Poster Reprint

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Real-time Electron Capture Dissociation Characterization of Antibody Subunits via Microdroplet Reactions

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Introduction

In-depth characterization of mAbs by mass spectrometry is crucial during therapeutic development to support understanding of product quality attributes that underpin safety and efficacy. Current analysis methods involve extensive offline reduction and digestion reactions which are time-consuming and increase the risk of introducing artifacts. The unique design of the Agilent Jet Stream (AJS) ion source enables fast, mAb characterization with microdroplet reactions which has been demonstrated to accelerate chemical reduction and digestion reactions.[1,2,3] Here, we further enhance microdroplet characterization by utilizing electron-capture dissociation (ECD) for sequence analysis. Combining microdroplet accelerated reactions with ECD fragmentation capabilities, the characterization of mAbs can be improved while reducing time and cost.

Experimental

A trispecific antibody, supplied by Johnson & Johnson was desalted using Amicon™ 10 kDa spin filters and diluted to 1 mg/mL in 5 mM ammonium bicarbonate. Lyophilized GlySERIAS® enzyme, obtained from Genovis, was dissolved to a concentration of 10 units/µL, desalted using AmiconTM 10 kDa spin filters, and then diluted to 1 unit/µL to be loaded into a vial to be placed on the LC multisampler. 10 mg/mL tris(2-carboxyethyl)phosphine (TCEP) was prepared in 5 mM ammonium bicarbonate and dispensed in a separate vial for loading onto the LC. The various reaction were conducted in similar manner as the following: using an autosampler injection program which drew up 5 µL of reagent/enzyme solution and then 5 µL of the trispecific antibody sample. The volume was then mixed in the autosampler needle and injected into the flow path of the instrument directly to the mass spectrometer ion source. Upon arrival at the ion source the mixture is aspirated, initiating the microdroplet reaction where TCEP reduces the disulfide bonds in microseconds. The resulting product ions are analyzed using the 6545XT AdvanceBio LC/Q-TOF (Figure 1). Reaction products were analyzed in MS1 mode to verify reaction efficiency then targeted MS2 was used to isolate the various subunits for ECD fragmentation analysis. ExDViewer software was then used to analyze ECD fragments for total fragmentation coverage. Table 1 and Table 2 delineate the conditions used for the LC/MS system and the injector program used to automate mixing of the reagents prior to injection. Figure 3 shows the schematic of the ExD cell lenses that were tuned.

Experimental

Figure 1. with Agilent Infinity II 1290 Bio UPLC paired with Agilent 6545XT AdvanceBio LC/Q-TOF fitted with Agilent ExD cell

Agilent 1290 Infinity II Bio UHPLC	
Column	Union
Thermostat	4 °C
Mobile Phase A	5mM Ammonium Bicarbonate
Column Temp	25 °C
Flow Rate	0.025 to 0.300 mL/min
Injection Volume	5 µL
Mass on Column	5 µg

Injector Program		
Function	**Parameter**	
Draw	10 µL of 10 mg/mL DTT	
Draw	5 µL of 1 mg/mL NIST mAb	
Mix	Mix from air 4 times	
Remote	Set remote line "start" for 125 ms	
Wait	Wait 0.1 min	
Inject	Inject	
Time (min)	**%A**	**Flow Rate (mL/min)**
0.0	100%	0.300
0.1	100%	0.300
0.2	100%	0.025
1.9	100%	0.025
2.0	100%	0.300
3.5	100%	0.300

Table 1. LC conditions and injector program used for microdroplet reactions.

Agilent 6545XT Q-TOF w/ExD Cell	
Source	Dual Agilent Jet Stream
Gas Temp	360 °C
Gas Flow	12 L/min
Nebulizer	60 psi
Sheath Gas Temp	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	5000 V
Nozzle Voltage	2000 V
Fragmentor	380 V
Skimmer	45 V
Octupole RF Voltage	750 V_{p-p}
Mass Range	200-3200 *m/z*
Scan Rate	2 scans/s
Slicer Position	High Resolution
Acquisition Mode	Positive, Standard (3200 *m/z*) Mass Range
Detector Mode	ADC: 2 GHz

Table 2. MS conditions used for microdroplet reactions with ExD Cell.

