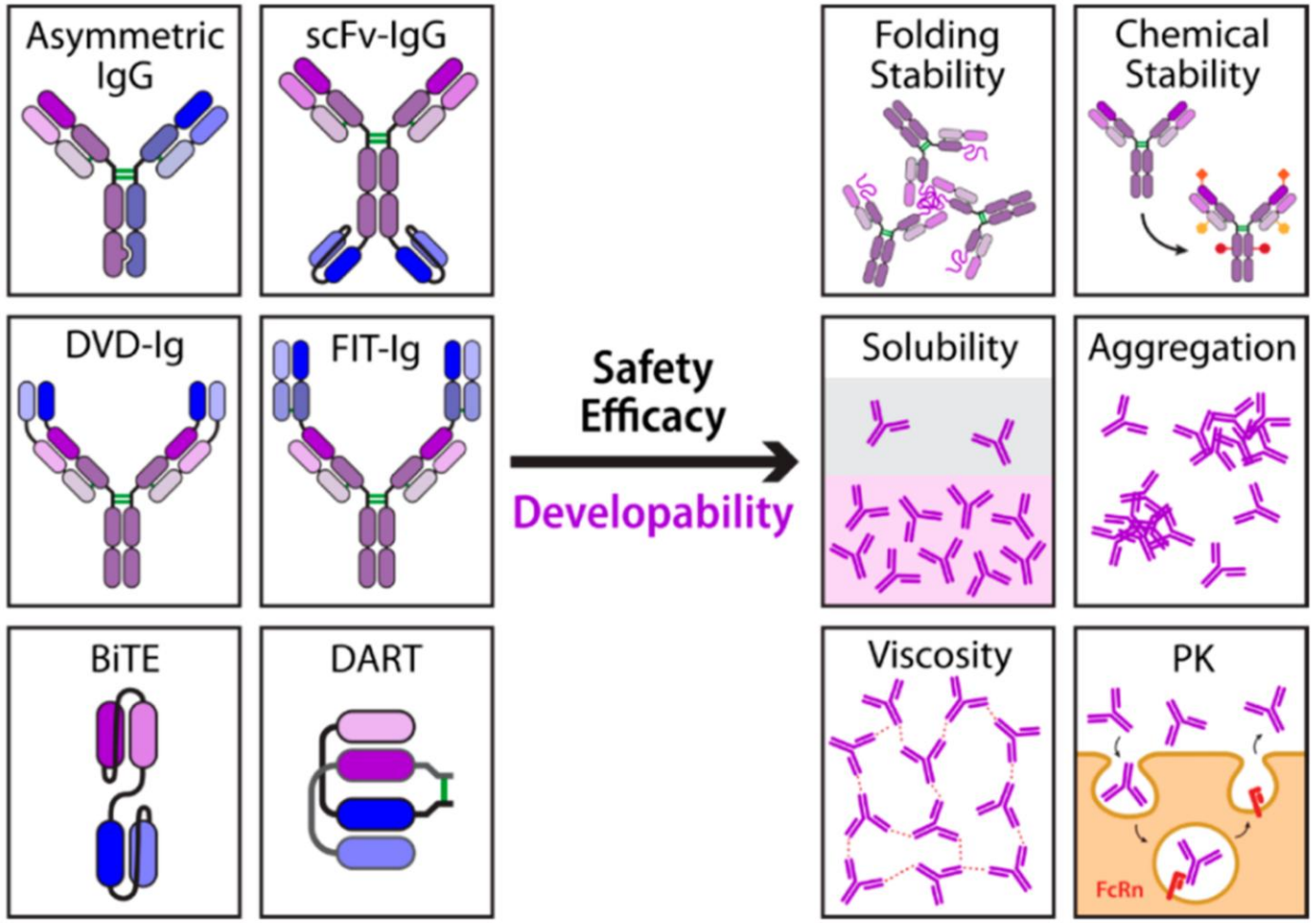




- More than 200 bispecific antibodies (bsAbs) are currently in clinical development.
- 50% of bsAbs in clinical development are in phase II and phase III or already approved.

a) Labrijn, A.F., Janmaat, M.L., Reichert, J.M., Parren, P.W.H.I. ***Nat Rev Drug Discov.* 2019, *18,*** 585-608. b) Tapia-Galisteo, A., Compte, M., Álvarez-Vallina, L., Sanz, L. ***Theranostics* 2023**, ***13*** (3), 1028-1041. c) Klein, C., Brinkmann, U., Reichert, J. M., Kontermann, R. E. ***Nat Rev Drug Discov.* 2024, *23*** (4), 301-319. d) Cao, Z., Osellame, L.D., Allan, L., Scott, A.M. ***Expert Opinion on Investigational Drugs*, 2025** accepted.

Physical and Chemical Challenges of Multispecific Abs (msAbs)

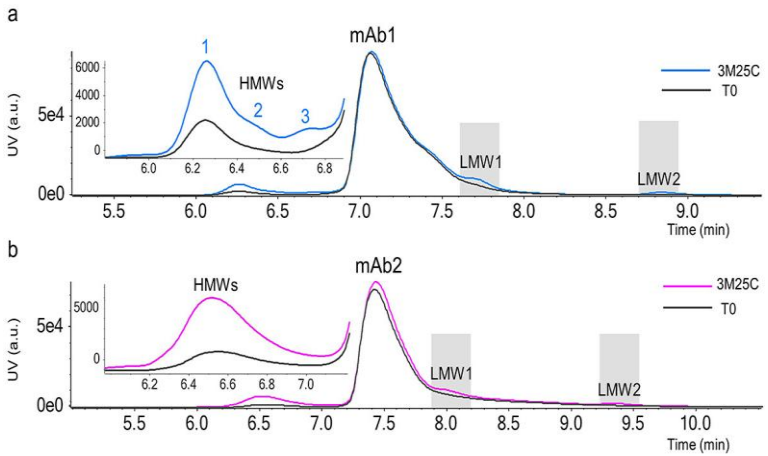


- x High levels of aggregate formation during protein expression and cell culture
- x Dimers, trimers, and higher order multimers can form during formulation and storage
- x Self-association has the potential to adversely affect shelf-life, pharmacokinetic properties, efficacy, safety and immunogenicity

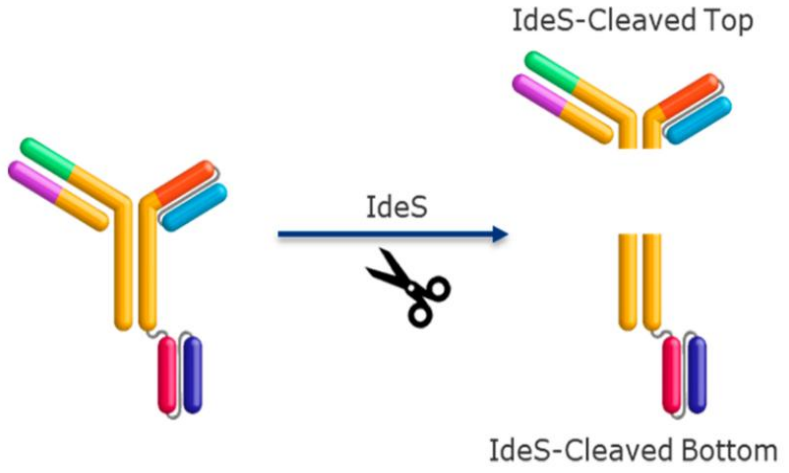
a) Sawant, M. S., Streu, C. N., Wu, L., Tessier, P. M. ***Int. J. Mol. Sci.*** **2020**, ***21*** (20), 7496. b) Moussa, E.M., Panchal, J.P., Moorthy, B.S., Blum, J.S., Joubert, M.K., Narhi, L.O., Topp, E. ***J. Pharm. Sci.*** **2016**, ***105***, 417–430. c) Ratanji, K.D., Derrick, J.P., Dearman, R.J., Kimber, I. ***J. Immunotoxicol.*** **2014**, ***11***, 99–109

Investigating Self-Association: Mass Spec as a Versatile Tool

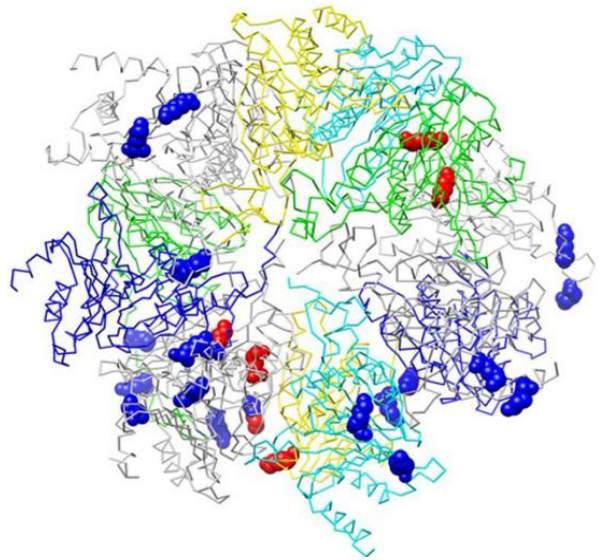
Native Mass Spec



Subunit analysis



Site-targeted analysis

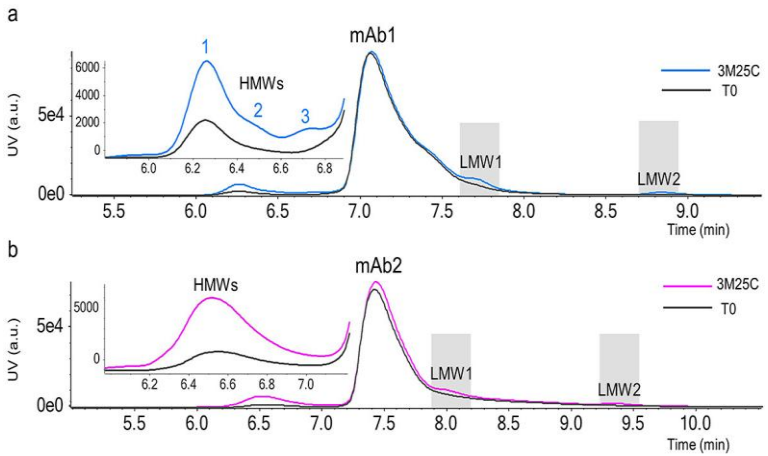


Structural information → **Resolution**

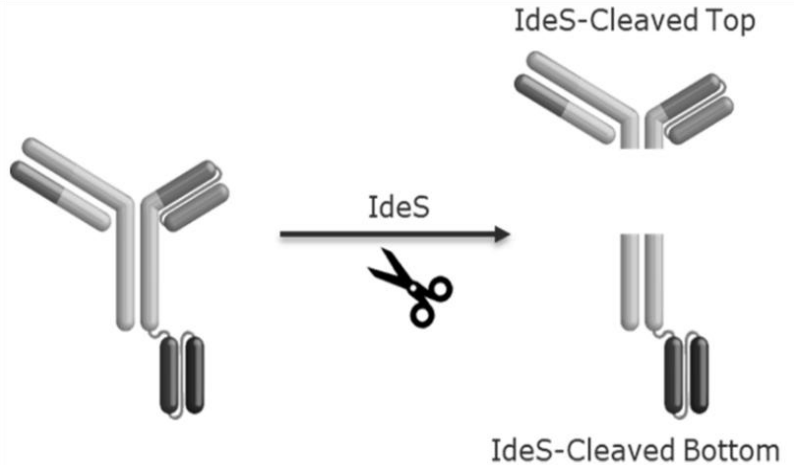
a) Xu, J., Coughlin, J.E., Szyjka, M., Jabary, S., Saluja, S., Sosic, Z., Chen, Y., Xu, C.F. ***mAbs***, **2024**, ***16*** (1) 2334783 b) Poltash, M.L., Srzentić, K., Beil, E., Gorre, E., Damoc, E., Mahan, A.D., Nanda, H., Chowdhury, P. ***J. Am. Soc. Mass Spectrom.*** **2023**, ***34***, 2654−2661. c) Kao, A., et al. ***Mol. Cell. Proteomics*** **2011**, ***10*** (1), M110-002170.

