

Rapid Characterization of Antibodies via Automated Flow Injection Coupled with Online Microdroplet Reactions and Native-pH Mass Spectrometry

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Revised for *Analytical Chemistry*

Abstract

Microdroplet reactions have aroused much interest due to significant reaction acceleration (e.g., ultrafast protein digestion in microdroplets could occur in less than 1 ms). This study integrated microdroplet protein digestion technique with automated sample flow injection (FI) and online mass spectrometry (MS) analysis, to develop a rapid and robust method for structural characterization of monoclonal antibodies (mAbs) that is essential to assess antibody drug's safety and quality. Automated sequential aspiration and mixing of an antibody and an enzyme (IdeS or Igde) enabled rapid analysis with high reproducibility (total analysis time: 2 min per sample; reproducibility: ~2% CV). Spraying sample in ammonium acetate buffer (pH 7) using a Jet Stream Source (JSS) allowed efficient digestion of antibodies and efficient ionization of resulting antibody subunits under native pH conditions. Importantly, it also provided a platform to directly studying specific binding of antibody and antigen (e.g., detecting the complexes of mAb/RSV antigen and F(ab')₂/RSV in this study). Furthermore, subsequent tandem MS analysis of a resulting subunit from microdroplet digestion enabled localizing post-translational modifications on particular domains of a mAb in a rapid fashion. In combination with IdeS digestion of antibody, additional tris(2-carboxyethyl)phosphine (TCEP) reduction and N-Glycosidase F (PNGase F) deglycosylation reactions that facilitate antibody analysis could be realized in "one pot" spray. Interestingly, increased deglycosylation yield in microdroplets was found, simply by raising the sample temperature. We expect that our method would have a high impact for rapid characterization of monoclonal antibodies.

Keywords: mass spectrometry; microdroplet reactions; antibody subunits; automated flow injection; tandem MS analysis.

Introduction

Reaction acceleration was recently found in micron-sized droplets (i.e., microdroplets),¹ which has been extensively investigated.²⁻⁷ Rates of various organic reactions are greatly increased in microdroplets, compared with those in bulk-phase solutions.^{2,3} Possible reasons of reaction rate acceleration in microdroplets include i) solvent evaporation that leads to the enhanced substrate concentrations on the microdroplet surfaces, ii) increased autoionization of water to provide proton and hydroxide ions, iii) partial desolvation of substrates on the microdroplet/air interface, iv) the presence of strong electric field at the interface, and v) restricted orientations of substrates in the microdroplets.⁸⁻¹¹ However, previous work focused on reactions of small organic molecules. Biochemical microdroplet reactions involving proteins,^{12, 13} lipids,^{14, 15} oligonucleotides¹⁶ and antibodies¹⁷ have not been reported till recently.

Therapeutic mAbs are one of the fastest growing classes of drugs. More than one hundred mAbs for treatment of cancer and autoimmune diseases have been approved or are in regulatory review in the US and EU.^{18, 19} This ever-growing trend has created a strong need for rapid technologies to characterize mAbs to secure drug product safety, quality, and efficacy.²⁰⁻²² Traditional mAb characterization methods by MS include intact and subunit mass analysis and peptide mapping.²³⁻²⁵ Typically, mAbs are subjected to enzymatic digestion into peptides prior to peptide mapping analysis. However, digestion is usually a time-consuming step that can take from 30 min to overnight incubation.^{26, 27} Besides, additional steps of protein denaturation, reduction, and alkylation are needed, which also lengthen the processing time and reduce analysis speed and throughput.²⁸ High-throughput MS analysis of mAbs could benefit from reducing the cycle-time between sample analysis via rapid sample introduction used in high-throughput screening methods such as matrix-assisted laser desorption ionization (MALDI), desorption electrospray ionization

(DESI), direct analysis in real time (DART) and RapidFire.²⁹⁻³⁴ We believe that rapid sample introduction coupled with very fast mAb digestion would be highly valuable for structural characterization of therapeutic antibodies.

Recently, we have reported an ultrafast enzymatic digestion (<1 ms) of various proteins in microdroplets in which antibody light and heavy chains were shown to be digested by trypsin in less than 1 ms.^{12, 35} Ultrafast digestion of intact antibody digestion was also demonstrated.¹⁷ However, most of the antibody digestion experiment¹⁷ involved offline MS analysis of the collected microdroplets resulting from spraying a manually mixed antibody and enzyme solution, making the total analysis time over 20 min. The coupling of microdroplet digestion with online MS analysis was also attempted¹⁷ but the ionization efficiency for the resulting antibody subunits was low, since protein digests from microdroplet reaction only contained water with no organic solvent and acid additives. To integrate such an ultrafast digestion protocol to antibody analysis in a fast and robust workflow, in this study, we coupled the microdroplet reactions of antibodies with automated rapid sample introduction and a Jet Stream Source (JSS) for sample ionization. Due to the use of high flow of hot nitrogen for nebulization, the JSS ion source not only allowed the microdroplet generation to effect fast mAb digestion, but also enabled direct and efficient ionization of the resulting mAb subunits from aqueous solution under native pH conditions, providing online MS analysis capability. The adoption of pre-programmed automated injections resulted in unprecedented sample throughput (2 min cycle time per sample) with high reproducibility of CV of ~2%. In this study, mAb was selectively cleaved by IdeS protease into antigen-binding fragment F(ab')₂ and single-chain, crystallizable fragment (scFc) non-covalent dimer or selectively cleaved by Igde to Fab and scFc x 2 (linked with disulfide bonds), respectively. When reductant TCEP was added into the spray solution, simultaneous disulfide bond reduction

occurred, giving rise to light chain (LC), Fd' and scFc. Because the native pH spray condition was used, the method also allowed observation of non-covalently bound dimer of LC and Fd'. More importantly, we detected the specific binding complex of NIST mAb and RSVF antigen. When IdeS was added, the specific binding complex of F(ab')₂ and RSVF was observed, suggesting the binding location of RSVF on the antibody F(ab')₂ subunit region. In addition, we achieved ultrafast deglycosylation of IgG1 by including PNGase F glycosylase in microdroplets, which constituted an alternative method to rapidly remove glycans from antibodies. In addition, temperature effects on mAb microdroplet digestion and glycan structure effects on mAb deglycosylation were also investigated in this study.

