Application Note

Biopharma

Agilent

Trusted Answers

Flash Characterization of Antibodies via Microdroplet Reactions in an Unmodified Jet Stream Source

Authors

Jim Lau, Hui Zhao, and Mike Knierman
Agilent Technologies, Inc.

Harsha Gunawardena
Janssen Research & Development,
The Janssen Pharmaceutical Companies of Johnson & Johnson
Springhouse, PA

Introduction

Traditionally, antibody characterization with IdeS enzyme digestion, reduction with tris(2-carboxyethyl)phosphine (TCEP), triethylphosphine (TEP), or dithiothreitol (DTT), and enzymatic deglycosylation require extended incubation time (minimum 30 minutes) in bulk solution. Recently, attention has been drawn to the use of microdroplet reactions for antibody analysis.[1] The microdroplet reactions are attractive due to the rapid reaction rate (microseconds) and high reaction yield achieved in the ESI spray chamber. The NIST IgG1 mAb was used to optimize microdroplet reaction conditions on two reactions, IdeS cleavage and disulfide bond reduction, using the Agilent Jet Stream ESI source. Optimized conditions were applied to several commercial antibodies to test robustness and broad applicability of the conditions. The results of the experiments demonstrated expected results and in addition to the time savings, the cost of analysis was dramatically lowered due to the reduction in enzyme and antibody consumption for characterization. This optimized workflow is referred to as "Flash Characterization".

Experimental

Materials

Ammonium bicarbonate, tris(2-carboxyethyl)phosphine, triethylphosphine, dithiothreitol, and antibodies (trastuzumab, bevacizumab, cetuximab, adalimumab, rituximab, vedolizumab, nivolumab, pembrolizumab, and SigmaMAb MSQC4) were purchased from Sigma. Fabricator (IdeS) was purchased from Genovis. The NISTmAb Monoclonal Antibody Reference Standard was procured from Agilent Technologies (part number 5191-5744).

Sample and reagent preparation

All mAbs including NIST IgG1 were diluted to 0.5 mg/mL in 5 mM ammonium bicarbonate and 50 µL was placed in an autosampler vial. The IdeS enzyme was diluted to 1 unit/µL in 5 mM ammonium bicarbonate, then 50 µL was placed in an autosampler vial. TCEP, TEP, and DTT were prepared at 10 mg/mL in 5 mM ammonium bicarbonate, and 50 µL was placed in an autosampler vial.

Instrumentation

LC System: The LC system used was an Agilent 1290 Infinity II LC consisting of a 1290 Infinity II Multisampler and a 1290 Infinity II High Speed Pump. The conditions are listed in Table 1. The injections were performed with the injector program in Table 2. The exit line of the autosampler was plumbed into the Jet Stream source with no column. The resulting samples were injected in flow injection (FIA) mode into the mass spectrometer.

Table 1. LC conditions.

Parameter	Value
UHPLC	Agilent 1290 Infinity II LC
Flow Injection Analysis (FIA)	
Column Oven Temperature	Ambient
Injection Volume	1 µL
Autosampler	5 ±2 °C
Mobile Phase A	5 mM Ammonium bicarbonate (ABC)
Gradient	Time (min) / Flow Rate (mL/min) / %A 0 / 0.3 / 100 0.1 / 0.3 / 100 0.2 / 0.025 / 100 1.9 / 0.025 / 100 2.0 / 0.3 / 100 3.5 / 0.3 / 100

Table 2. Injector program.

Function	Parameter
Draw	Draw 1.0 µL from reagent vial
Draw	Draw 1.0 µL from mAb vial
Mix	Mix 2 µL from air and repeat two times
Remote	Set remote line "Start" for 125 ms
Wait	Wait 0.1 min
Inject	Inject

For testing various ammonium bicarbonate concentrations, solvent A was 5 mM ammonium bicarbonate, and solvent B was 100 mM ammonium bicarbonate; the conditions tested are listed in Table 3.

Table 3. Ammonium bicarbonate conditions tested for microdroplet IdeS reaction.

Condition	A (100 mM ABC)	B (5 mM ABC)	Final (mM ABC)
1	100	0	100
2	90	10	90.5
3	70	30	71.5
4	50	50	52.5
5	30	70	33.5
6	10	90	14.5
7	0	100	5

Flow rates tested were 15, 25, 30, 50, 100, and 200 µL/min at 5 mM ammonium bicarbonate.

The IdeS ratio was tested by mixing 1, 2, or 3 µL of the diluted IdeS with 1 µL of mAb in the autosampler loop.

MS system

The MS system used was an Agilent 6546 QTOF mass spectrometer with a Jet Stream ESI source operated in high mass mode with the settings in Table 4.