Investigating Self-Association: Mass Spec as a Versatile Tool

Native Mass Spec



Subunit analysis



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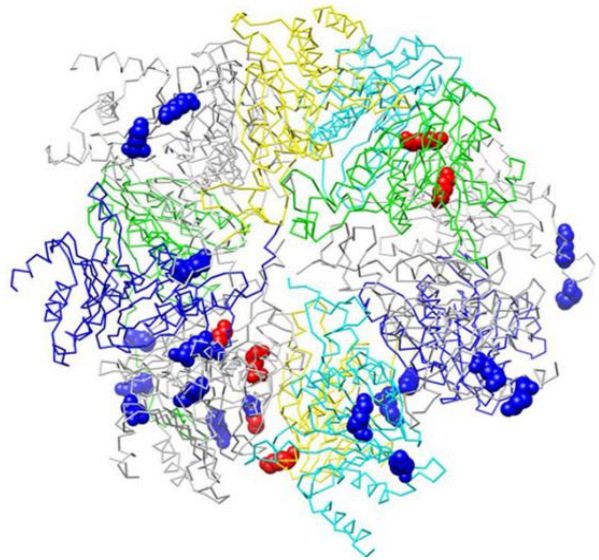

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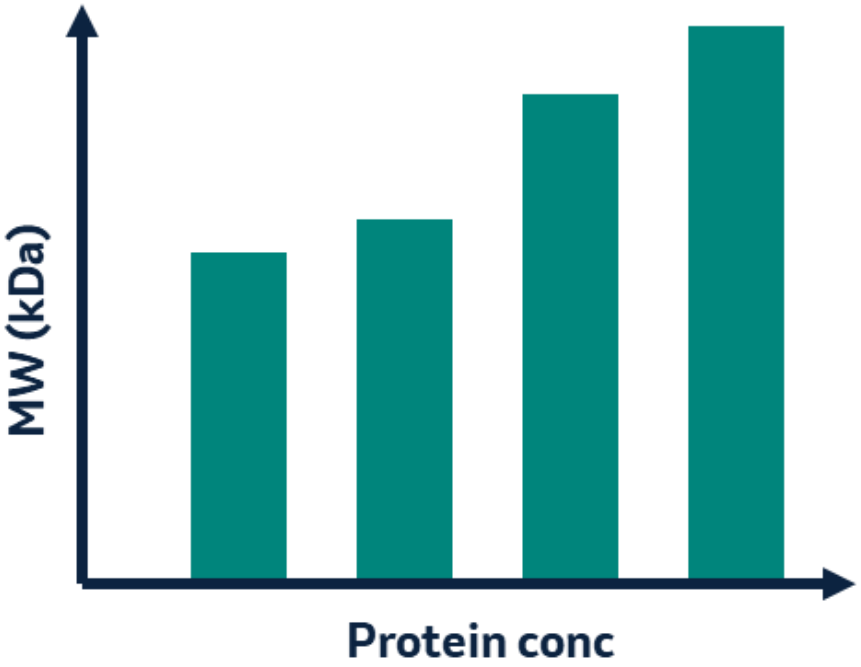
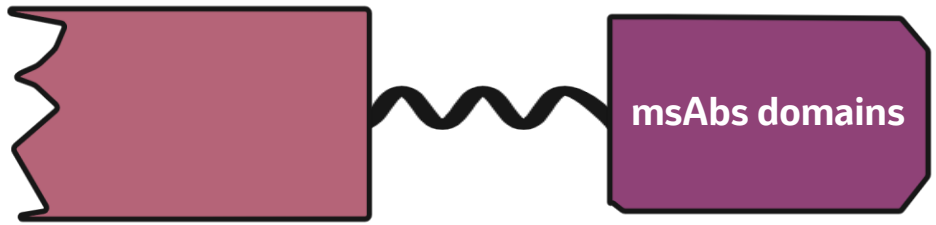
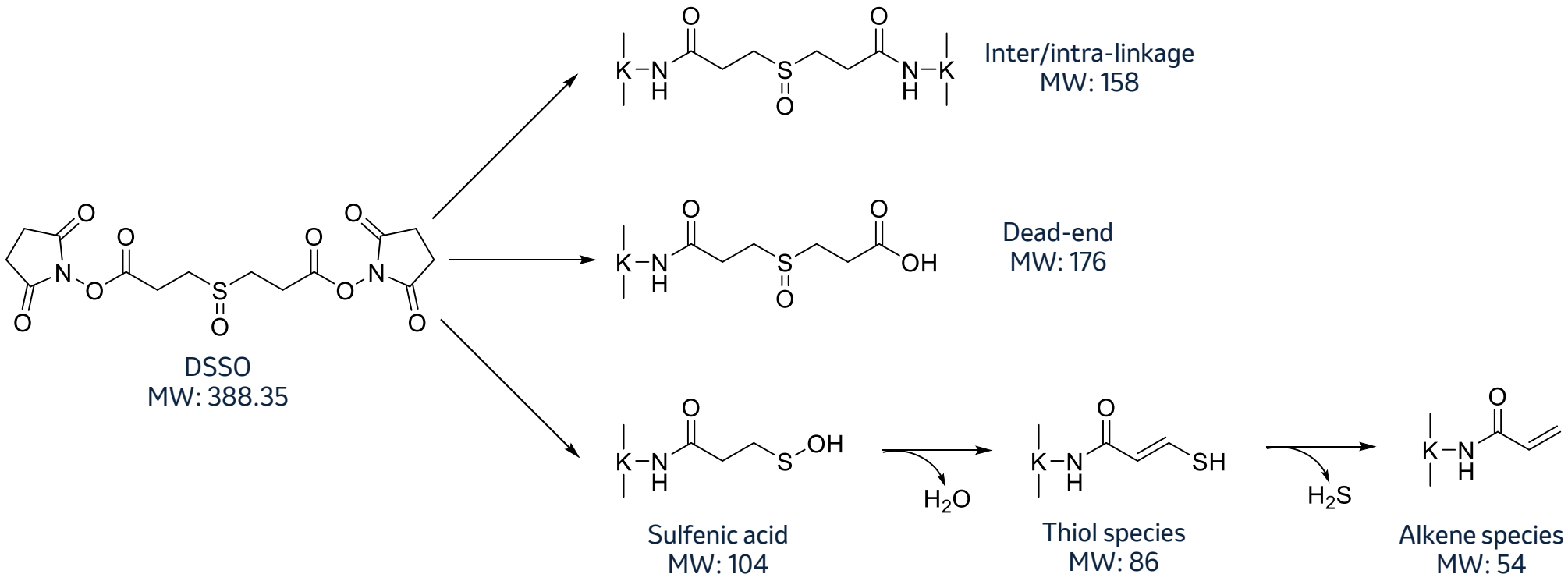
Understanding Self-Association Mechanism in the Pipeline





Using a Cross-Linking Reagent to Understand Aggregation



Kao, A., et al. ***Mol. Cell. Proteomics*** **2011**, 10 (1), M110-002170.

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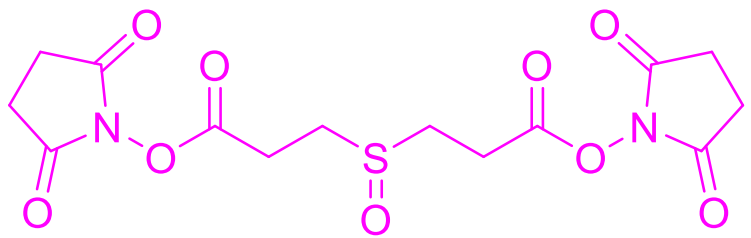
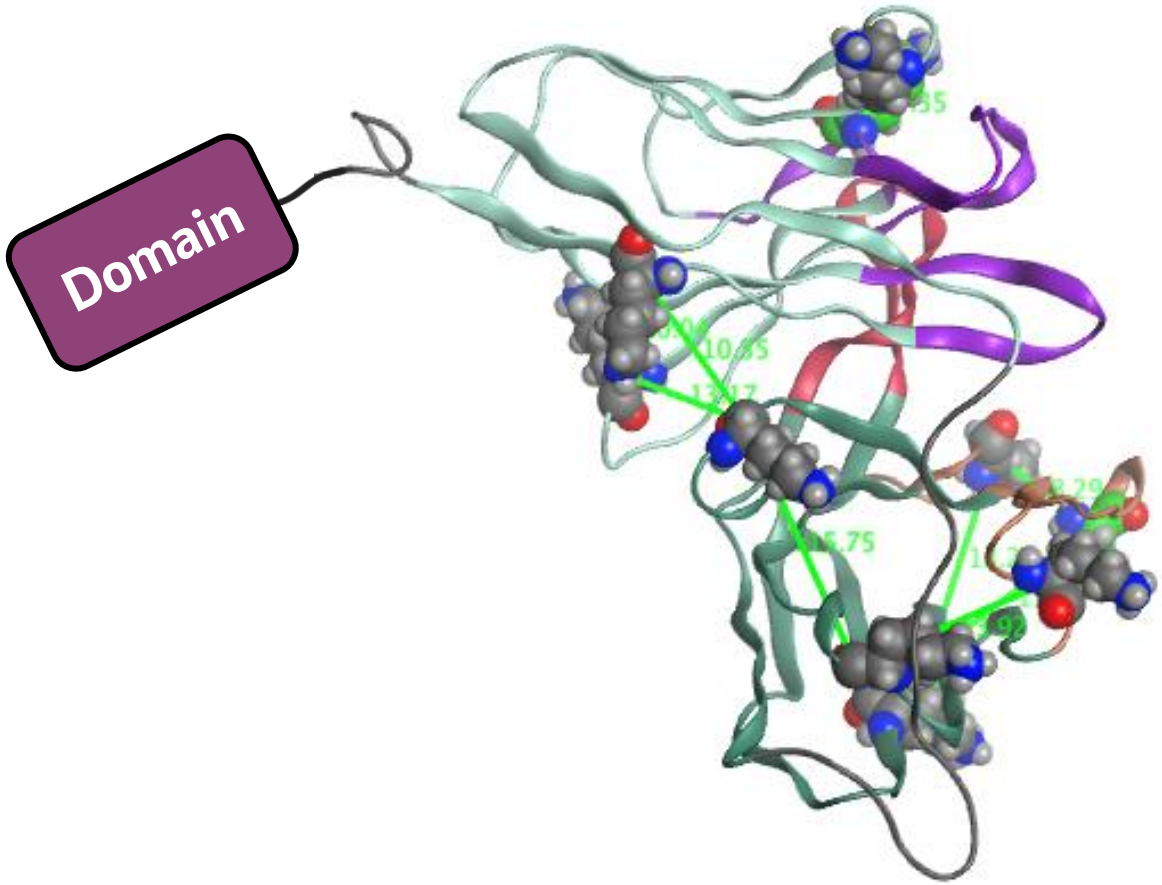