Experimental Section

Microdroplet Generation via Flow Injection Analysis (FIA)

Microdroplets were generated by spraying an aqueous sample solution consisting of antibody (mAb (NIST monoclonal antibody reference material 8671), mAb-1 or mAb-2 tool antibodies) and enzyme (IdeS, IgdE, or PNGase F) using a pre-programmed injection sequence of an AS1290 autosampler and isocratic flow generated from the 1260 Quaternary pump (Agilent Technologies, Santa Clara, CA) as illustrated in Figure 1. All mAbs used in this study were IgG1 based (See chemical information in Supporting Information). For the experiment shown in Figure 2, the valve position on the injection port was initialized such that 100 μ L/min flow of 200 mM NH₄OAc buffer from the isocratic pump was by-passing the injection loop. Then 5 μ L of 1 mg/mL NIST mAb in 200 mM NH₄OAc buffer (pH 7) was aspirated to the sampling loop by the sampling syringe. Next, 5 μ L of 2 units/ μ L IdeS enzyme in 200 mM NH₄OAc buffer (pH 7) was aspirated to the sampling loop by the sampling syringe at default speed of 100 μ L/min. Both reactants were next fully ejected to an empty well position and the 9 μ L of the mixture was further mixed twice

at the default speed. Then 5 μ L of the reaction mixture was aspirated at the default speed. The reactants were further mixed in 100 μ L sample loop 5 x at maximum-speed. Finally, the needle was rinsed and placed in the low-pressure needle port and the valve position was switched from by-pass to main pass whereby the isocratic flow was in-line with the sample loop for the injection of the antibody sample mixed with enzyme. The flow entered the column compartment that was either heated or kept at room temperature via a programmable heat exchanger, followed by spray ionization by using a JSS. All FIAs were performed for a total run time of 2 min. Detailed procedures for flow injections of different antibodies and enzymes with or without TCEP are shown in the Supporting Information.

Mass Spectrometry

MS analysis was performed on a 6545XT Q-TOF (Agilent Technologies, Santa Clara, CA) with a JSS Source. The JSS consists of a stainless-steel capillary (130 μ m i.d., 312 μ m O.D, 9 cm in length) and a coaxial capillary ($\sim$ 3.95 mm o.d. and a tapered 667 μ m i.d.). The small size difference between two capillaries was capable of providing the nebulizing gas at high velocity. The nebulizer nitrogen gas was operated at 60 psi. The JSS sheath (9 mm O.D) was operated at a nitrogen gas flow of 12 L/min and a gas temperature of 400°C. The Agilent sprayer was grounded while the nozzle was maintained at 2 kV. The spray orifice drying gas was operated at 12 L/min and a gas temperature of 350°C. The mass spectrometer TOF analyzer was tuned and calibrated for high mass-mode of operation (up to 10 kDa upper mass range). MS1 spectra were collected at m/z range of 2400-9000 Th, with an acquisition rate of 0.5 spectra/s at a collision energy (CE) of 0 eV. MS2 spectra were collected with m/z range of 100-4000 Th, with an acquisition rate was set to 0.5 spectra/s. Targeted MS/MS was performed on selected m/z at CE ranging from 20-100 eV

using a narrow mass selection window of ~ 1.3 Da in the MassHunter (Ver. B08.01) data acquisition software. Detailed data analysis information is shown in the Supporting Information.