Table 4. MS conditions.

Parameter	Value
Drying Gas Temperature	365 °C
Drying Gas Flow	13 L/min
Nebulizer Gas	60 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	5,000 V
Nozzle Voltage	2,000 V
Ion Mode	AJS ESI positive
Fragmentor	380 V
Skimmer	45 V
MS Range	*m/z* 1,350 to 10,000
Acquisition Rate/Time	2 spectra/s

Data processing

Data processing was performed with Agilent MassHunter Qualitative Analysis v10 and BioConfirm v10.

Results and discussion

Optimization experiments for the IdeS microdroplet reactions were performed for ammonium bicarbonate concentration, flow rate, and IdeS concentration. The source settings were optimized for the intact antibody spectra. Figure 1 shows an intact NIST antibody spectrum and Figure 2 shows NIST antibody mixed with 1 U of the IdeS enzyme in 5 mM ammonium bicarbonate analyzed in a flow injection mode under the final conditions optimized for microdroplet reactions.

Different concentrations of ammonium bicarbonate for the mobile phase and different flow rates were evaluated for the conversion efficiency of IgG1 IdeS microdroplet. Conversion

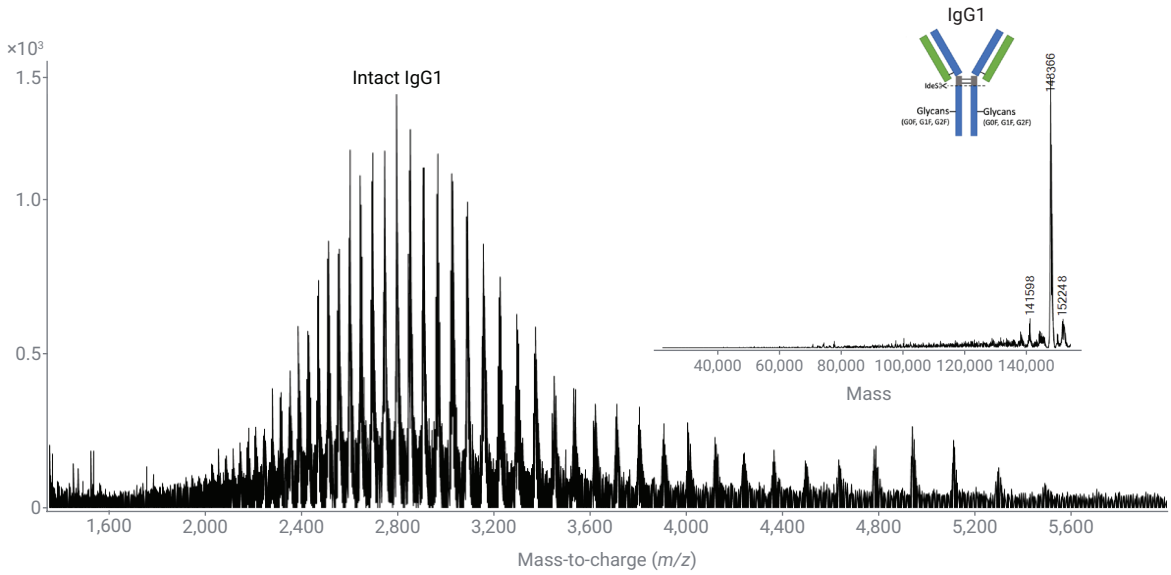

Figure 1. MS spectrum of intact NIST mAb. Inset is the deconvoluted MS spectrum of the intact NIST mAb.