DSSO

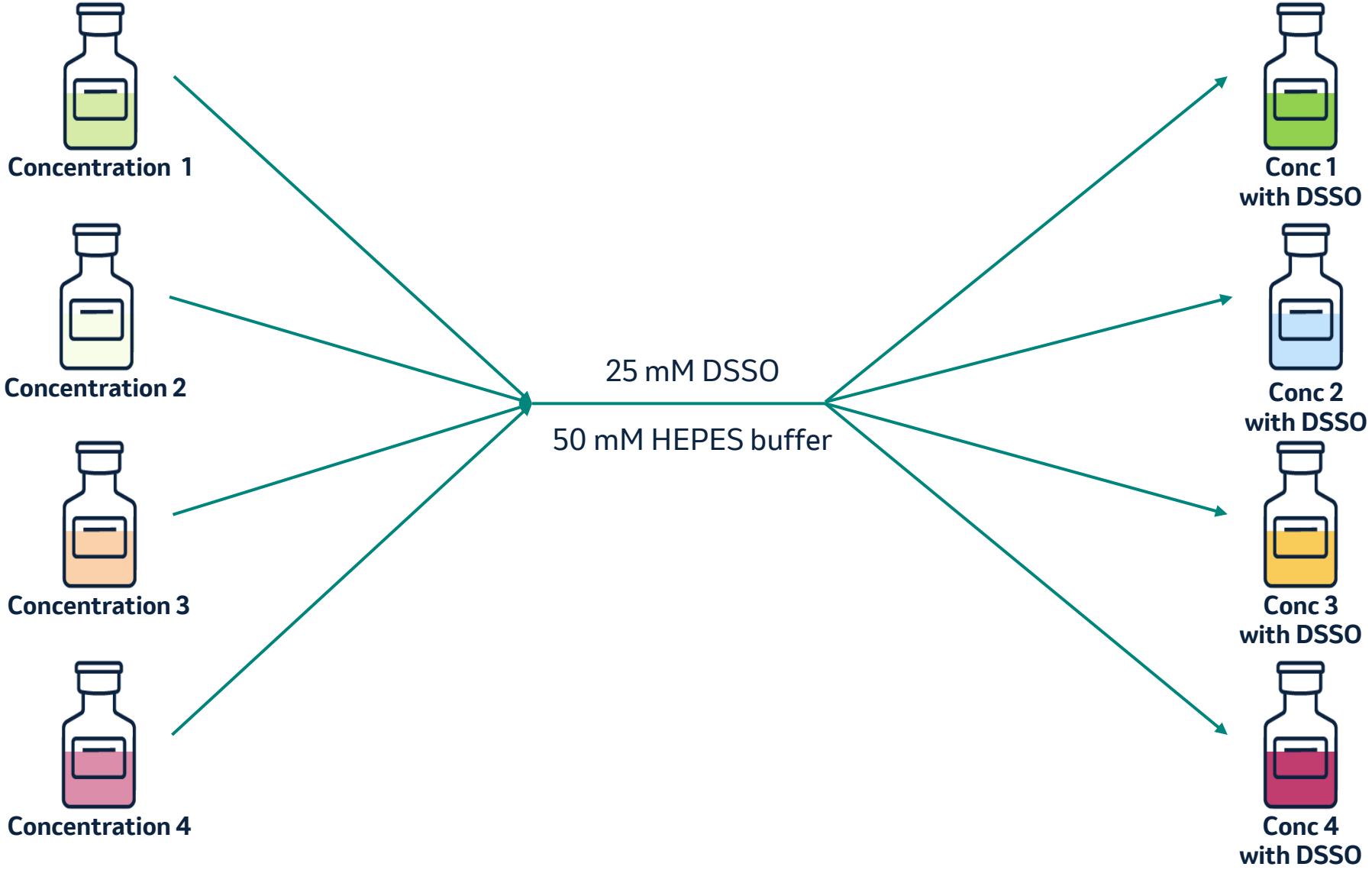
Targets Primary Amines such as Lysine
K to K cross-link (XL)

Spacer length: 10.1 Å



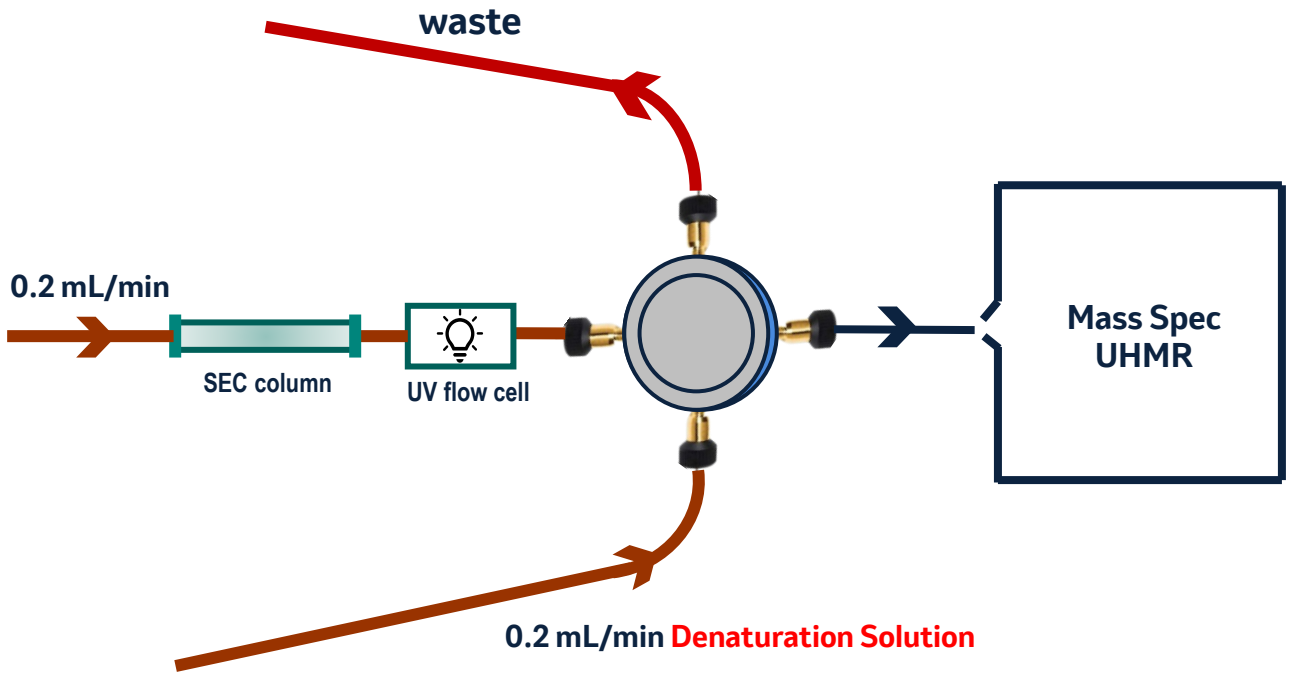
Kao, A., et al. ***Mol. Cell. Proteomics*** **2011**, 10 (1), M110-002170.

Preparation of DSSO-Cross Linked Samples



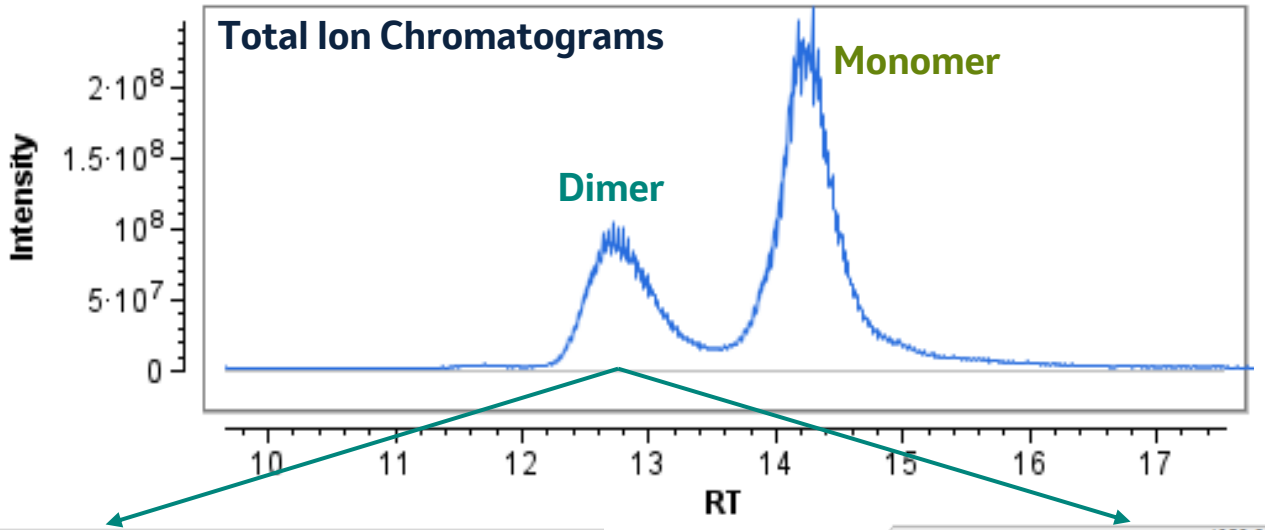
Native SEC-MS with Post-Column Denaturation (nSEC-PCD-MS)

