Optimization and Characterization of Disulfide Linkages in the Complex Cysteine Engineered Stapled scFv for Bispecific Antibodies

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Introduction

- Multispecific antibodies are the next generation of biotherapeutics. Their single-chain fragment variable (scFv) domains composed of variable light chain (VL) and heavy chain (VH) are critical and known to have a lower stability and a tendency to aggregate.
- We (mAbs. 2023) revealed a stapling strategy by introducing two engineered disulfide bonds between VL and VH domains. The stapled scFv (called spFv) technology for bispecific achieves higher thermal stability and minimal aggregation.
- Here, we demonstrate a workflow to improve the challenge of a comprehensive disulfide mapping of stapled bispecific antibodies.

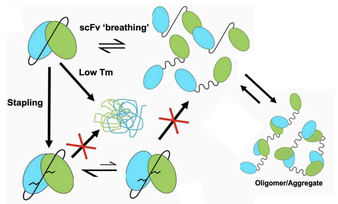
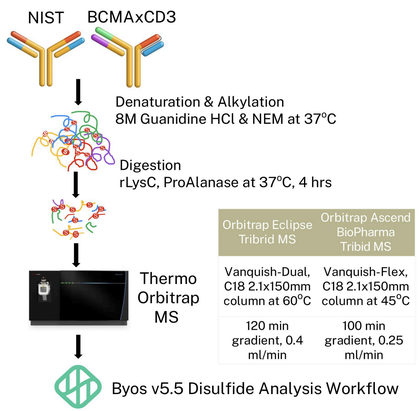


Figure 1. Stapling scFv improves stability and minimizes breathing-mediated aggregation in scFv (mAbs. 2023)

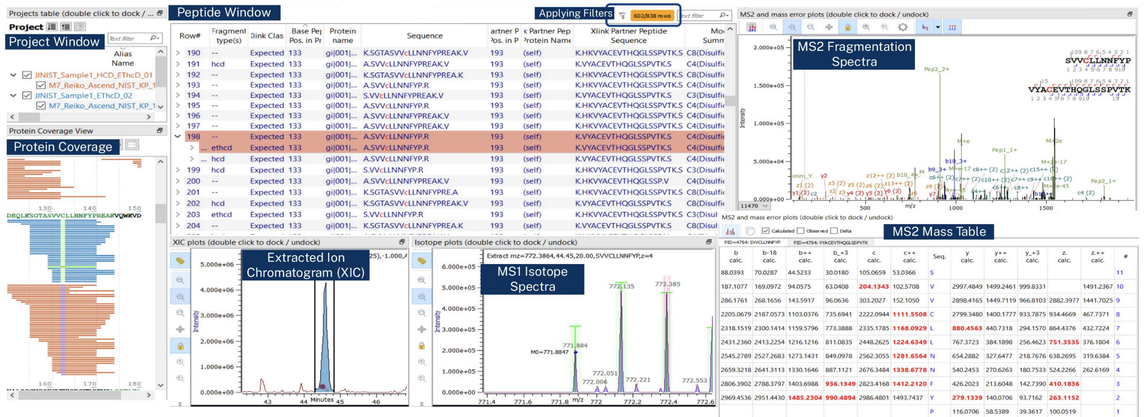
Methods



	Orbitrap Eclipse Tribrid MS	Orbitrap Ascend BioPharma Tribid MS
	Vanquish-Dual, C18 2.1x150mm column at 60°C	Vanquish-Flex, C18 2.1x150mm column at 45°C
	120 min gradient, 0.4 ml/min	100 min gradient, 0.25 ml/min

Results & Discussion

Byos Results and Inspection



Disulfide Validation

Off by X=0 | Score > 150 | Delta Mod Score > 10 | MS1 Correlation > 0.8 | PPM Standard Deviations =3 | PEP 2D | Xlink Score

MS Methods: EThcD vs. Combination (EThcD + HCD)

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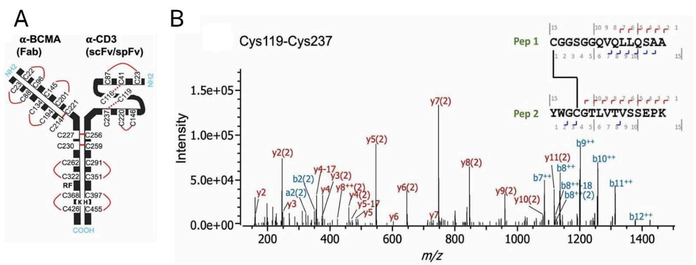


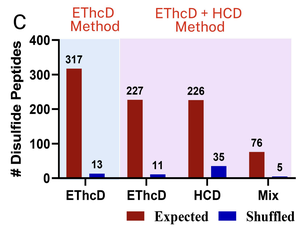
Figure 3. Bispecifics with spFv show expected disulfide formation in the stapled linker. A) Schematic of BCMA (Fab) x CD3(spFv) bispecific molecular architecture. B) MS2 spectrum of the expected stapled disulfide linkage peptides between Cys 119-Cys237 (mAbs. 2023).

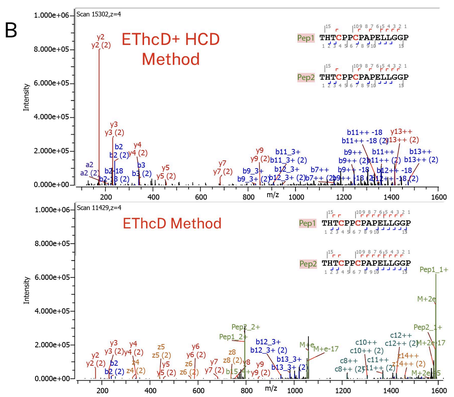
MS Methods: EThcD vs. Combination (EThcD + HCD)

Figure 2. EThcD only fragmentation method results in higher quality spectra. A) MS parameters of EThcD+ HCD and EThcD only methods. B) An example spectra of expected stapled disulfide linkage peptides from NIST antibody using the two methods. C) Number of expected or shuffled disulfide peptides identified using Byos software. Mix indicated the peptides have identified with both EThcD and HCD fragmentation.

A

Parameters	EThcD + HCD	EThcD
MS OT	120K Resolution, 250% AGC target, 40% RF lens, 2-8 Charge state	120K Resolution, 250% AGC target, 40% RF lens, 2-8 Charge state
ddMS2 OT	EThcD (Charge state 2-8)	EThcD (Charge state 3-8)
	HCD (Charge state 2-4)	HCD (Charge state 2)





Conclusions

- A better fragmentation of disulfide-linked peptides was observed using EThcD only compared to the combination of EThcD and HCD.
- The updated disulfide workflow improved the identification of the expected stapled disulfides in the stapled bispecifics. The next step will be testing with the optimized MS acquisition methods.
- Disulfide analysis workflow in Byos allows efficient identification, inspection, and relative quantification of disulfide linkages.

Reference and Acknowledgments

Boucher, L. E., Prinslow, E. G., Feldkamp, M., Yi, F., Nanjunda, R., Wu, S. J., ... Luo, J. (2023). "Stapling" scFv for multispecific biotherapeutics of superior properties. mAbs, 15(1).

The authors declare no competing financial interest.