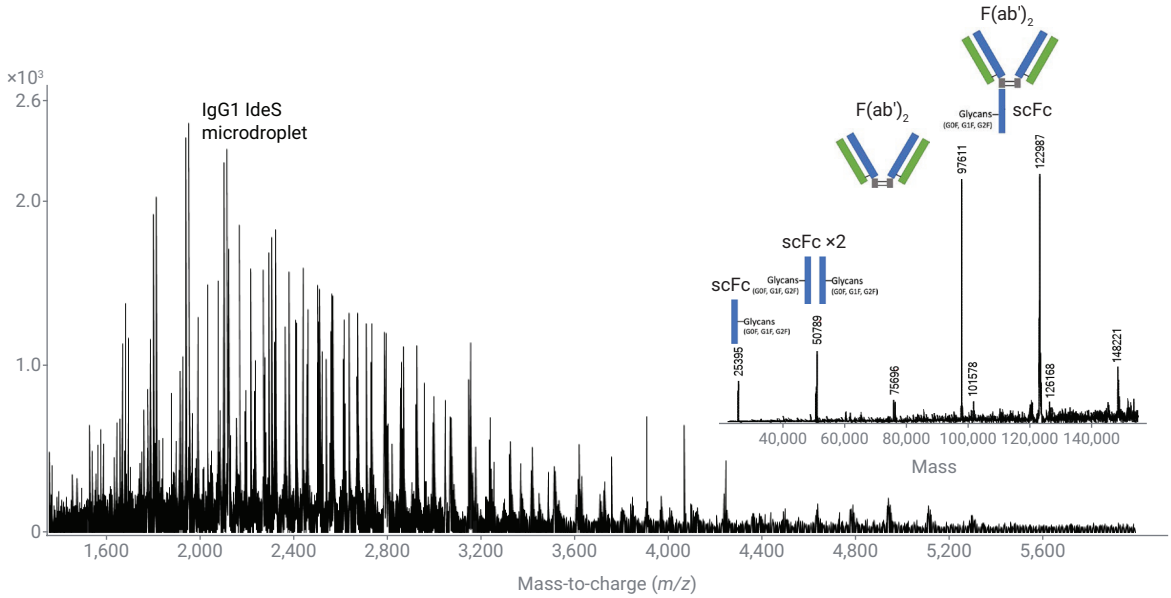

Figure 2. MS spectrum of the NIST mAb and IdeS under microdroplet reaction conditions. Inset is the deconvoluted MS spectrum of the products of the NIST mAb and IdeS microdroplet reaction with assignments.

efficiency was calculated as one minus the remaining intact signal in the microdroplet reaction deconvoluted spectrum divided by the signal of the intact antibody in buffer alone (Figure 4). Five millimolar ammonium bicarbonate was found to be optimum for the mobile phase and sample diluent. A flowrate of 25 µL/min was found to yield the optimal signal (Figure 3). A nebulizer pressure setting of 60 was found to work best at the optimal flow rate and source conditions.

Figure 5 shows the microdroplet reaction results for IdeS and Herceptin (heavy chain LLGGPS) and Figure 6 shows a negative control with IdeS and Vedolizumab that lacks the cleavage motif for IdeS (heavy chain LAGAPS). This demonstrates that the reaction observed is occurring because of the IdeS enzyme reaction and is not related to another effect.

The effect of the IdeS to mAb ratio on microdroplet reaction efficiency is demonstrated in Figure 7 and shows that increasing the amount of IdeS enzyme in the reaction reduces the presence of a single heavy chain IdeS cut on the antibody (122,978 Da) and leads to more of the fully cleaved mAb (97,611 Da).

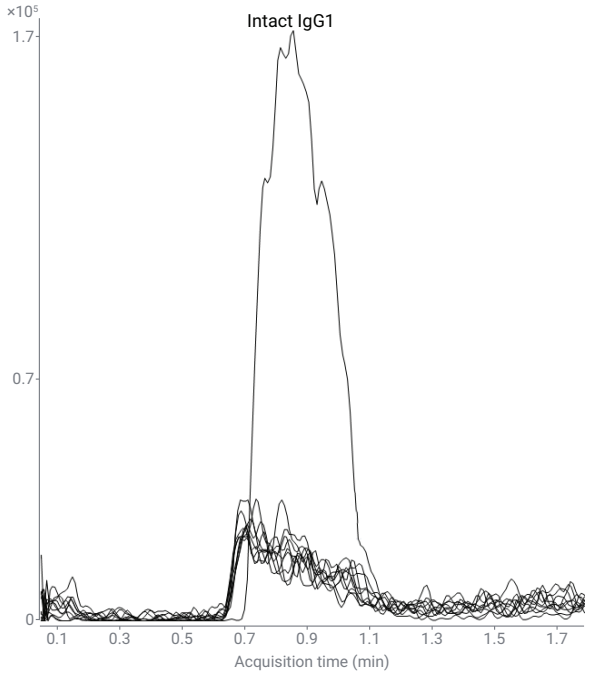

Figure 4. Stacked extracted ion chromatograms of intact IgG1 and 10 runs of the residual IgG1 signal after the microdroplet reaction with the IdeS enzyme.