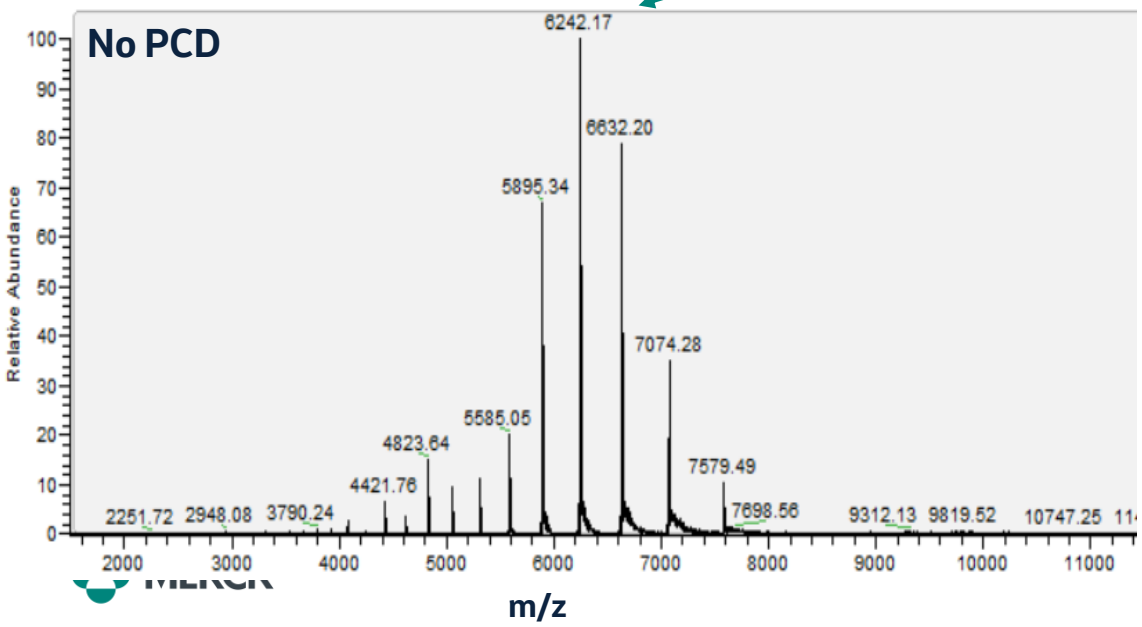
Column	Waters Acquity Protein BEH SEC 4.6x300 mm
Mobile Phase A	300 mM Ammonium Acetate
Mobile Phase B	—
Denaturation solution	**60% ACN, 4% Formic Acid**
Gradient	Isocratic over 30 min
Flow rate	0.2 mL/min
Column Temp	20 °C
Injection amount	10 µg
UV detection	280 nm

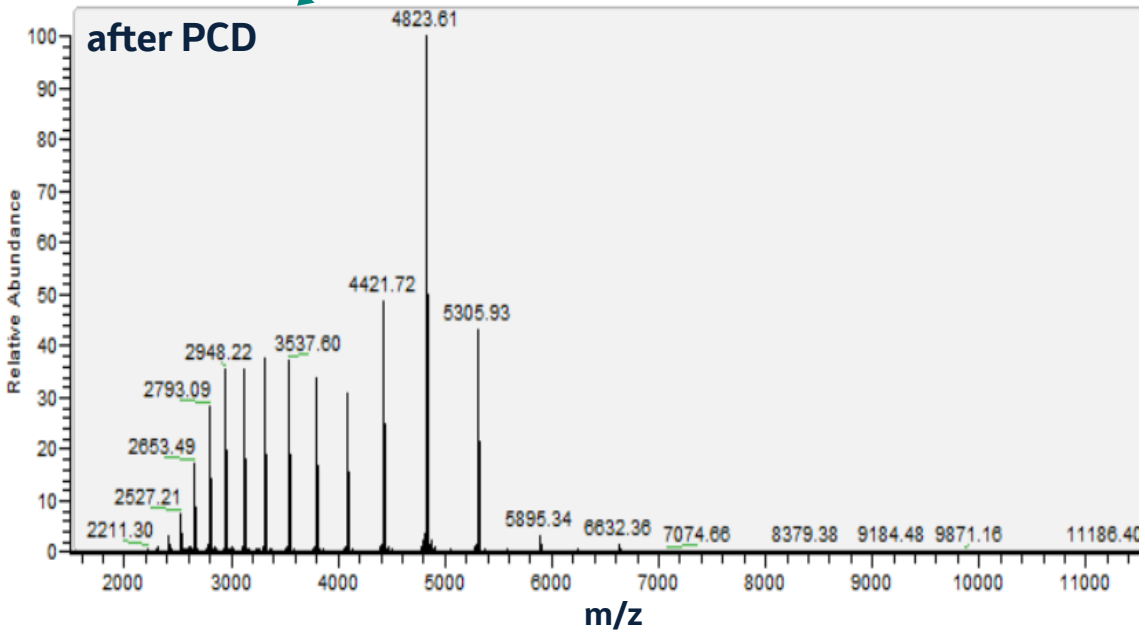


Zhang, Z., Liu, A. P., Wang, H., Schuessler, H. A. ***J. Pharmaceut. Biomed.*** **2025**, ***255***, 116635.

nSEC-PCD-MS as a Powerful Tool to Investigate the Nature of Dimers







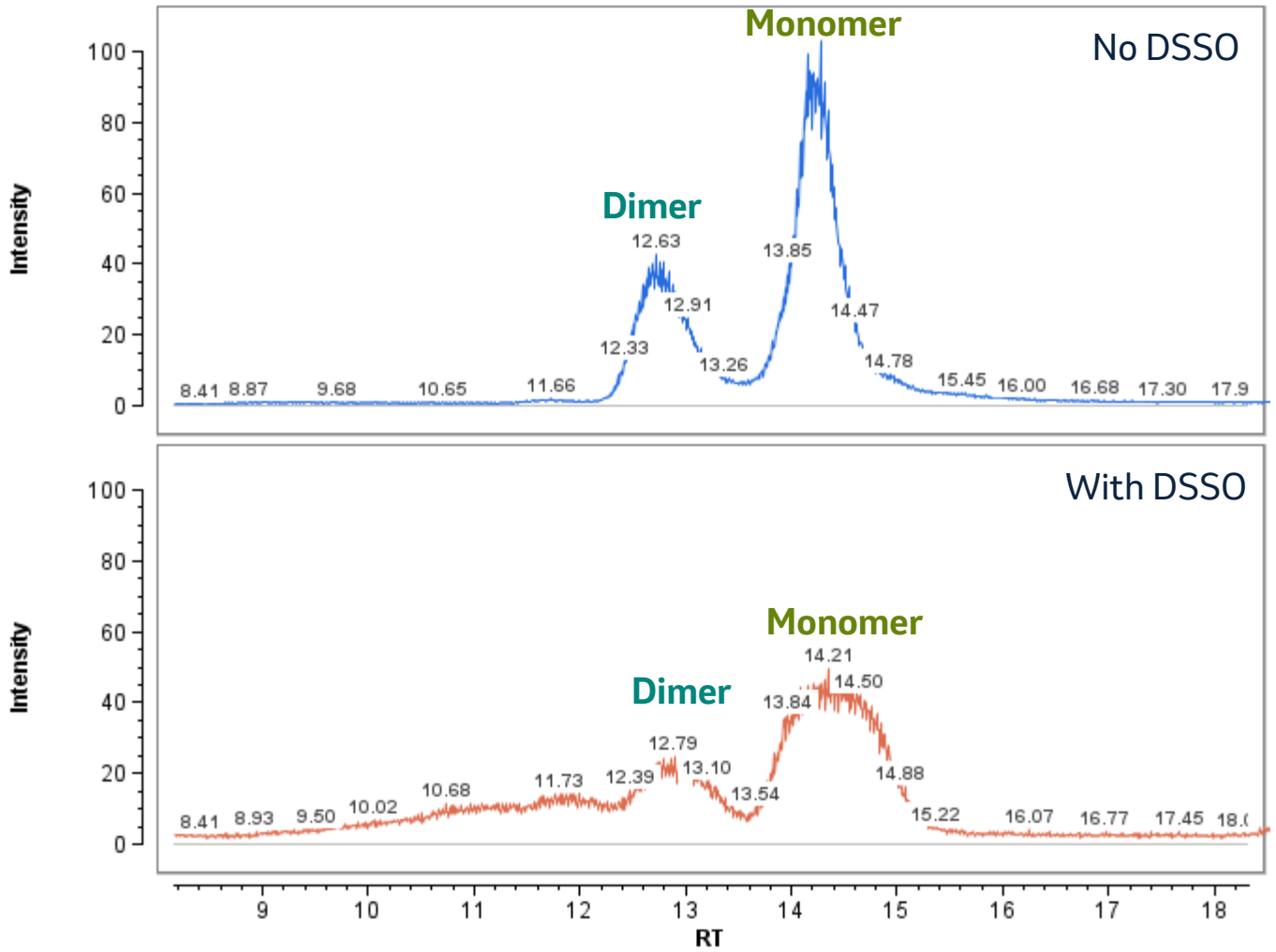
Using nSEC-PCD-MS to Investigate Protein Cross-Linking