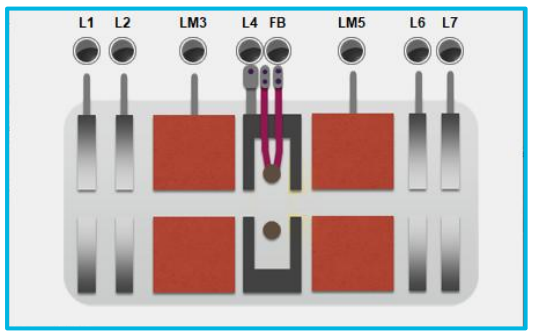

Figure 3. Schematic of ExD Cell lenses and filament

ECD Fragmentation of Trispecific Antibody LC with ExD Cell

The trispecific antibody (Figure 4) was reduced using microdroplet reduction (Figure 5) with TCEP and the 15^{+} charge state was isolated at 1561.49 *m/z*. These ions were fragmented using the ExD cell and the coverage was analyzed using ExD Viewer (Figure 6) and 65% coverage was obtained. Notably coverage was present in regions of the LC in between intra-chain disulfide bonds.

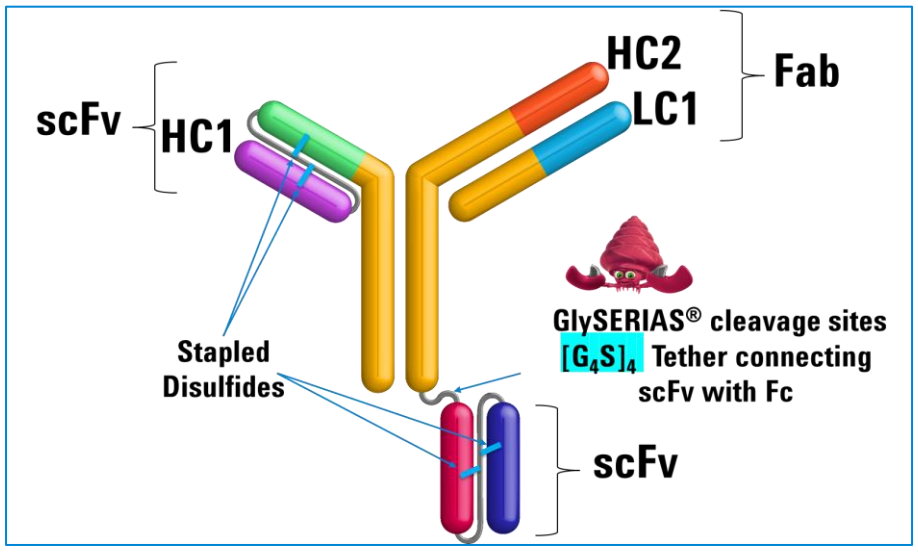

Figure 4. Structure of the trispecific antibody provided by Johnson and Johnson.