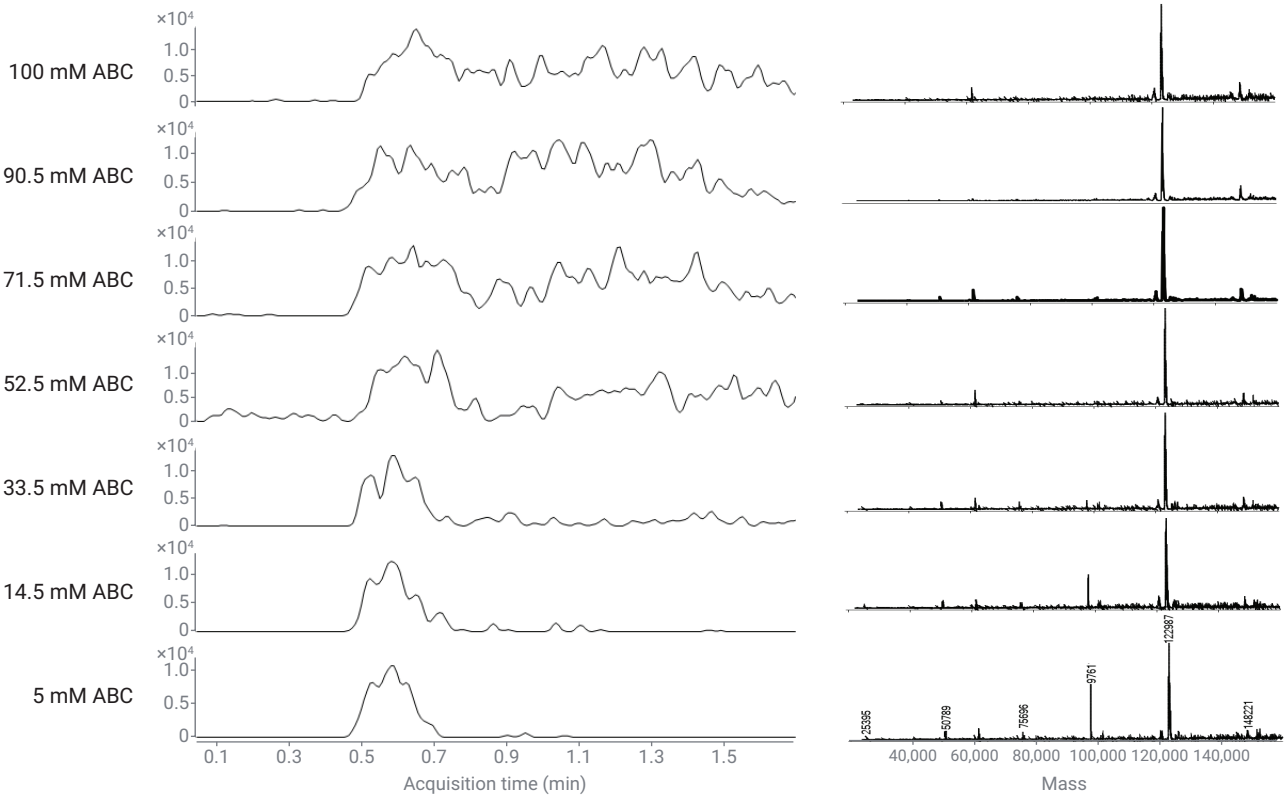

Figure 3. TIC and deconvoluted MS spectrum of the NIST mAb/IdeS products at different concentrations of ammonium bicarbonate.

The universality of this reaction is demonstrated with 7 antibodies and a negative control antibody that were analyzed under optimum conditions. Table 5 shows the efficiency of the microdroplet IdeS reaction on the 7 antibodies and the negative control antibody. The robustness of the system was demonstrated using ten injections of NIST mAb with IdeS. The remaining antibody signal was calculated and analyzed for the 10 runs (Table 6). The results show a conversion efficiency of 85% with a precision of 4.3%.

Reduction of disufide bonds in antibodies also works under microdroplet reactions conditions in 5 mM ammonium bicarbonate. Figures 8, 9, and 10 show NIST mAb under microdroplet conditions with TCEP, DTT, and TEP respectively and show similar reaction efficiency to IdeS for each of the reducing reagents.