Control
No DSSO

Direct Injection

msAb
With DSSO

Total Ion Chromatograms



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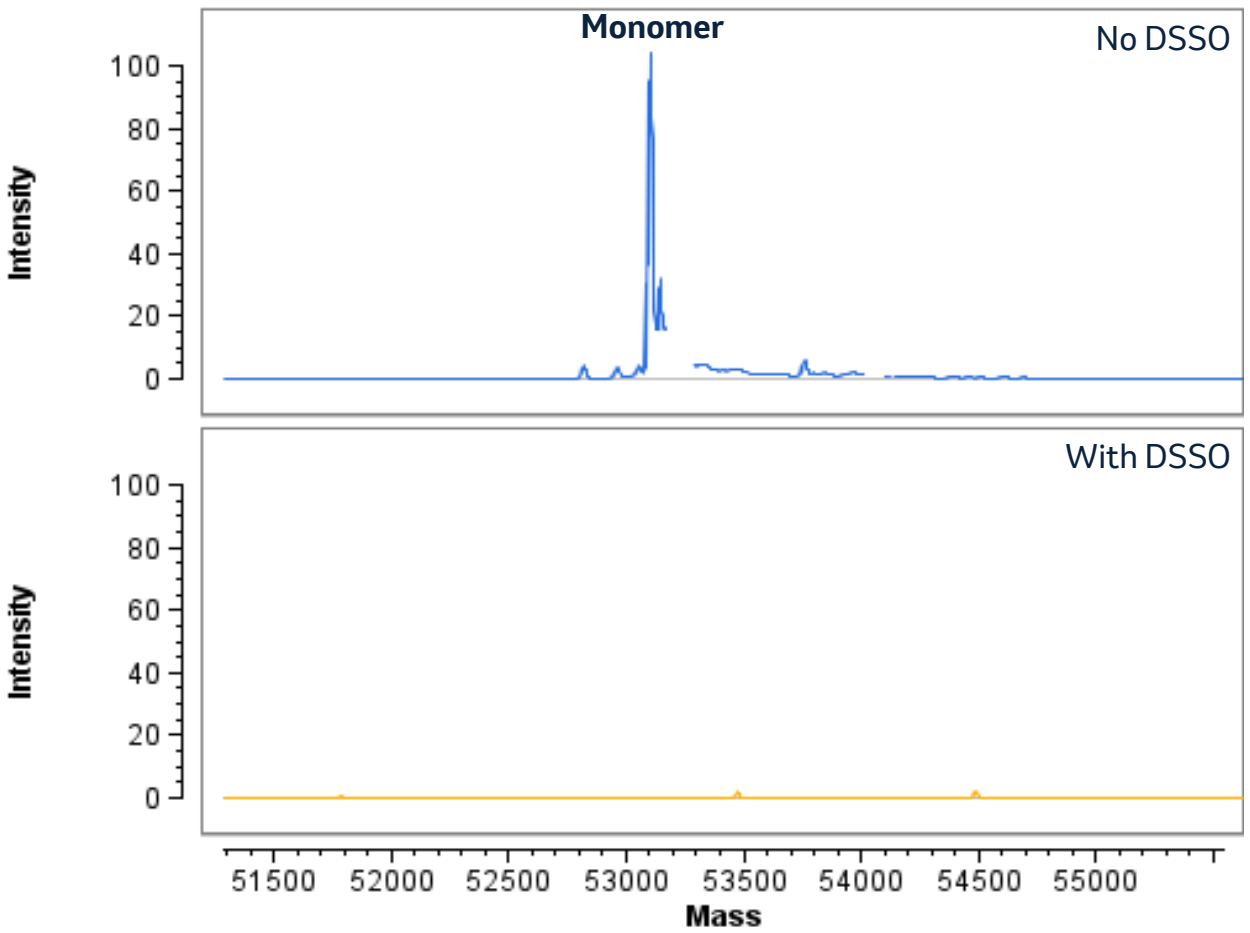
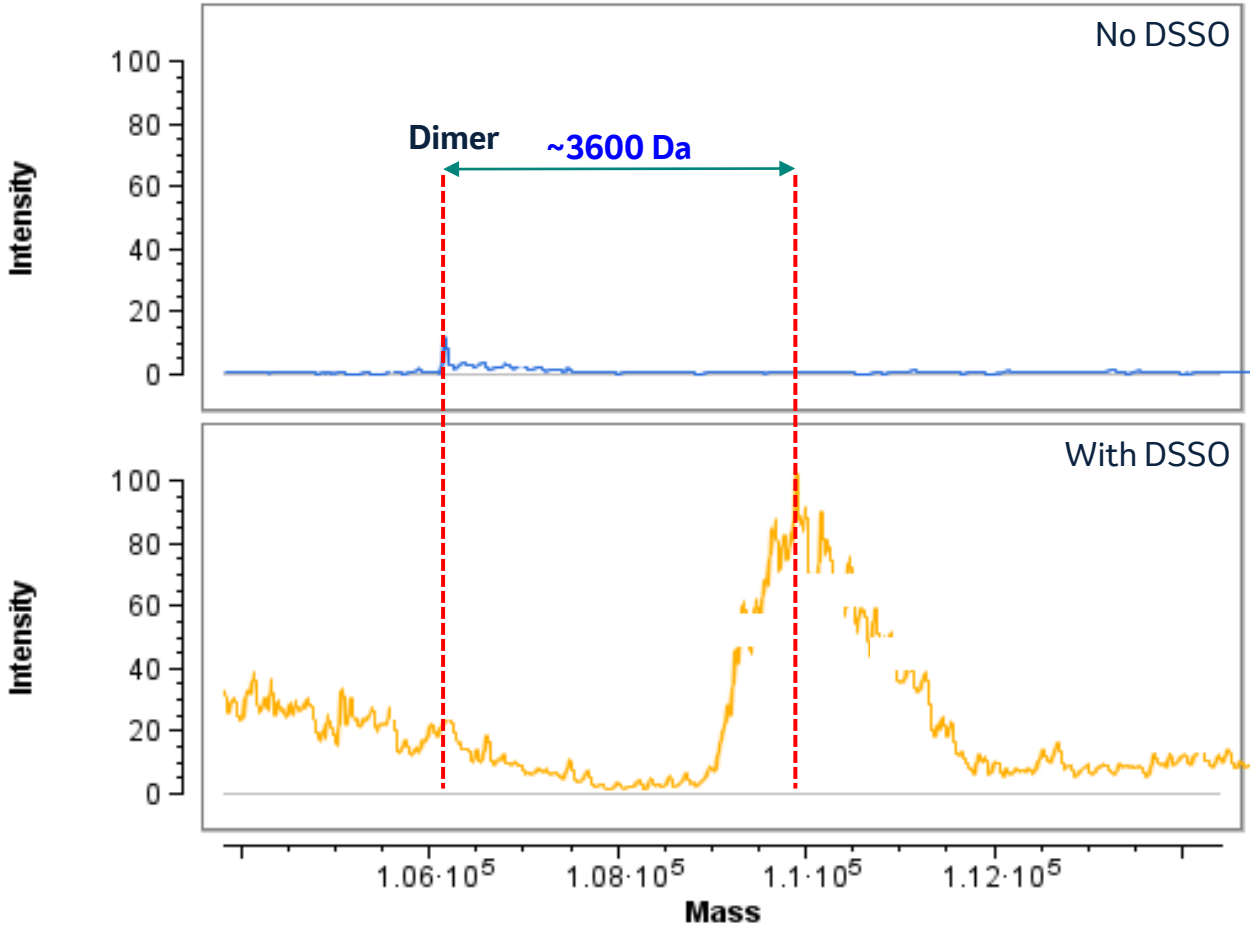
A Closer Look at **Dimer** Peak following nSEC-PCD-MS





- Dimers in the control sample were denatured into monomers whereas the dimers present in the cross-linked sample stay intact.
- A mass shift of 3600 Da at the expected dimer mass may correspond to DSSO cross-linked msAbs.