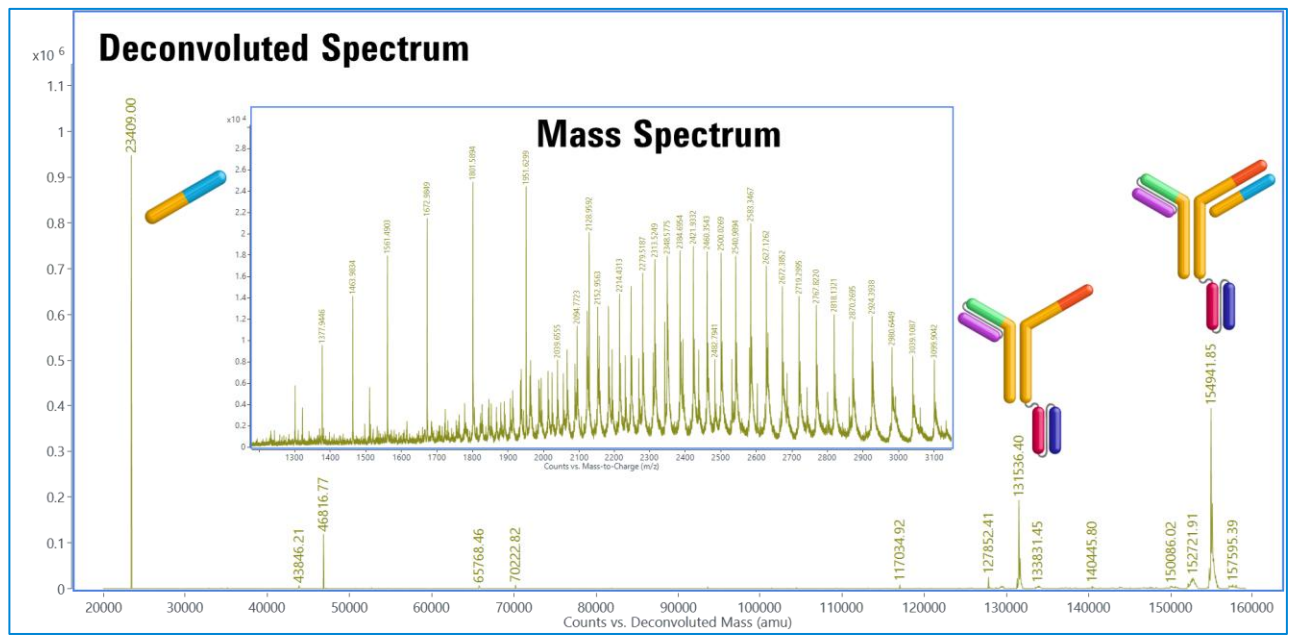

Figure 5. Mass spectrum and deconvoluted spectrum of the products resulting from the reduction microdroplet reaction.