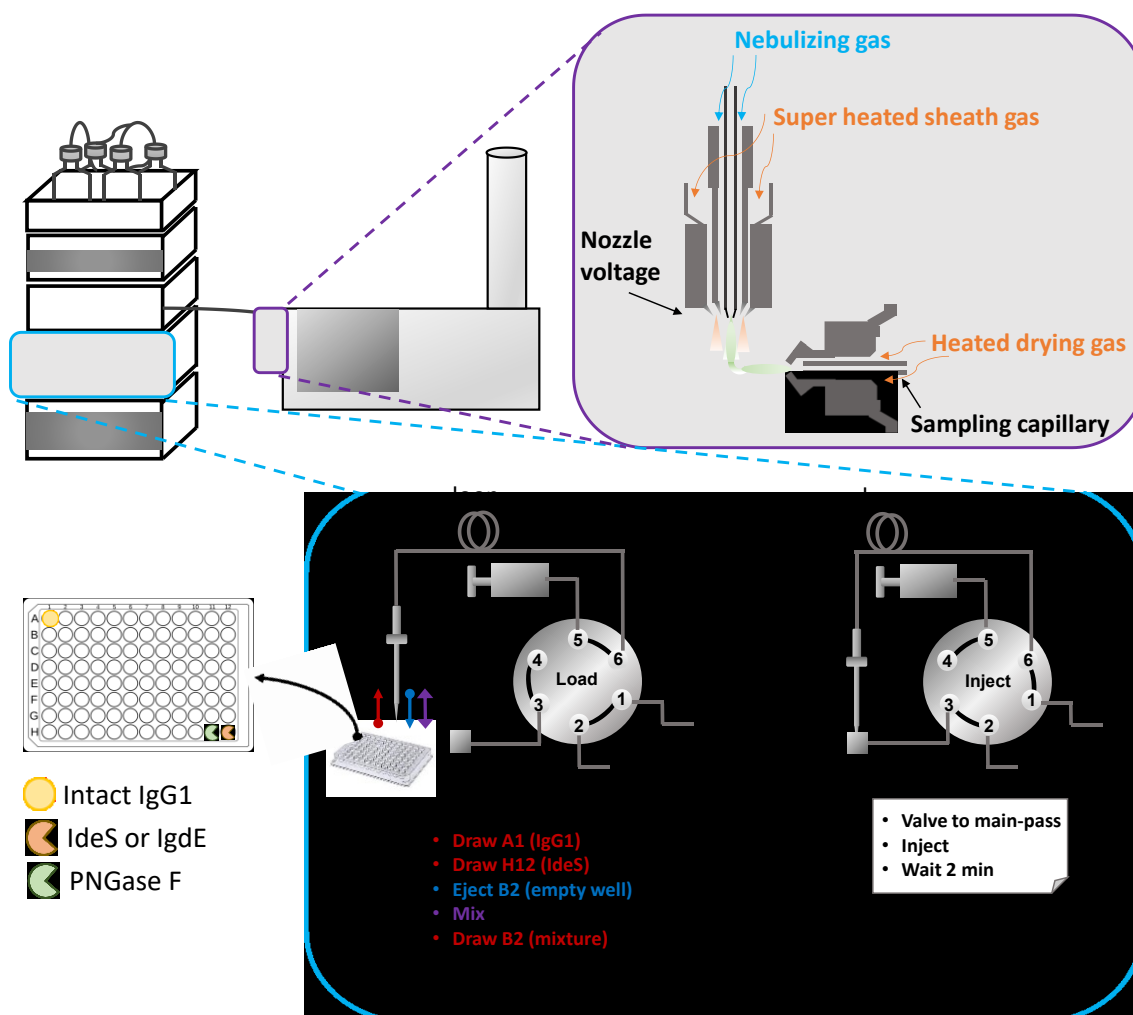


Figure 1. A General scheme for performing microdroplet reactions via autosampler injector programmed to sequentially draw, mix and inject mAb and enzymes (IdeS, Igde or PNGase F) at 0.1 mL/min isocratic flow of 200 mM ammonium acetate coupled with a Q-TOF mass spectrometer tuned for intact mass analysis (MS1) and tandem mass spectrometry (MS2). Note: The Zoomed views on the top and at the bottom show the JSS Source and the valve and injection positions during the steps of autosampler injector program, respectively.

Results and Discussion

Our previous study¹⁷ showed ultrafast digestion of antibodies with enzymes using home-built sprayer can occur during the spray time period (<1 ms) but manual injection of samples

along subsequent desalting and offline MS analysis took more than 20 min. This study aimed to examine the coupling of automated flow injection with antibody microdroplet digestion and online MS monitoring, which would allow for robust and reproducible introduction of reactants for microdroplet digestion. It would also save time in comparison to manual mixing of samples and offline MS analysis of microdroplet-digested sample. Furthermore, online MS monitoring, enabled by JSS ionization in this study, would produce antibody subunits under native-pH conditions since sample solutions were prepared in ammonium acetate solution with no addition of organic solvent. Figure 1 shows a pre-programmed injection sequence to aspirate samples and reagents from two well positions before mixing and injecting the mixture to the JSS spray source that was tuned for microdroplet chemistry. We first investigated the IdeS enzyme, cloned from *Streptococcus pyogenes* and expressed in *Escherichia coli*, which specifically digests IgG1 below the hinge region and generates F(ab')₂ and scFc fragments (Figure 2a). In our experiment, NIST mAb and IdeS were placed on two separate wells on a 96-well sample collection plate that was stored at 4°C. IgG1 and IdeS were sequentially aspirated and brought to an empty well for mixing. The mixed sample was injected into the flow path and was kept at room temperature before introducing to the JSS nebulizer. The flow injection total run time was set at 2 min. The distance between sprayer and the spray orifice was ~2 cm, and the speed of the sprayed microdroplets formed within the first 6 mm from the nebulizer tip had a median droplet size of 10 µm and a median velocity of 105 m/s (personal communication from Agilent Technologies).³⁶ Thus, the flight time of microdroplets where majority of the microdroplet reactions take place is estimated as 57 µs [$0.6 \text{ cm}/(105 \text{ m/s}) \cong 57 \text{ µs}$]. Even in such a short spray time, we achieved high digestion efficiency of an antibody in microdroplets.