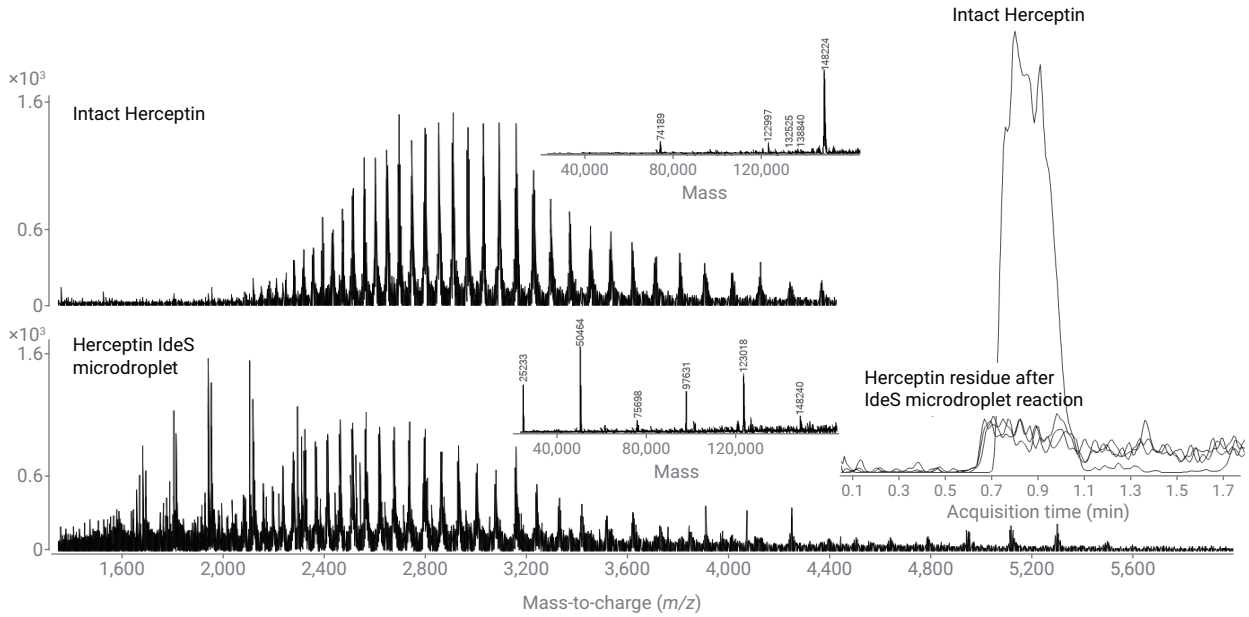

Figure 5. MS spectrum and deconvoluted spectrum of Herceptin alone and after the microdroplet reaction with the IdeS enzyme. Plot on the right is the stacked extracted ion chromatograms for intact Herceptin of Herceptin alone and three runs of the microdroplet reaction of Herceptin with the IdeS enzyme.

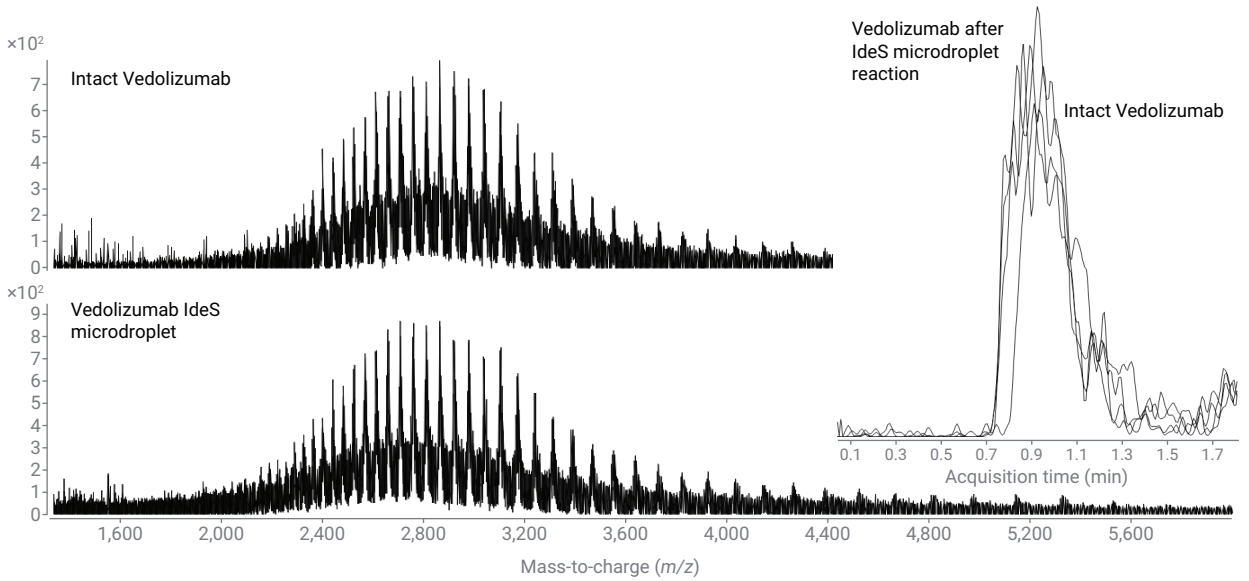

Figure 6. MS spectrum and deconvoluted spectrum of Vedolizumab alone and after the microdroplet reaction with the IdeS enzyme. Plot on the right is the stacked extracted ion chromatograms for intact Vedolizumab of Vedolizumab alone and three runs of the microdroplet reaction of Vedolizumab with the IdeS enzyme. The lack of a reaction with the IdeS enzyme is due to the missing cleavage motif for IdeS in Vedolizumab.