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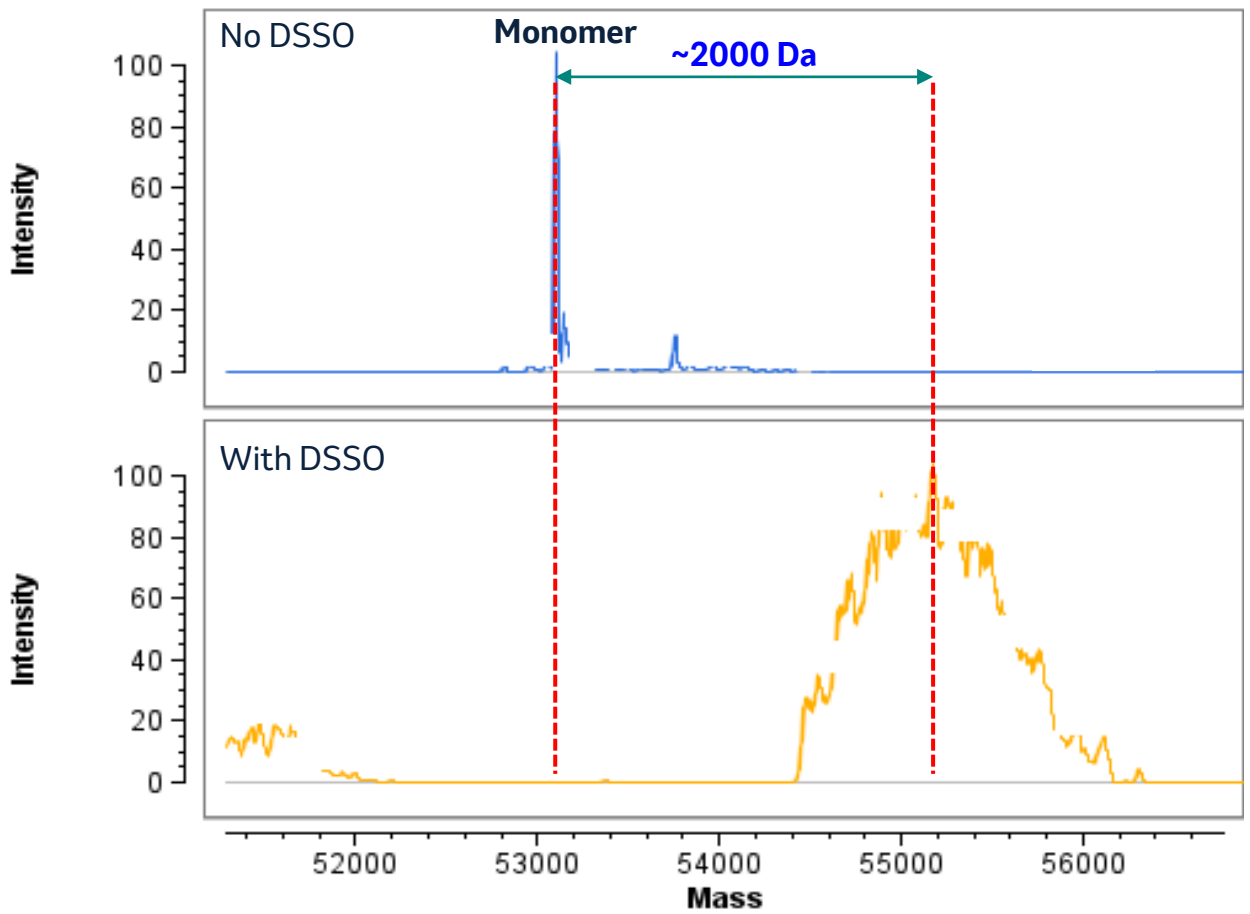
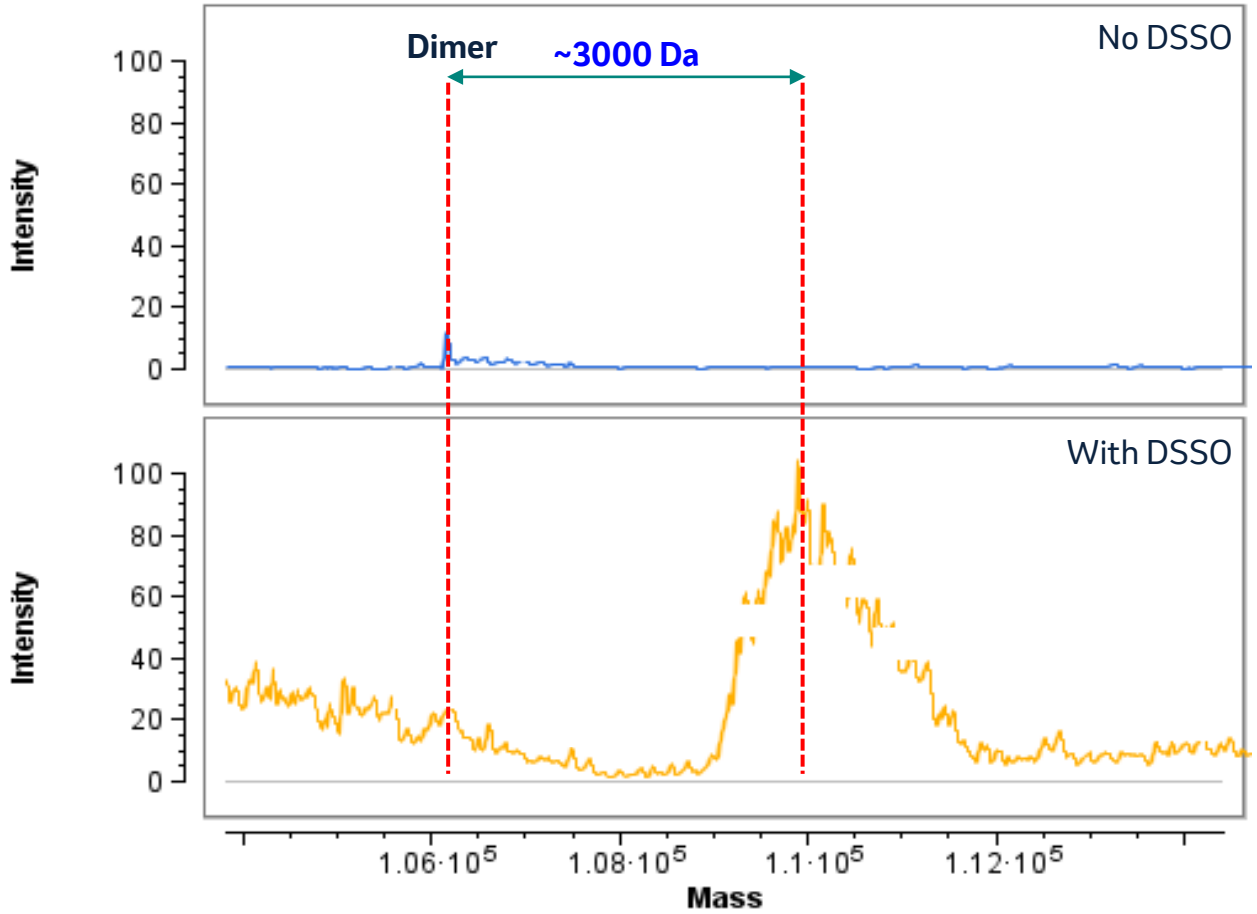
A Closer Look at **Monomer** Peak following nSEC-PCD-MS





- The monomer mass in the cross-linked sample shows a notable mass shift of ~2000Da compared to the non-cross linked which may indicate the presence of various dead-end species.
- We still observed the presence of cross-linked peptide at higher masses.

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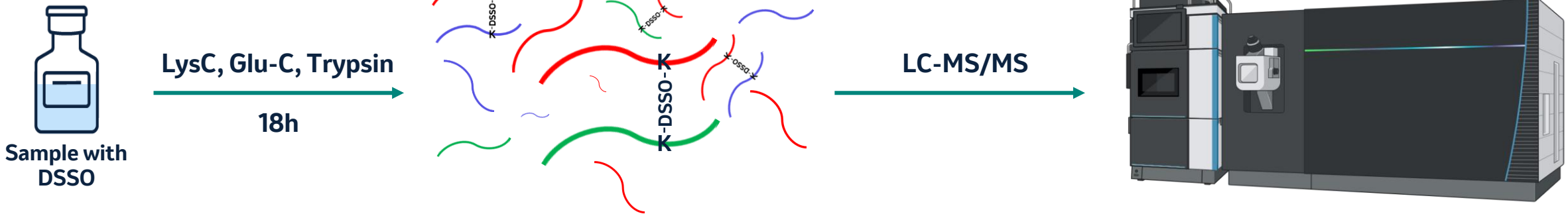
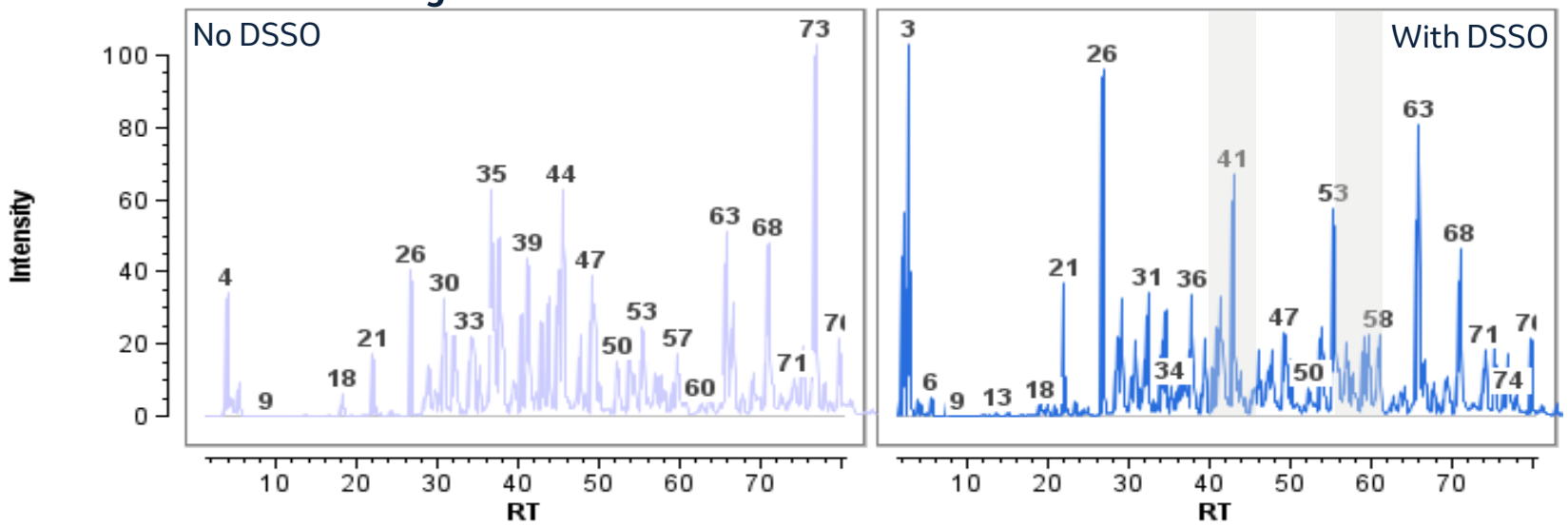
Cross Linking Identification at the Peptide Level



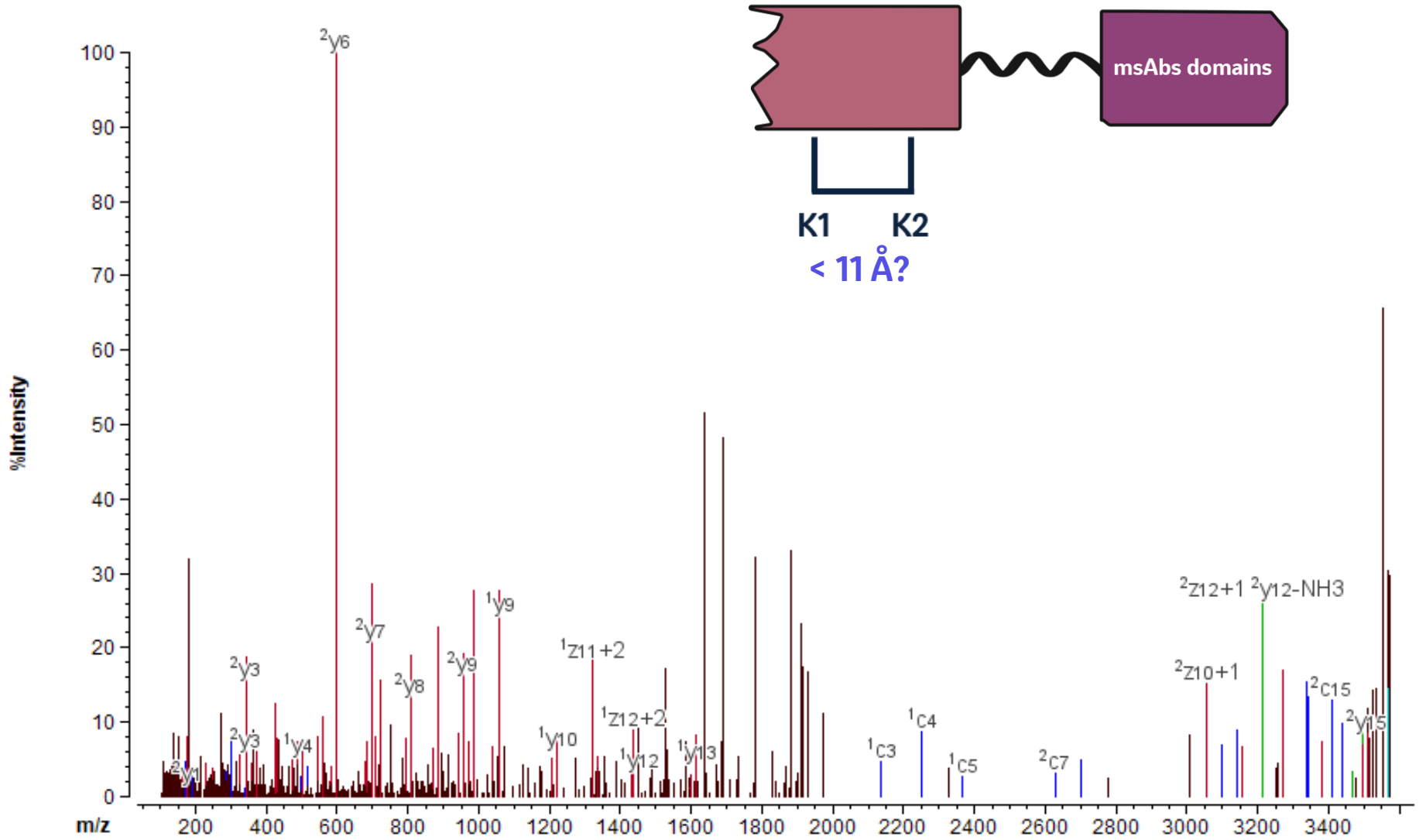
Electron transfer with supplementary HCD (EThcD)