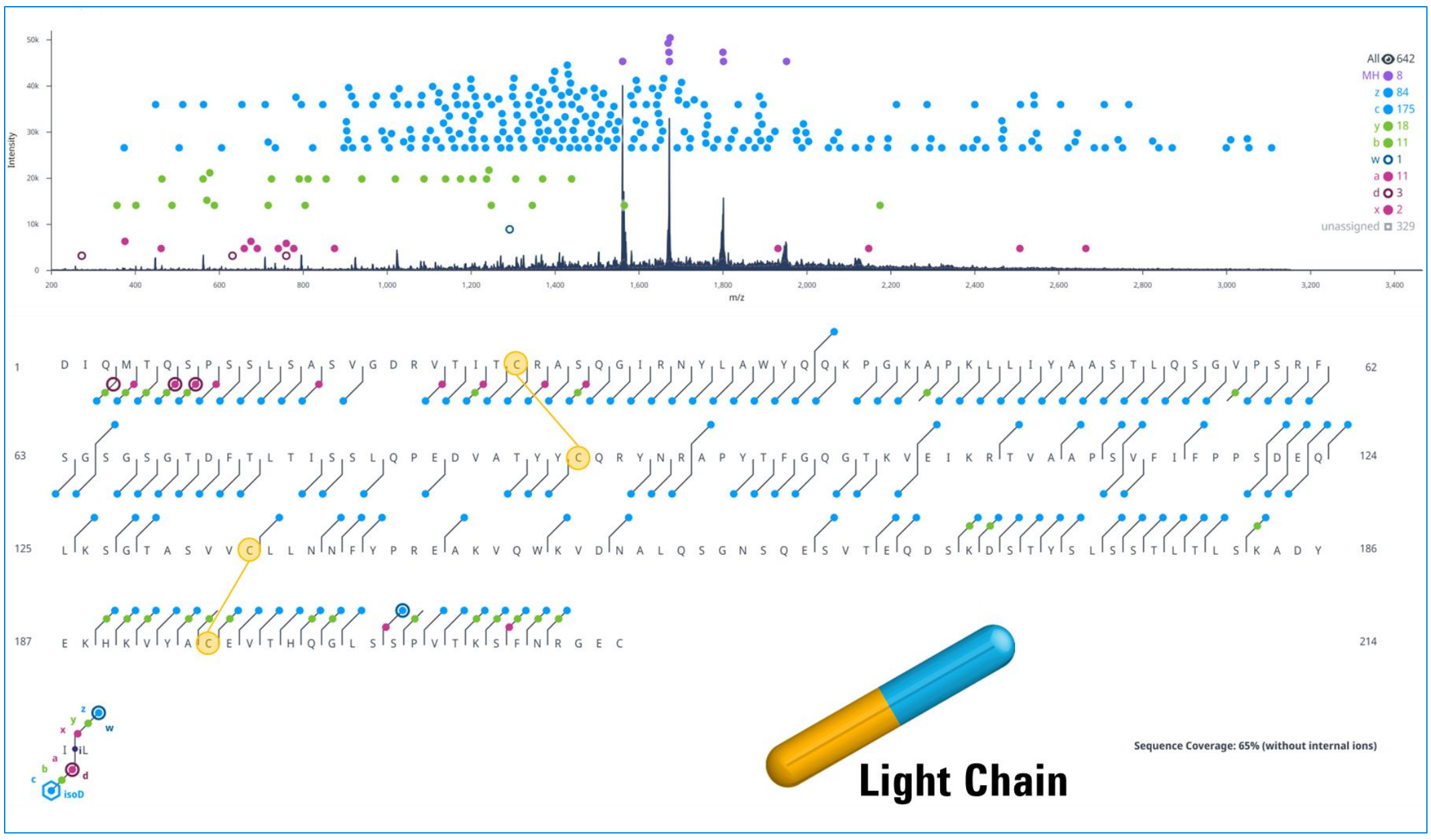

Figure 6. Coverage map of the trispecific antibody light chain after microdroplet reduction and ECD fragmentation. Results show 65% coverage using “restrictive scoring” parameter in ExD Viewer software. Potential disulfides are highlighted in yellow

Results and Discussion

GlySERIAS Enzymatic Microdroplet Digestion of scFv

The trispecific antibody was mixed with GlySERIAS® enzyme in the multisampler and digested using microdroplet reactions in the Agilent AJS source. Figure 7 shows the products of the reaction where GlySERIAS® cleaved the $[Gly_4Ser]_4$ tethering peptide at various locations. The deconvoluted spectrum shows the species that are present due to the variability in the cleavage site. The products of the digestion, when conducted offline, were comparable to the microdroplet reaction products. Also to note is that the offline digestion requires an hour-long incubation step, whereas no incubation step is used in the results shown in Figure 7.