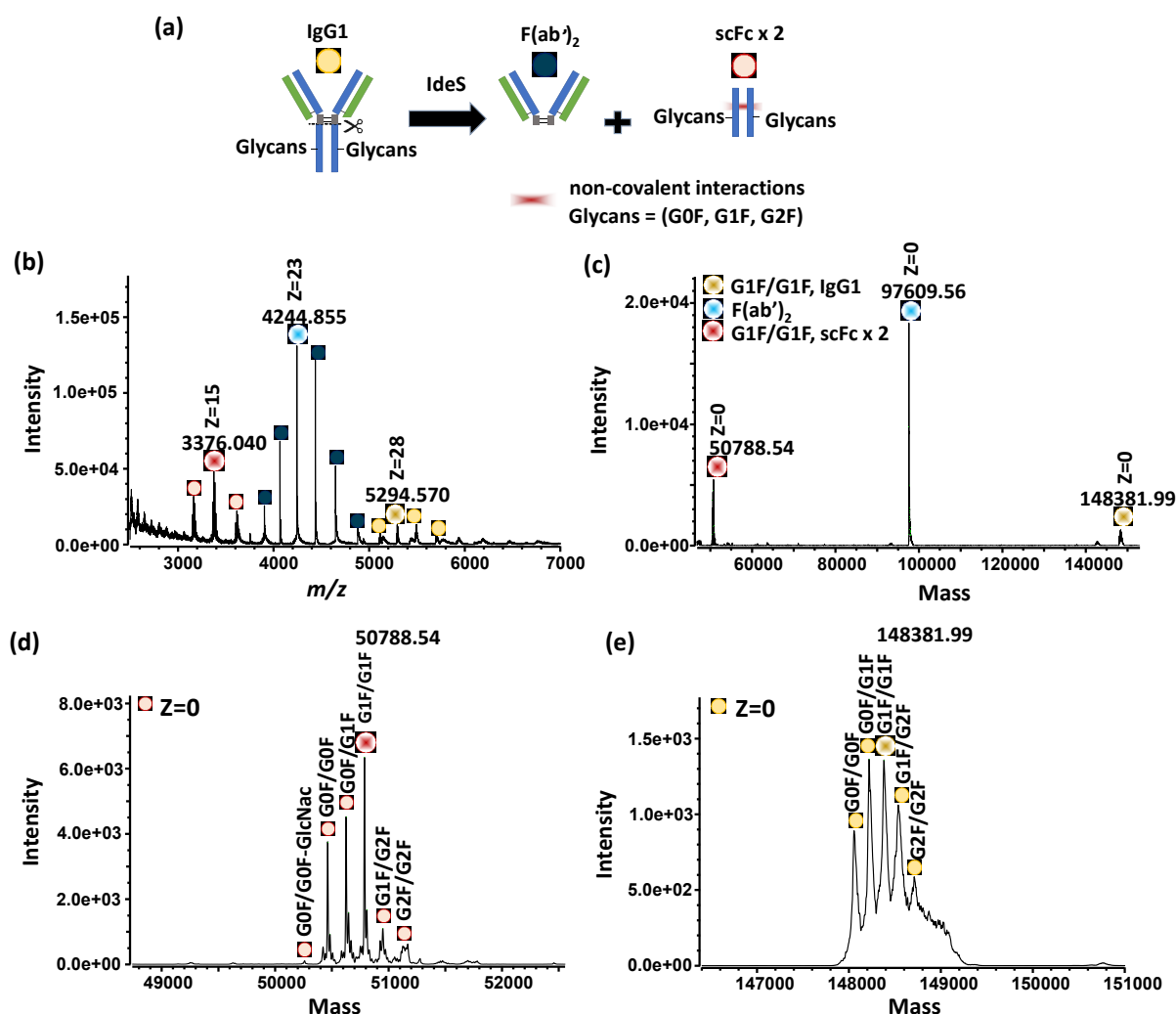


Figure 2. NIST mAb IdeS microdroplet reaction under flow injection analysis-mass spectrometry (FIA-MS). (a) mAb digestion at a single point below the hinge of the heavy chain with IdeS; (b) Post-IdeS digestion MS spectrum showing dimerized-scFc (scFc x 2), F(ab')₂ and residual mAb; (c) Post-IdeS digestion deconvoluted MS zero-charge spectrum of (b); (d) Zoomed view of dimerized-scFc (scFc x 2) domain in (c); (e) Zoomed view of the residual intact NIST mAb. Note: Most abundant charge states and representative m/z (or mass) values are annotated in the mass spectra.

Figure 2b shows that all the IdeS-cleaved subunits of NIST mAb (i.e., F(ab')₂ and dimer of scFc (scFc x 2)) were clearly observed in the MS spectrum with minor amount of residual intact mAb. Indeed, the clear observation of these subunit ions in high intensity does show that the JSS ion source employed in this study is capable of efficiently ionizing samples from aqueous solution without organic solvent or acid additives. Interestingly, scFc fragments were observed as a non-

covalently held dimer (scFcx2). Figure 2c shows the charge deconvoluted spectrum showing relative abundances of the digested products and the residual antibody. We clearly detected and resolved different glycoforms (G0F-GlcNac, G0F/G0F, G0F/G1F, G1F/G1F, G1F/G2F and G2F/G2F) of dimerized scFc fragments (Figure 2d) and F(ab')₂ with high mass measurement accuracy (Figure S1, Supporting Information) and residual mAb (Figure 2e).

In this case of using the commercial JSS sprayer, a digestion efficiency of ~ 90% (based on the relative change on intact mAb ion signal before and after digestion, Figure S2, Supporting Information) of an intact antibody at 25°C represented a 31 million-fold speed improvement, in comparison with traditional digestion in bulk solution that requires at least 30 minutes and up to 1 hour at 37°C.²⁶ Indeed, no noticeable digestion was reported for bulk in-solution digestion when mAb was incubated with IdeS at 37°C for 5 min.¹⁷ Note that, in Figure 2 of the microdroplet experiment, the contact time of the NIST mAb and IdeS in the 130 cm flow tubing after rapid mixing was only ~ 10.8 seconds (See calculation in Scheme S1, Supporting Information), during which no noticeable digestion would occur. Furthermore, automated sample injection in this study allowed a fast workflow (total time: 2 min) and reproducibly digested antibody samples. Our results showed a highly reproducible antibody digestion in 5 runs within 10 min (digestion efficiency variation: ~2%; Figure S2, Supporting Information), which suggests the potential of our new approach for high throughput antibody analysis.