Total Ion Chromatograms



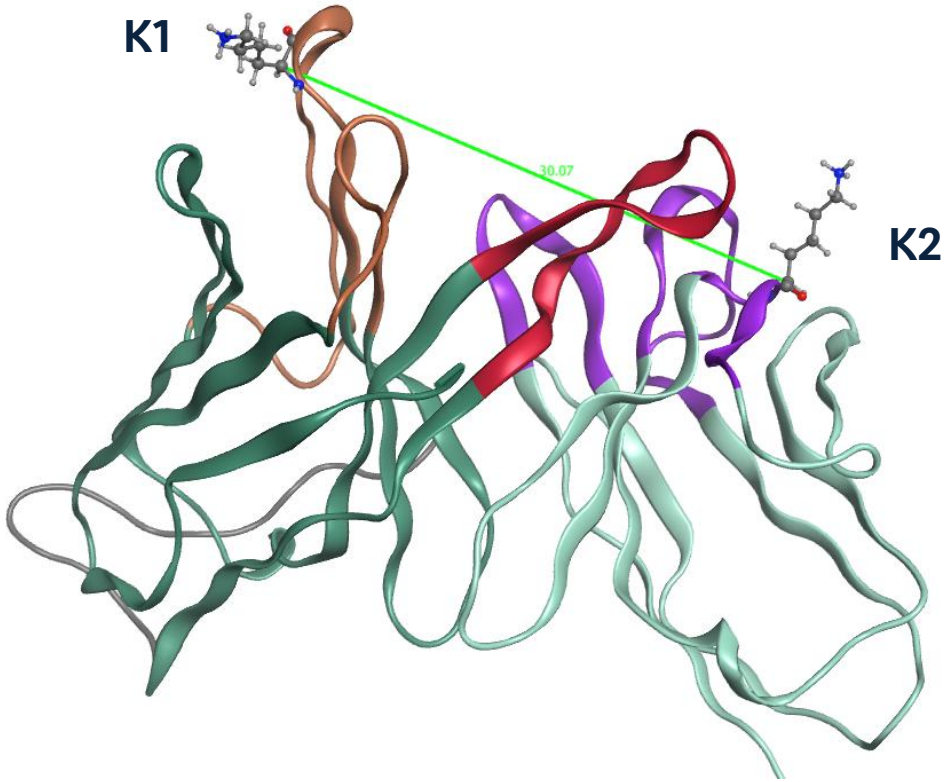
a) Giese, S.H.; Belsom, A.; Rappsilber, J. ***Anal. Chem.*** **2016**, 88 (16), 8239-8247. b) Kolbowski, L.; Mendes; M.L., Rappsilber, J. ***Anal. Chem.*** **2017**, 89, 5311–5318. c) Zhao, B.; Reilly, C.P.; Reilly, J.P. ***J. Am. Soc. Mass Spectrom.*****2019**, 30 (9), 1631-1642.

Confirming Cross-Linking Identity by MS/MS



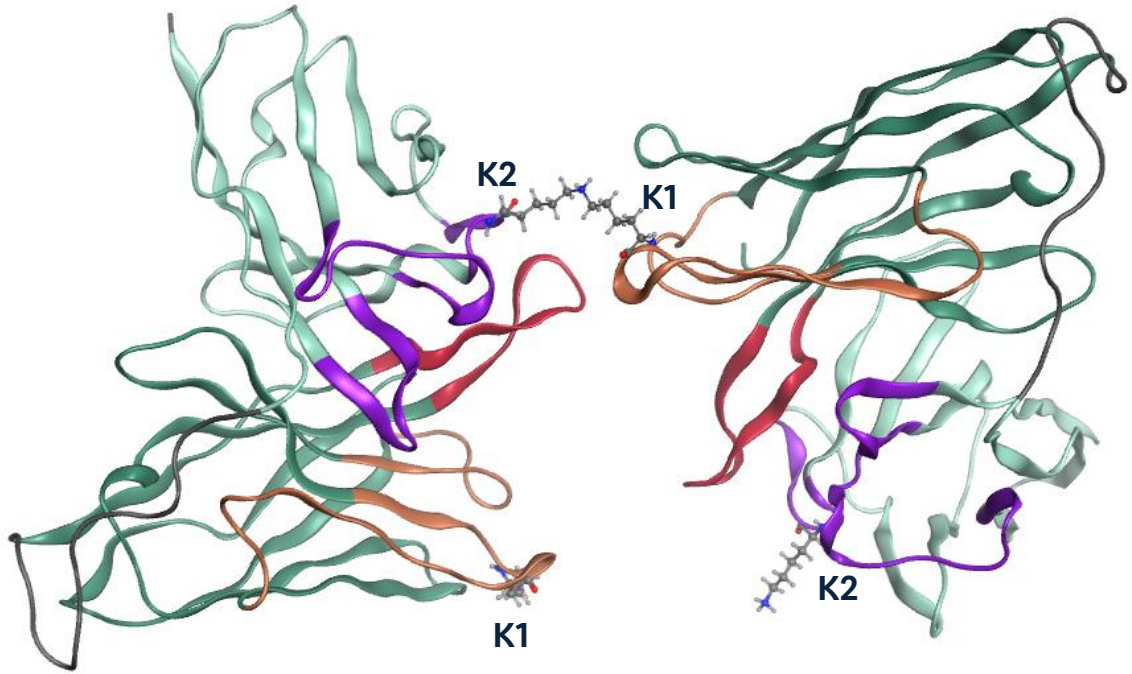
Digging Into Molecule Geometry Using Computational Modeling

Intra-molecule cross-linking



- Cα-Cα distance between K1 and K2 in the monomer is about 30 Å
- **The msAb is too large** for K1 and K2 to form an intra-protein covalent bond

Inter-molecule cross-linking



Both K1 and K2 are:

1. Fully solvent exposed
2. On the tips of the predicted CDR loops

➢ **Inter-protein cross-linking plausible if loops face each other.**

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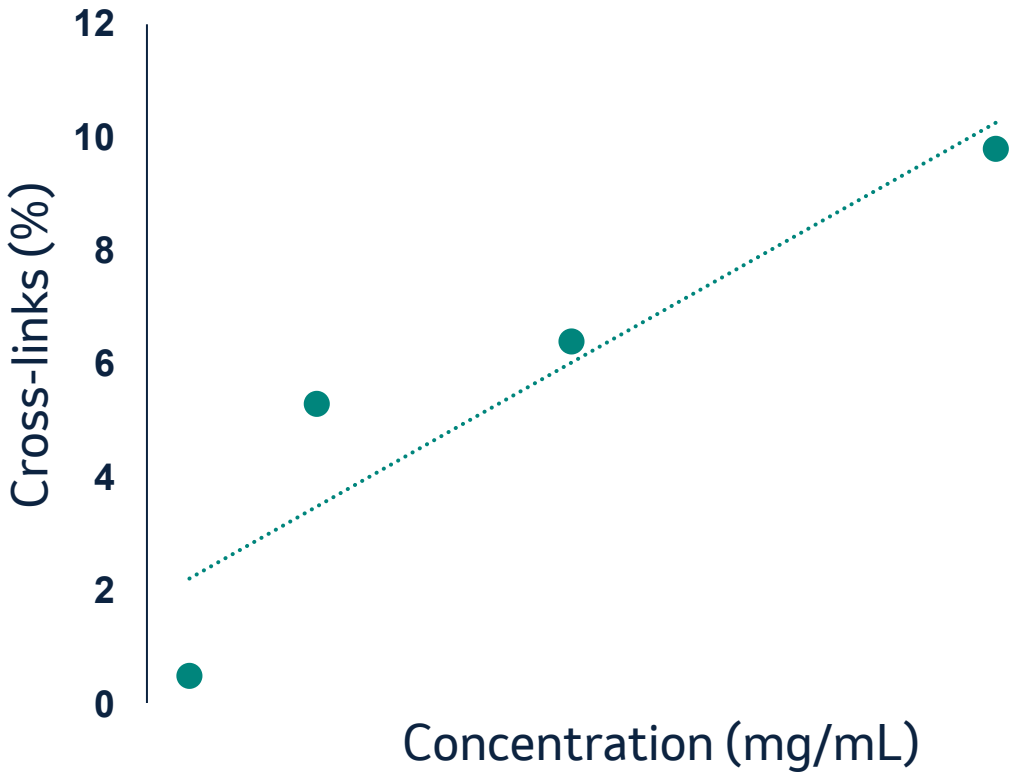
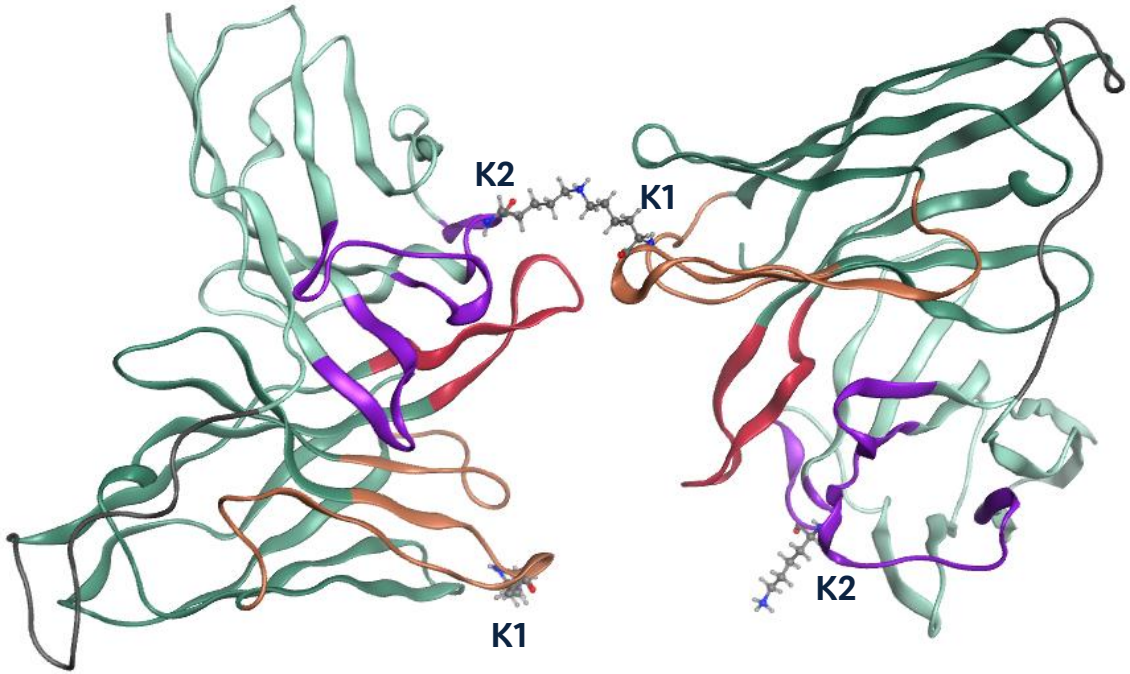
Digging Into Molecule Geometry Using Computation Modeling