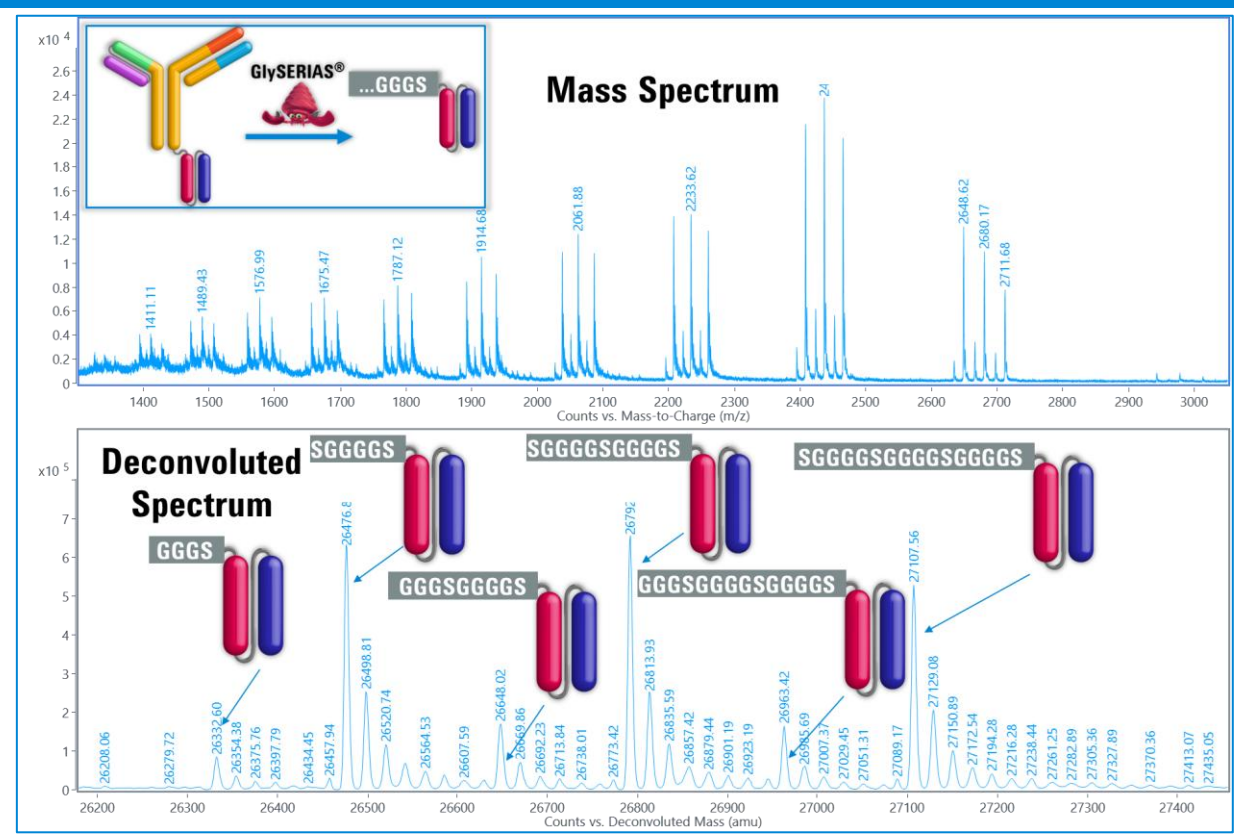

Figure 7. Mass spectrum and deconvoluted spectrum of the products resulting form the GlySERIAS® microdroplet reaction.

ECD Fragmentation of Trispecific Antibody scFv with ExD Cell

The products of the reaction are diverse due to the nature of the cleavage sites on the tethering peptide of the scFv. The 12^+ charge state at 2207.34 *m/z* was isolated and fragmented via the ExD cell to examine ECD fragment coverage. The overall coverage was 27% and is likely caused by the presence of intact intra-chain disulfide bonds that are displayed in yellow in Figure 8. This observation is confirmed by the measured mass and the sparse MS/MS coverage in regions bounded by the disulfide bonds. Despite additional runs using TCEP, the intra-chain disulfide bonds are resilient to reduction and did not yield higher coverage. Other charge states were also examined but yielded less coverage due to precursor intensity or precursor having a lower charge state.