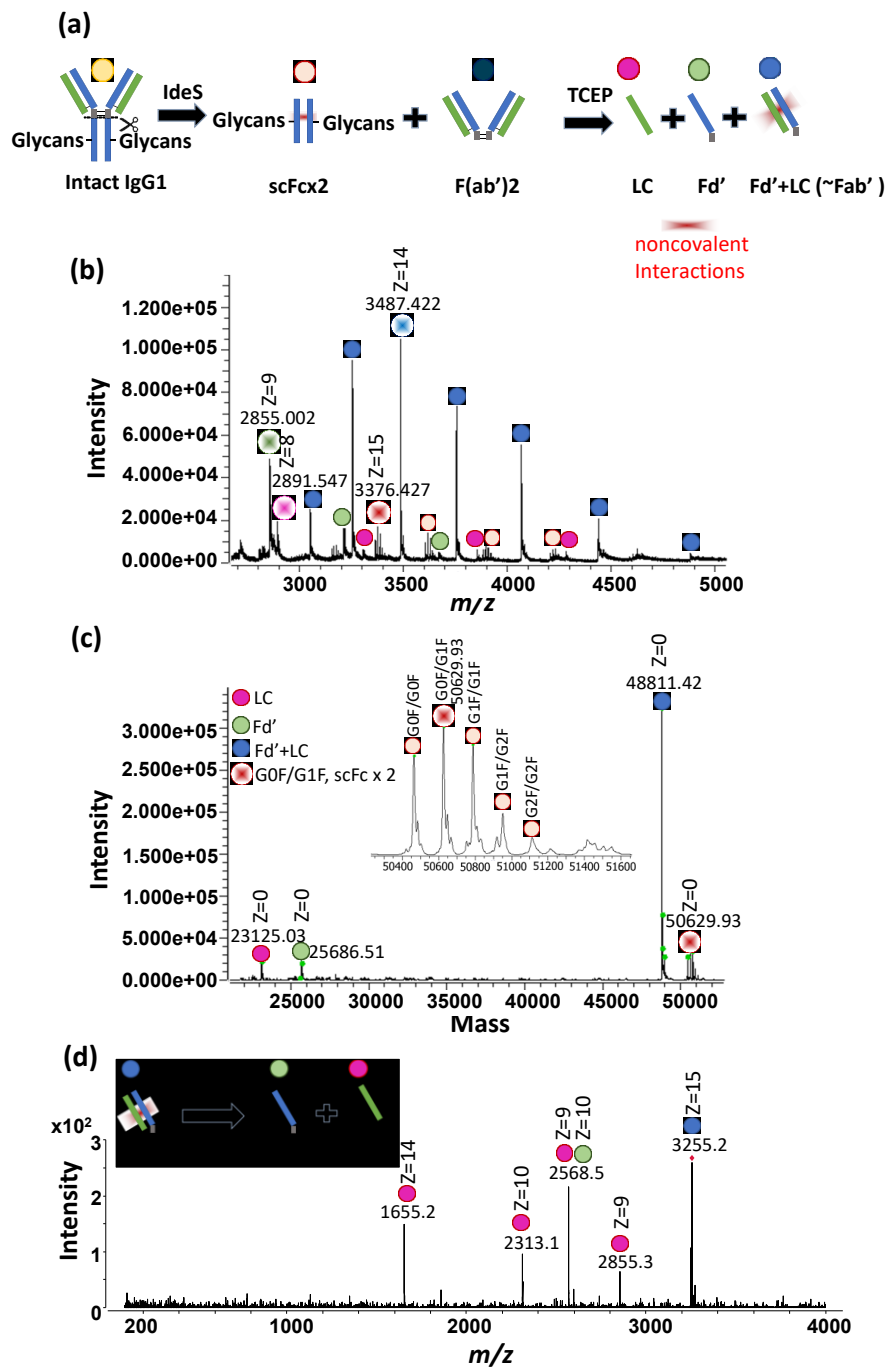


Figure 3. NIST mAb one-pot microdroplet reactions using IdeS and TCEP reducing agent under FIA-MS condition (a) A scheme depicting observed subunits post-IdeS digestion and TCEP reduction reactions in native-like buffers; (b) Post-IdeS digestion and TCEP reduction MS spectrum; (c) Corresponding deconvoluted MS spectrum of (b). Dimerized Fc α 2 is shown in inset of (c); (d) MS $_2$ -CID spectrum of Fd'+LC dimer at 40 eV collision energy. Note: Most abundant charge states and representative m/z (or mass) values are annotated in the mass spectra.

Addition of a reducing reagent TCEP in the spray solution containing IdeS and NIST mAb accelerated simultaneous digestion and disulfide bond reduction in the microdroplets. In our experiment, NIST mAb, IdeS and TCEP were sequentially aspirated and brought together in an empty well where mixing occurred. The mixed sample was injected into the flow path with the column compartment kept at 60°C before introducing to the JSS nebulizer. Figure 3a illustrates the possible IdeS-digested and TCEP-reduced subunits. Indeed, we clearly observed in the MS spectrum (Figure 3b) all the digested and reduced fragments, LC, Fd', and dimerized-scFc's (scFcx2) including glycoforms (G0F, G1F, G2F). In addition, to our surprise, we also observed a non-covalently bound dimer between Fd' and LC (equivalent to Fab', Figure 3b and the deconvoluted MS spectrum in Figure 3c).