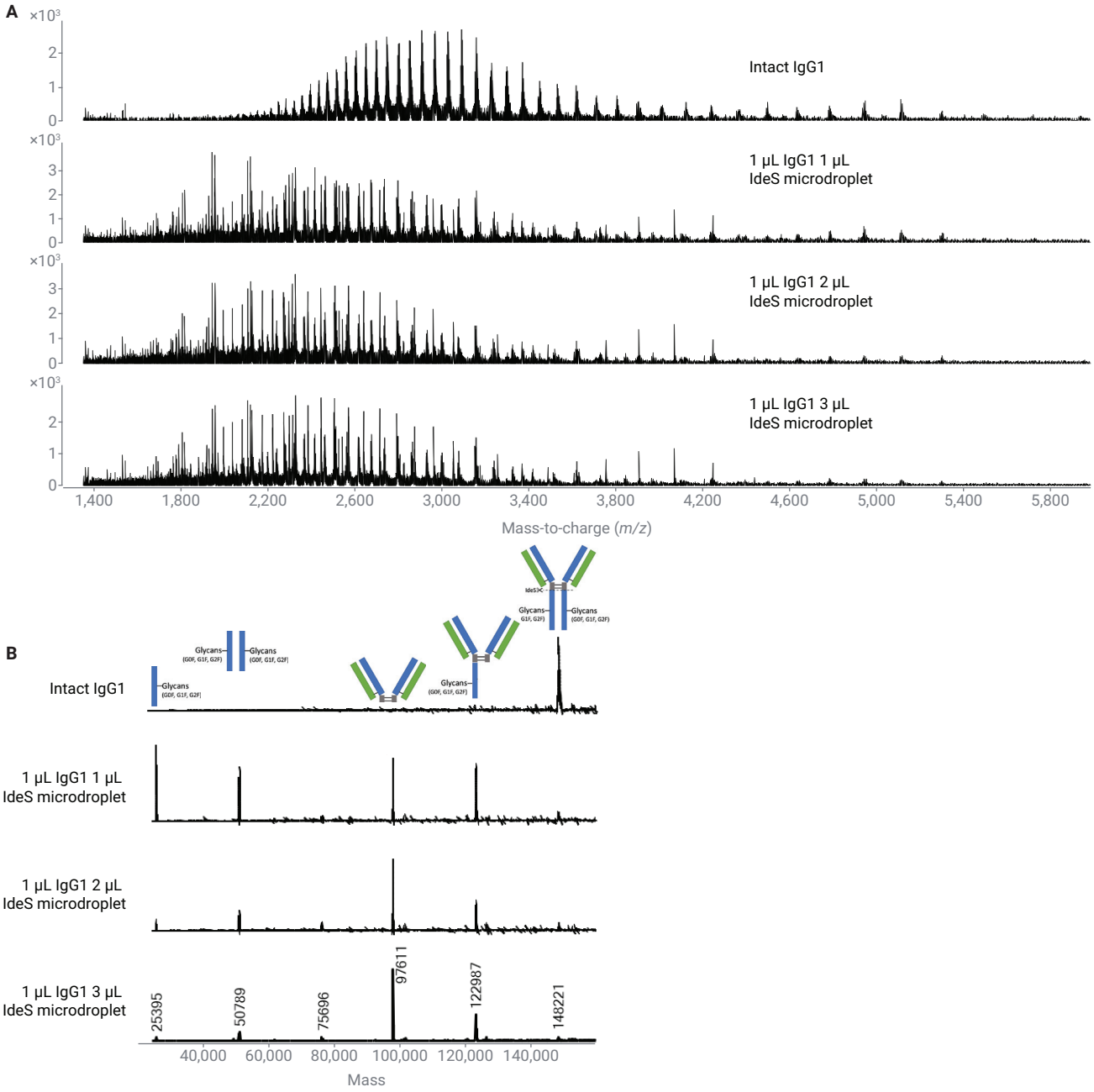

Figure 7. Panel A is the raw spectra and panel B is the deconvoluted spectra showing the effect of the IdeS ratio on the microdroplet reaction efficiency with NIST mAb. One unit of IdeS (1 µL) was sufficient to cleave most of 0.5 µg (1 µL) of NIST antibody, but there was some reaction product with one heavy chain left uncleaved (122,987 Da). Increasing the amount of IdeS to 2 or 3 units (2 or 3 µL) increased the complete reaction product with both heavy chains cleaved (97,611 Da).