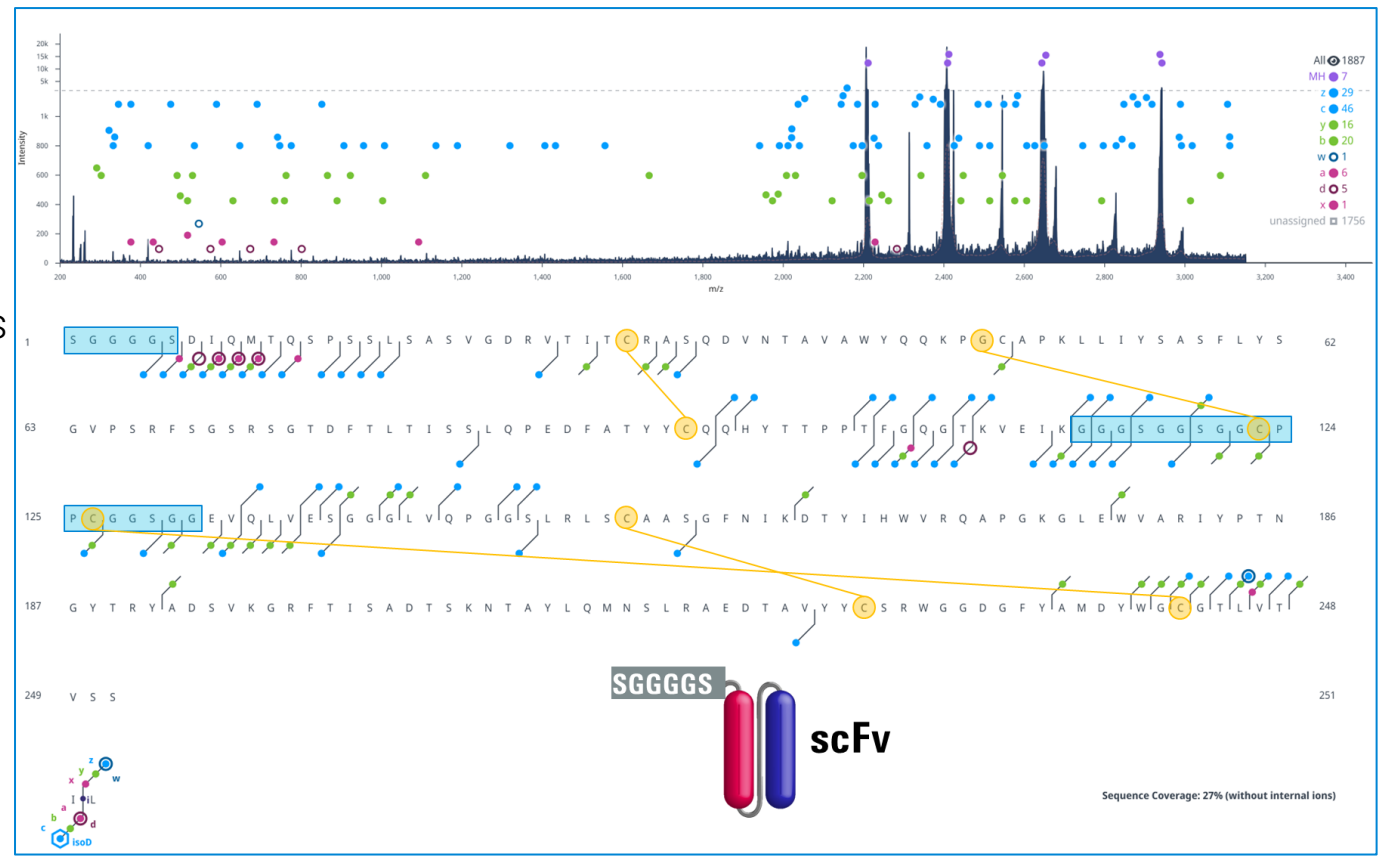

Figure 8. Coverage map of the scFv fragment resulting from the GlySERIAS® microdroplet reaction. Potential disulfides are highlighted in yellow, and tethering peptide domains are highlighted in blue.

Conclusions

These experiments demonstrate the capabilities of the Agilent 6545XT AdvanceBio QTOF with the ExD cell to do “middle-down” characterization of antibodies. The unique design of the Agilent Jet Stream source enables efficient acceleration of reactions in the low-entropy microdroplet environment. Microdroplets have been shown to accelerate reagent and enzymatic reactions, and the list of potential reactions is ever-growing.

References

[1]Agilent App note: 5994-6752EN

[2]Gunawardena, H. P.; Ai, Y.; Gao, J.; Zare, R. N.; Chen, H. Rapid Characterization of Antibodies via Automated Flow Injection Coupled with Online Microdroplet Reactions and Native-pH Mass Spectrometry. Anal. Chem. 2023, 95 (6), 3340– 3348, DOI: 10.1021/acs.analchem.2c04535

[3]Yang, Y.; Xiao, M.; Lau, J.; Knierman, M.; Zhao, H.; Qiu, X.; Luo, K.; Sausen, J.; Gunawardena, H. P.; Chen, H. Ultrafast Microdroplet Digestion of Antibodies with Fc-Silencing Mutations. Anal. Chem. 2025, 97 (24), 12813– 12823, DOI: 10.1021/acs.analchem.5c01856

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