In antibody characterization, IdeS-digested and TCEP-reduced fragments, also known as antibody subunits, are valuable because they are amenable to MS/MS techniques that provide high-sequence coverage and localization of structural modifications. For the NIST mAb subunits, their peaks in the acquired mass spectrum were under the 4000 Th m/z -limit. This allowed us to perform single precursor ion charge state isolation by the resolving quadrupole and subsequent beam-type CID on the collision cell, prior to TOF mass analysis. We next examined real-time digestion in tandem with beam-type CID for structural confirmation of +15 ion of the Fd'+LC dimer. At the collision energy of 40 eV, the dimer dissociated with expected charge asymmetric dissociation³⁷ where LC was observed at +9, +10 and +14 and Fd' was observed at +9 and +10 (Figure 3d). Note that the complementary subunits for each of these LC and Fd' are expected but were not observed due to the 4000 Th m/z -limit of the quadrupole mass analyzer. At higher collision energies of 60 eV and 80 eV, we saw complementary ion pairs (e.g., observation of LC +8 and Fd' +7 in Figure S3,

Supporting Information). We believe that tandem MS analysis of non-covalently held complexes will complement other analytical strategies for mapping interactions due to aggregation.

Aggregate analysis at the subunit-level is required to localize domain-specific interactions rather than the entire antibody.^{38,39} Tandem MS analysis combining microdroplet chemistry allows the localization of interaction to particular domains of a mAb in a rapid fashion. Indeed, taking one step further, our online microdroplet digestion can be used to study specific bindings of antibody and antigen, under native-pH conditions. For instance, we detected the specific binding complex of NIST mAb and RSVF antigen by JSS ionization of a mixture of mAb and RSVF. When IdeS was added into the solution for spray, the specific bound complexes of F(ab')₂ and RSVF (1:1 and 1:2 complexes) were observed, suggesting the binding location of RSVF on the F(ab')₂ subunit of the antibody (Figures S4 and S5).

Antibody Fc chain mutations (i.e., LALA and PG mutations) make IdeS digestion less effective.⁴⁰ We therefore extended our workflow to investigate another enzyme IgdE derived from the pathogen *Streptococcus agalactiae*, and recombinantly expressed in *E. coli*,⁴¹ which specifically digests mAb above the hinge region (KSCDKT/HTCPPC), generating intact Fab and scFc x 2 fragments (Figure 4a). In our experiment, 5 μ L of 1 mg/mL NIST mAb sample and 5 μ L of 5 units/ μ L IgdE were sequentially aspirated and then injected for spray by JSS. The heat-exchanger was kept at room temperature. Figure 4b shows the acquired MS spectrum of IgdE-cleaved subunits of mAb-1 (i.e., Fab and scFc x 2). Figure 4c shows the corresponding deconvoluted mass spectrum. It is important to note that Fab consists of LC and Fd' fragments linked by a single disulfide bond. Structural interrogation of Fab is important as they possess the variable regions of mAb that binds to a specific antigen. Figure 4d shows the MS2-CID product ion spectrum of +15 ion of the Fab at a collision energy of 80 eV, acquired within the FIA time

of 2 mins, which was confidently assigned with *a*-, *b*-, and *y*-type ions mapped to the theoretical NIST mAb sequence (Figure 4e). Among these fragment ions, b_{42} (Fd'), b_{102} (LC) and b_{165} (Fd') resulted from N-terminal proline cleavage during CID. Different from the aforementioned CID result for non-covalently bound Fd'+LC dimer ion (equivalent to Fab', Figure S3c, 80 eV collision energy MS2-CID, Supporting Information), backbone cleavages of the Fab in this case were observed, which was attributed by a single interchain disulfide linkage between LC and Fd' in Fab.

Further structural elucidation of modifications on the Fab region is imperative in assessing critical product quality attribute in many therapeutic antibodies, as elucidating post-translational modification masses (i.e., glycation, trisulfides, or oxidation) are more accurate at the subunit-level than the entire antibody.^{26, 42} Antibody subunits characterization of modifications in combination with microdroplet chemistry was also performed on the NIST mAb that was first stressed with glucose and subjected to FIA combined with IgDE microdroplet digestion. The resulting Fab fragment consisted of up to two resolvable sugar adducts (See Figure S6, Supporting Information). We next subjected +15 charge state of the glycated Fab with a single sugar to MS2-CID in a similar manner to the +15 charge state of unmodified Fab. We confirmed and localized the single glycation specifically to either lysine-38 or lysine-41 of the the light chain, as fragment ions carrying hexose were identified as $[b_{204}+Hex]^{15+}(LC)$, $[b_{196}+Hex]^{9+}(LC)$, and $[b_{80}+Hex]^{3+}(LC)$ due to N-terminal proline cleavage. Furthermore, all fragment ions associated with Fd' showed the absence hexose modifications, confirming that glycation was exclusive to the light chain at aforementioned residues (Figure S7, Supporting Information). We believe that microdroplet-generated subunit analysis combining any type of MS/MS dissociation method to further localize glycation would complement bottom-up peptide mapping with unprecedented speeds.