Table 5. Microdroplet reaction results with IdeS on eight different antibodies.

mAb	Mass of $F(ab')_2$ (Da)	Response of Intact mAb	MS response of mAb after IdeS Microdroplet Reaction			Average Conversion % (n = 3)	Precision % (n = 3)
			1	2	3		
Adalimumab	97,719	4,826,294	854,558	880,255	784,104	83	5.9
Bevacizumab	98,770**	593,679	59,623	64,895	55,712	89	7.7
Herceptin	97,631	2,603,028	333,711	351,471	347,472	87	2.7
Nivolumab	95,824**	578,475	75,817	81,608	71,283	87	6.8
Rituximab	96,714	1,154,326	140,117	147,817	144,427	88	2.7
SigmaMAb	96,300**	499,829	124,836	128,147	114,772	77	5.7
Trastuzumab	97,629	1,207,714	123,903	121,589	115,809	90	3.5
Vedolizumab*	–	801,472	857,924	890,857	878,026	0	1.9

* Vedolizumab sequence (LAGAPS) does not contain the LGGP motif for IdeS cleavage like the other antibodies tested such as Rituximab (LLGGPS) and Nivolumab (FLGGPS).

** With 2- or 3-unit IdeS.

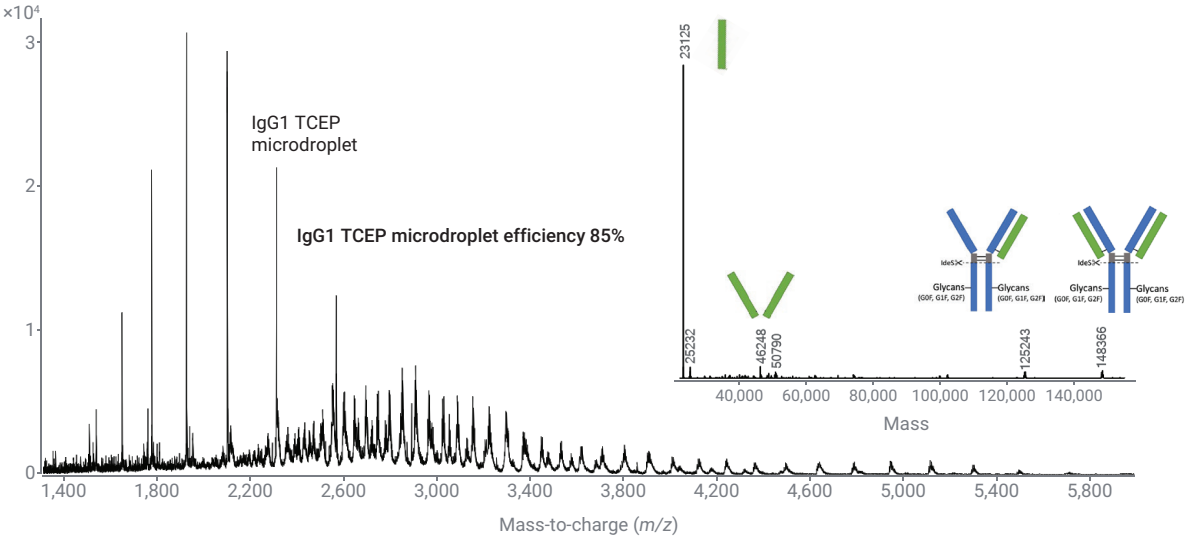

Figure 8. Microdroplet reactions with NIST mAb and TCEP to form free heavy and light chains.

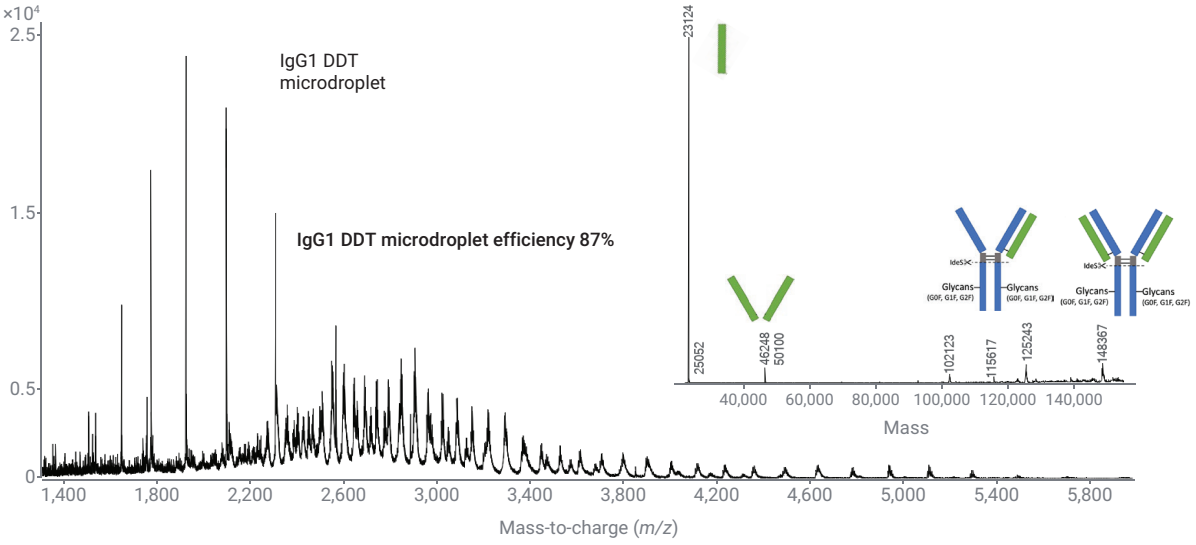

Figure 9. Microdroplet reactions with NIST mAb and DTT to form free heavy and light chains.