Inter-molecule cross-linking



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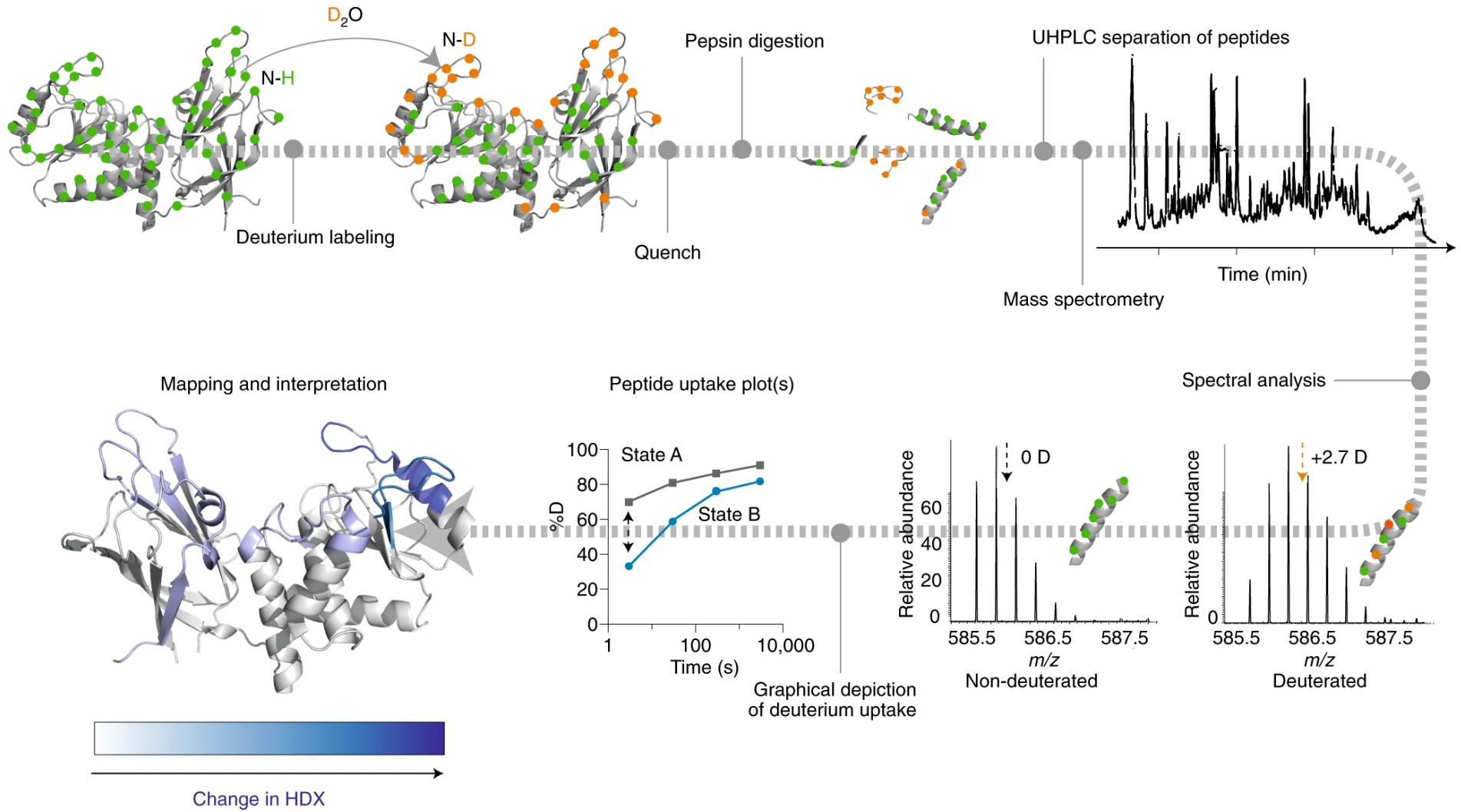
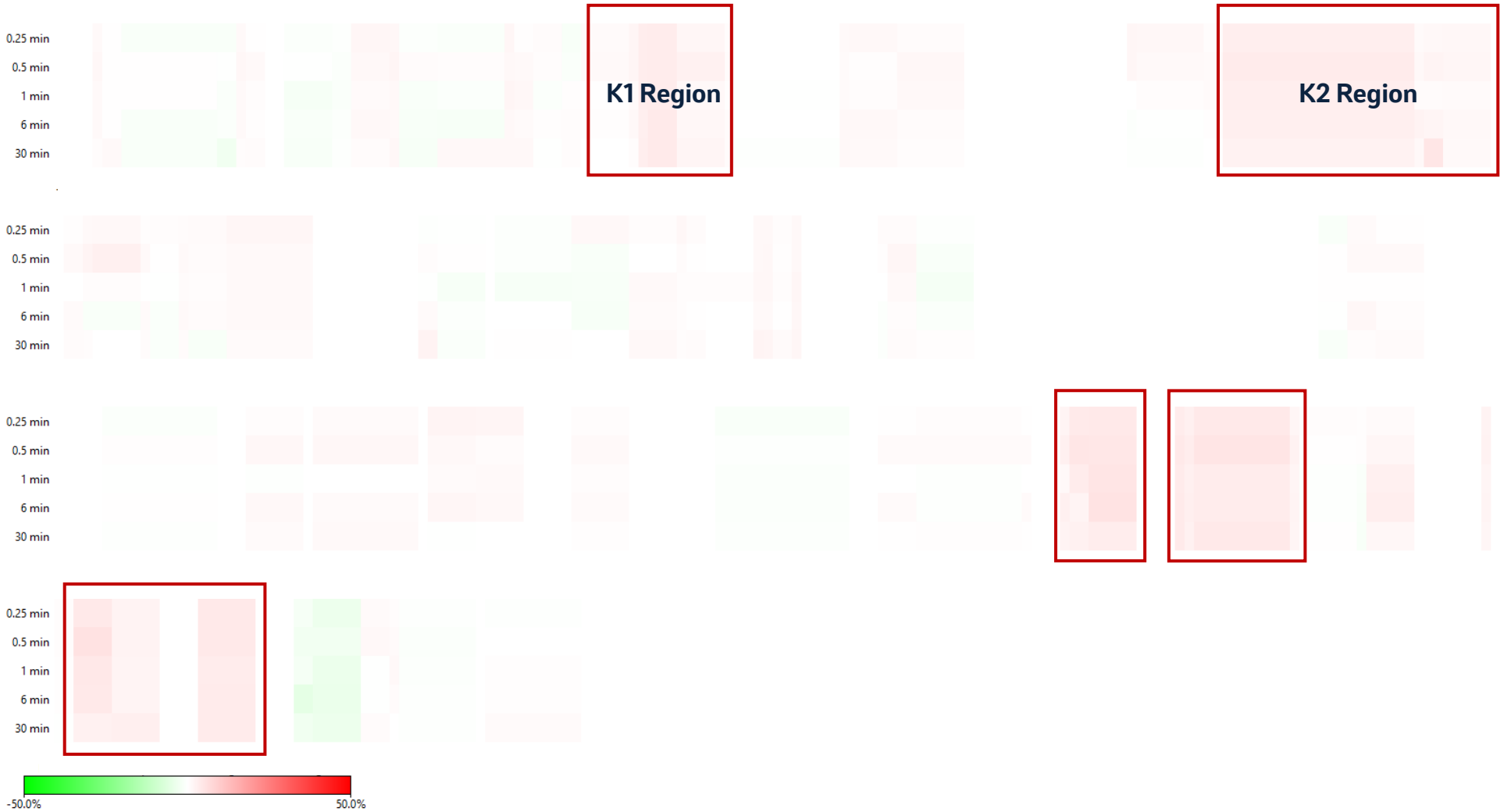
Using HDX-MS Workflow as a Confirmation Tool



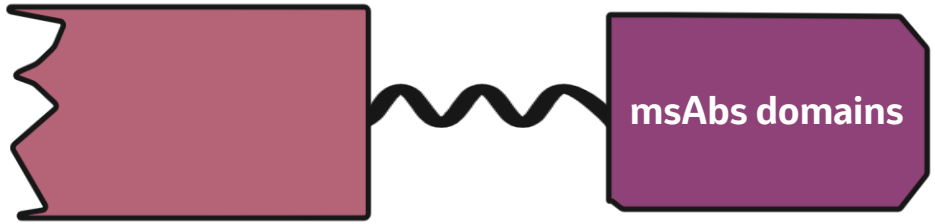
Masson, G.R., Burke, J.E., Ahn, N.G. et al. ***Nat Methods*** **2019**, 16, 595–602.

Concentration-Dependent Uptake Changes: HDX Heat Map



Summary and Conclusions

- nSEC-PCD-MS highlighted that cross linking of the msAb using DSSO was effective.
- Using an overnight enzymatic digestion and EThcD ionization, we revealed many cross-linked peptides, the primary one being Domain 1 residues K1 and K2.
- Computational chemistry confirmed that the molecular geometry enables interactions between K1 and K2 residues from ***two distinct molecules***.
- Additionally, HDX confirmed that the region around K1 and K2 show deuterium uptake, pointing to these residues as cross-linking hot spots.



Acknowledgments

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Hillary Schuessler

Discovery - Computational Chemistry

Michael Hartmann

AR&D - Biologics

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Yannan Lin
Xiaoqing Hua

Thank you

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