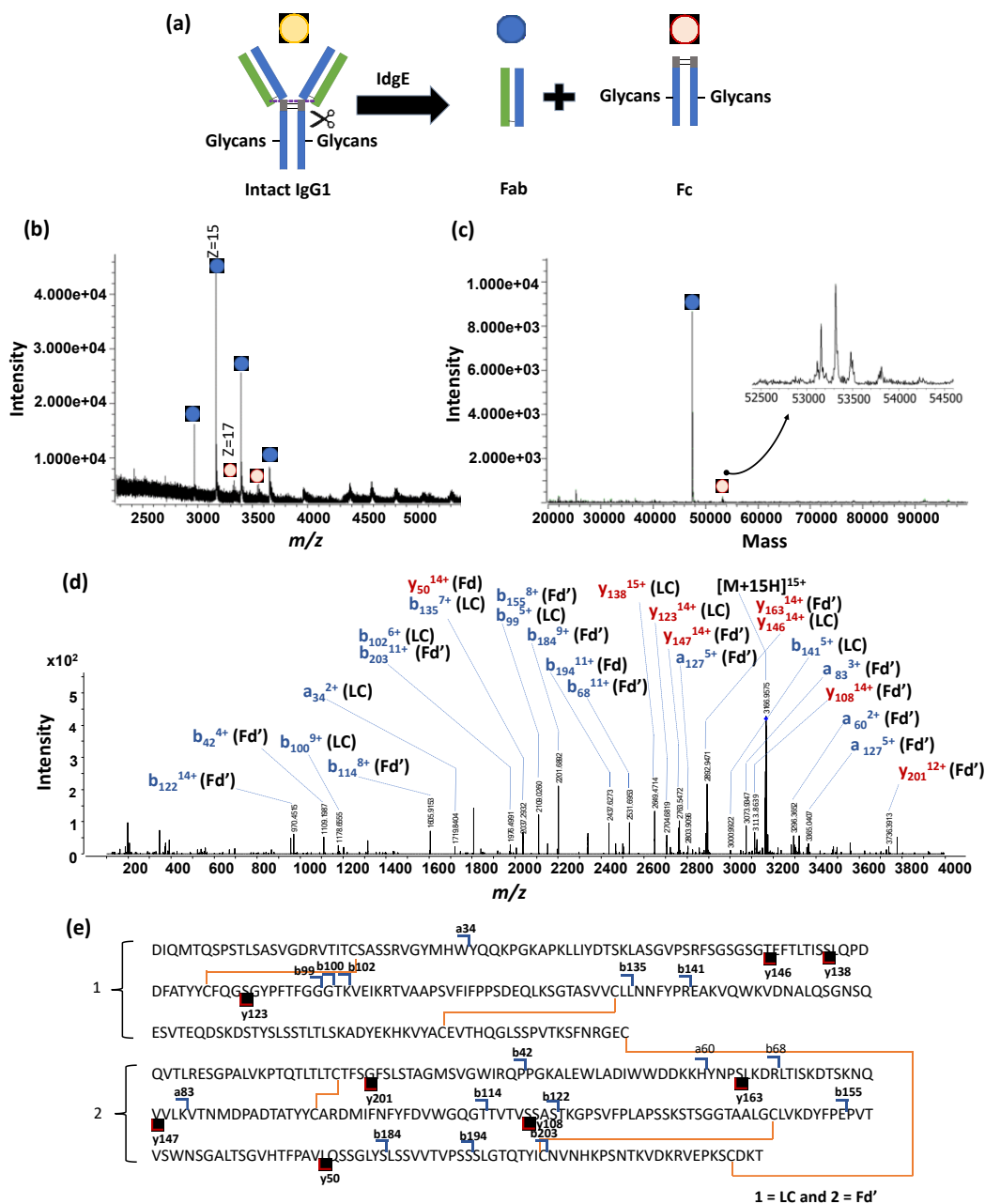


Figure 4. NIST mAb IgDE microdroplet reactions coupled with FIA-MS and FIA-MS/MS structural characterization of subunits. (a) General scheme of IgDE digestion; (b) post-IgDE MS spectrum of NIST mAb; (c) Deconvoluted MS spectrum of (b); (d) MS2-CID product ion spectrum of NIST Fab which was mass selected using a 1.3 Da isolation window and subjected to beam-type CID at 80 eV collision energy; (e) Fragmentation map of NIST Fab.

Furthermore, we examined the utility of IgDE enzyme to generate Fab fragments from mAb-1 that had LALA and PG mutations⁴⁰ on the Fc region by performing microdroplet reactions

on mAb-1 using IgDE and IdeS (Figure S8a, Supporting Information). Figure S8b (Supporting Information) shows the acquired MS spectra IgDE-cleaved subunits of mAb-1 (i.e., Fab and scFc x 2). We clearly detected and resolved different glycoforms (G0F/G0F, G0F/G1F, G1F/G1F) on the intact mAb-1 and the Fc+G0F/G0F, Fc+G0F/G1F, and Fc+G1F/G1F subunits (Figure S8c, deconvoluted mass spectrum, Supporting Information). In contrast, mAb-1 does not show any microdroplet reactions with IdeS due to LALA and PG mutations in Fc (Figures S8d and S8e, Supporting Information).