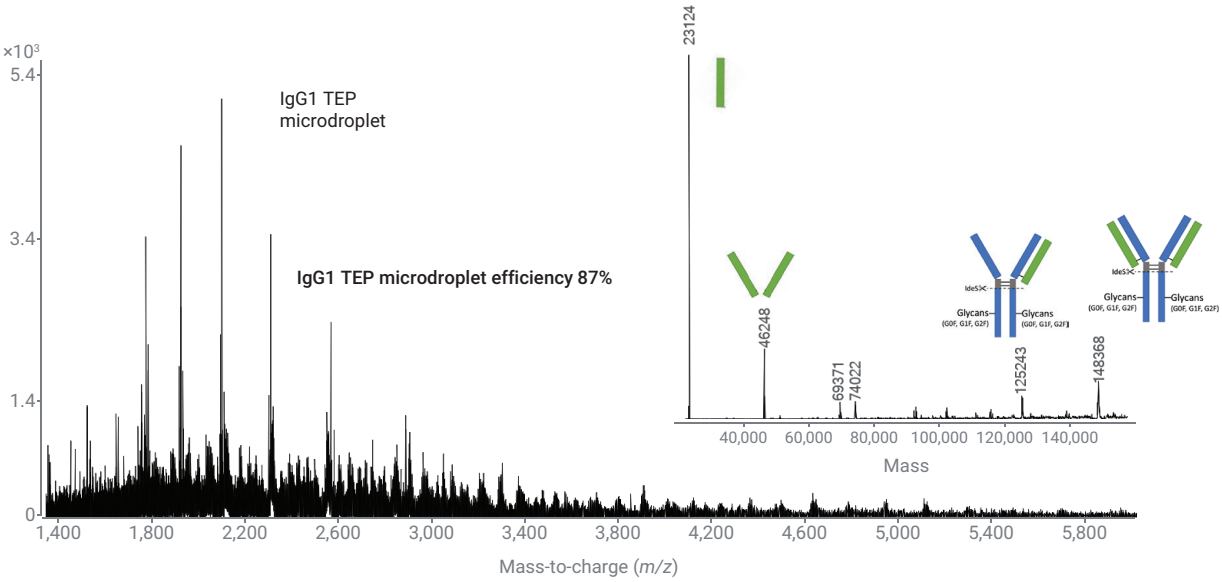

Figure 10. Microdroplet reaction with the volatile reducing reagent triethylphosphine (TEP) and NIST mAb to form free heavy and light chains.

Table 6. Reproducibility of microdroplet reactions of NIST mAb and IdeS.

mAb	Response of mAb Residue After IdeS Microdroplet Reaction
IgG1	2,598,747 (Intact IgG1)
IgG1 Residue-1	403,094
IgG1 Residue-2	417,371
IgG1 Residue-3	380,727
IgG1 Residue-4	382,958
IgG1 Residue-5	437,117
IgG1 Residue-6	404,127
IgG1 Residue-7	395,262
IgG1 Residue-8	418,881
IgG1 Residue-9	403,459
IgG1 Residue-10	390,050
Average	403,305
Precision %	4.3
Average Conversion (%)	85

Conclusion

The ultrafast microdroplet reactions with IdeS and reduction reagents were achieved with excellent peak shape, high conversion efficiency (≥85% except one mAb), and exceptional reproducibility (≤10%) for different monoclonal antibodies using the Agilent Jet Stream source and an Agilent 6546 Q-TOF. Optimum reaction conditions were found with 5 mM ammonium bicarbonate (pH 7) as the mobile phase, 25 µL/min flow rate, the MS source conditions in Table 4, and the LC conditions in Tables 1 and 2. The conditions outlined give a starting point for exploring other biomolecule characterization reactions.

Reference

1. Gunawardena, H. P. *et al.* 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.

www.agilent.com

DE93238932

This information is subject to change without notice.

© Agilent Technologies, Inc. 2023
Printed in the USA, October 12, 2023
5994-6752EN

Agilent
Trusted Answers