One more important modification for antibody is glycosylation, which can be heterogeneous, and variable in cell culture. Removing glycosylation during pharmaceutical production analysis would be beneficial to ease the characterization of antibodies and to obtain the correct N-linked oligosaccharide (N-glycan) profiles. N-Glycosidase F (PNGase F), a glycosidase from *Elizabethkingia miricola*, is one of the most effective enzymes to cleave N-glycans from proteins⁴³ (illustrated in Figure S9a, Supporting Information). Figure S9c (Supporting Information) shows the NIST mAb MS spectrum from PNGase F microdroplet digestion performed when the column compartment was kept at room temperature. The deconvoluted spectrum (Figure S9e, Supporting Information) consists mostly of undigested mAb with both glycans still kept intact, and mAb with one glycan remaining on its heavy chain, and a minor mAb species with removal of both glycans on its heavy chains. Figure S9b (Supporting Information) shows the MS spectrum of the NIST mAb after PNGase F microdroplet digestion acquired when the column compartment was heated at 60 °C. The deconvoluted spectrum (Figure S9d, Supporting Information) consists mostly of the deglycosylated NIST mAb (deglycosylation reaction efficiency of ~60% was estimated based on the relative peak intensities of deglycosylated IgG1 and remaining glycosylated peaks). Heating of the solvent flow substantially (setting the heat exchanger to 60 °C) improved

microdroplet deglycosylation efficiency in our flow injection setup. The enhanced deglycosylation yield in this case could be ascribed to either heat denaturation of antibody or heat acceleration of microdroplet reaction. For clarity, we carried a separate heating experiment which did show that pre-heating of mAb at 70°C prior to spray does increase deglycosylation efficiency (Figure S10, details shown in Supporting Information).

In addition, we further investigated the intrinsic effects of antibody structure and glycan structure on microdroplet deglycosylation reactions. Overall, no significant effect of both antennary galactose and fucosylation on PNGase F deglycosylation reactions was observed while IgG1 structures appeared to have a large effect on deglycosylation reaction yields (Figures S11-13 and Table S1, Supporting information).

We further tested mAb-2 that had high-levels of galactose for microdroplet reactions with PNGase F and IdeS. We first tested each reaction independently (experimental details are shown in Figure S14). Finally we performed an ‘one-pot’ reaction where IdeS digestion and PNGaseF deglycosylation were performed triggering simultaneous deglycosylation and digestion in microdroplets. We introduced the samples and enzymes to the flow path by sequentially aspirating 5 μ L of 1 mg/mL mAb-2 sample, 5 μ L of 2 units/ μ L IdeS and 5 μ L of 5 units/ μ L PNGase F enzyme into the mixing loop where they were mixed and sprayed (with the column compartment being heated at 60°C). Figure S14a (Supporting Information) shows the MS spectrum of mAb-2 (pre-digestion) that was used as a reference. Figure S14b is MS spectrum collected post-PNGase F microdroplet reaction, which shows ions of mAb-2 without any glycans (G0F, G1F, and G2F) in addition to PNGase F ions. Thus, deglycosylation efficiency in the microdroplets appeared to be 100%, as confirmed in the deconvoluted MS spectrum (Figure S15, Supporting Information). Figure S14c shows the MS spectrum of mAb-2 post-IdeS microdroplet reaction. IdeS-cleaved

subunits of mAb-2 (i.e., F(ab')₂ and scFc) were clearly observed in the MS spectrum with minor amount of residual intact mAb-2. Figure S14d shows the MS spectrum of mAb-2 post-IdeS and post-PNGase F microdroplet reactions. The intact scFc fragment was observed without any glycans (G0F, G1F, and G2F), showing complete deglycosylation under these conditions.

Conclusions

In summary, this work represents the first report of combining ultrafast antibody microdroplet chemistry with automated and rapid sample flow injection and JSS ionization under native-pH conditions. The total analysis workflow only takes 2 min, including MS1 and MS2 analysis of antibody subunits. Coupling flow injection vastly improves the efficiency and robustness of microdroplet reactions where sample introduction is hands-off. The setup is therefore amenable to high-throughput automated analysis of mAbs with minimum sample preparation. Furthermore, ultrafast reduction is feasible by doping the spray solvent with TCEP, leading to the IdeS-cleaved and TCEP-reduced fragments (LC, Fd' and scFc with different glycoforms). In addition, as antibody is digested, its subunits can be structurally interrogated under native conditions with targeted tandem MS analysis where m/z is not too high for quadrupole isolation, thus enabling localization of domain-specific modifications. Besides, we found that heating of mAb and PNGase F can further enhance microdroplet reaction efficiency. Also, native-pH MS conditions would be useful to study antibody-antigen specific binding interactions. All of these applications can be implemented as a high-throughput mass spectrometry screening experiment where microdroplet enables subunit generation in real time.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

HPG acknowledges the support from Janssen Research & Development. HC and YL thanks NIH (1R15GM137311-01) and NSF (CHE-2203284) for funding. Authors thank Christian Klein and Kenneth Newton (Agilent Technologies) for useful discussion on the Agilent Jet Stream Source and Bob Giuffre (Agilent Technologies) for technical assistance with autosampler injector programming.

Supporting Information: Additional MS spectra, chemical information, experimental procedures and setup are included.

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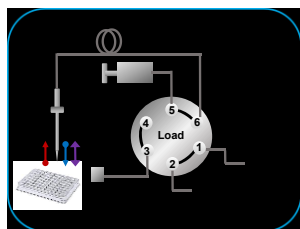
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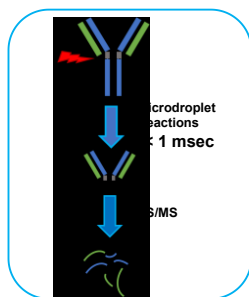
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